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Short communication: A survey of grass-clover ley management and creation of a Near Infra-Red Reflectance Spectroscopy equation to predict clover concentration

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Summary

The purpose of the present study was, firstly, to examine current practice for the agronomy of grass-clover mixed swards used for silage-making in the UK, and secondly, to develop and validate a Near Infra-Red Reflectance Spectroscopy (NIRS) equation capable of predicting clover concentration (CC) in undried and unmilled grass-clover silage samples. A calibration set of 94 grass-clover (white, *trifolium repens*, and red, *trifolium pratense*) mixture silage samples were sourced from UK farms and an accompanying questionnaire was used to obtain information on the sward agronomy used to produce each sample. Questionnaire data highlighted that (i) reducing the use of fertiliser inputs (ii) increasing uptake of new varieties, and (iii) increasing the farmer's ability to measure botanical composition as potential strategies for improving the utilisation of clover in grass swards. Botanical composition was measured by hand separation for each sample and a new NIRS equation was created and assessed using blind validation with an independent set of 30 grass-clover samples. The relative standard error of cross validation (SECV, as a percentage of the measured mean) of the optimised equation produced was 36.8%, and, in an independent validation test, the ratio of standard error of prediction to the standard deviation of the reference data set (RPD) was 1.56. The equation could be improved by increasing accuracy at high CCs but showed promise as a simple tool to assist growers in sward management decisions.

Introduction

The use of mixed grass-clover swards for both grazing and silage production is now relatively wide-spread across temperate European agricultural systems and particularly in the UK where 70% of grass swards on dairy farms are thought to contain clover (DEFRA, 2015). Grass-legume swards offer a sustainable approach to reduce fertiliser input into grasslands, as the atmospheric nitrogen (**N**) fixed by clover can be utilised by grass, an example of niche complementarity between two species (Nyfeler et al., 2011, Phelan et al., 2015). Utilising niche complementarity in this way is becoming an area of increasing interest for both binary and complex (3+ species) sward mixtures. Combining species of different functional groups, can not only increase productivity and minimise the need for inputs, but may also supply different beneficial nutrients, minerals, and secondary plant compounds to livestock (Provenza et al., 2007). A key determinant of the success of mixed swards is determining and maintaining the correct concentration ratio of species or functional groups so that they work in harmony. Previous research has shown that, in general, the best results can be achieved by an even distribution of species within a sward, with no single species becoming dominant (Finn et al., 2013). Where one species is over-dominant the others may not reach their production potential or fulfil their niche functionalities, and the productivity of the whole sward could be reduced (Kirwan et al., 2007, Lüscher et al., 2014).

To date, we are not aware of any published surveys that document the management strategies farmers utilise for grass-clover leys, and to what extent these conform to best practice guidelines for maintaining species evenness. Additionally, there is a need for increased development of practical methods by which growers can manage species evenness within a sward, beginning with simple binary mixtures. The first step to improved management is the ability to measure the botanical composition of a sward with ease. Near Infra-Red Spectroscopy (**NIRS**) analysis offers a quick and inexpensive method, already routinely used for silage analysis, by which the composition of a mixed sample might be determined. Prediction equations for NIRS analysis of clover in a mixed grass-clover silage sample have been successfully reported previously using dried samples for calibration (Wachendorf et al., 1999, Cougnon et al., 2014) however no prediction equations currently exist which are appropriate for the UK where silage analysis is performed on undried (fresh) and unmilled samples. Once the botanical composition of a sward is known, management may be adjusted to suit one species or another by varying cutting height, cutting frequency, fertilisation or grazing intensity (Yarrow and Penning, 1994, Phelan et al., 2014).

The objectives of the present study were therefore to develop an NIRS equation to measure the botanical composition of fresh grass-clover silages appropriate for uptake by laboratories in the UK, and secondly, to assess current management practices of grass-clover swards to better understand where further research into management of botanical composition is required.

Material and methods

Experimental design

Ninety-four grass-clover silages (58 baled and 36 clamped) were sourced from 50 commercial farms spread throughout England, Scotland, and Wales, and brought to the University of Reading's Centre for Dairy Research (**CEDAR**; Arborfield, UK) for processing. A further 95th sample was created by combining one of the original 94 samples with additional grass silage to create a new sample, this was done to create a greater quantity of material for other *in vivo* analyses. The samples were obtained to evaluate the use of NIRS analysis for nutrient concentrations as described previously (Thomson et al., 2018) over three consecutive years (2012/13, 2013/14, and 2014/15). The quantity of each silage collected was approximately 500 kg. Where the clover species was known (n = 65) 66% of samples were red clover (*trifolium repens*), 20% were white clover (*trifolium pratense*) and 14% were a mixture of both. The number of samples that were first, second, third and fourth cuts were 36, 20, 16, and 4 respectively (22 samples unknown). Sample processing is described in detail by Thomson *et al.* (2018), however, in brief, samples were mixed either in a feeder wagon containing knives (Hi-Spec Mix Max, Hi Spec Engineering, Co. Carlow, Ireland for 45 min), or in a DataRanger feed mixer without knives (American Calan, Northwood, NH, USA) for unchopped and pre-chopped samples respectively. After mixing, representative subsamples of each silage sample were stored separately at -20ºC for future analysis by manual separation and NIRS.

Silage Questionnaire

A questionnaire was given to each farmer who donated a silage sample to the study. The questionnaire comprised 17 questions (Supplementary Table 1) relating to the timing of establishment, fertiliser applications, and harvesting; the composition of seed mixtures used; and ensiling practices. For the botanical composition of the seed mixture, the variety sown was recorded for ryegrass and clover whereas any other components were simply recorded at the species level as variety was rarely provided. In addition farmers were asked to retrospectively estimate the percentage of clover in the sward at the time of harvest (Question 9, Supplementary Table 1). Farms were permitted to contribute more than one silage sample to the study provided the samples originated from differing cuts, years, or swards. Separate questionnaires were completed for each of the samples. Questionnaire forms were returned for 64 of the 94 samples however not all questions were answered on all returned questionnaires and in some instances answers were insufficiently detailed to be included. These 64 completed questionaires originated from 36 individual farms, reflecting that a number of farms returned more than one questionnaire, each relating to a different crop of silage.

Sample analysis and NIRS scanning

Approximately 200 g of silage was manually aspeciated into clover, grass and other species as a means of determining the clover concentration (**CC**) in the silage. Resulting fractions were then oven dried at 60ºC for 72 h to determine CC on a DM basis. A second 2 kg subsample of frozen material was sent to the Agri-Food and Biosciences Institute (**AFBI**; Hillsborough, Northern Ireland) where all samples were hand-chopped to 2.5 cm length. Two separate packages were created from each sample, each containing 100 g of undried and unmilled silage wrapped in non-PVC cling film which were placed in coarse transport cells for scanning (Park et al., 1999). NIRS spectra for each scan, recorded as Log 1/Reflectance over a 400-2498 nm range (2 nm gaps), were obtained using a Foss NIRSystems 6500 machine (Foss, Hillerød, Denmark) and ISI v.3.10 software (Infrasoft International, Port Matilda, PA, USA).

Data Analysis

Statistical analysis. Survey data are summarised and reported as percentages of recieved responses for each question. A one-way ANOVA followed by a post-hoc Tukey test was used to determine the effect of cut number in the year (1st, 2nd, 3rd or 4th) on CC, where CC was the independent variable, in Genstat 16th Edition (VSN International, Hemel Hempsted, UK). P values < 0.05 were considerred statistically significant. As the survey only concerns one group of farmers (rather than contrasting multiple groups) no further statistical analysis was deemed necessary.

Data pre-treatment and production of new NIRS equations. Creation of a new prediction equation for CC was performed as described by Thomson *et al.* (2018) using WinISI III v1.50 (Infrasoft International, Port Matilda, PA, USA). Calibration was carried out as Modified Partial Least Squares regressions over the range 1100-2498 nm using a 2 nm gap. To prevent any sub-sampling error the root mean square difference of each sub-sample was calculated (an upper limit of 5000 was used to judge poor replication however none of the samples in the calibration set were above this limit). Raw data and two derivatives were tested (Raw (0,0,1,1), 1st Derivative (1,4,4,1) and 2nd Derivative (2,10,5,1)) and three scatter corrections (Standard Normal Variate Detrending (**SNVD**), Normal Multiplicative Scatter Correction (**NMSC**) and Weighted Multiplicative Scatter Correction (**WMSC**)) for each of the derivatives. The maximum number of terms set for each equation was 11. For cross validation, three elimination passes were carried out and the cross validation value was set at 6 (i.e. the calibration set was divided into six groups with one group removed sequentially and predicted using a calibration formed using the remaining samples) and the combined standard error of cross validation (**SECV**) was obtained in addition to the standard error of calibration (**SEC**). The optimal equation (lowest SECV) was compared against an equation produced using the UK industry standard data-pretreatment method: first derivative (1,4,4,1) SNVD scatter correction and a repeatability file (a file containing multiple spectra from the same sample measured under different conditions) (Park et al., 1997). For the purposes of a blind validation test, 30 independant grass-clover silage samples of known CC (for which NIR spectra had been previously obtained at AFBI in a prior study) were used to assess prediction accuracy of the new equation using root mean square error of prediction (**RMSEP**) and the ratio of the standard error of predicition to the standard deviation of the measured sample set (**RPD**) (Williams, 2014).

Results and Discussion

Grass-clover management questionnaire

The final set of 95 samples had a mean concentration of clover, grass and other species of 310, 640 and 50 g/kg DM, respectively (median CC = 280 g/kg DM). The distribution of CC, shown in Figure 1a, indicated that low CC silages were more common than high CC silages within the sample set. Ploughing or subsoiling were the most common forms of cultivation prior to establishment with 48% of those providing cultivation information employing these methods. A further 34% of respondents used light cultivation such as discs, tines or a power harrow and 18% reported using a minimum or no tillage approach. In 18 instances (31% of responses) the sward was established by under-sowing. The timing of crop establishment (where known) was roughly evenly split between spring and autumn with 20 sown in March, April or May, 4 sown in June or July, and 22 sown in August or September.

All farms providing seed information reported sowing more than one variety of grass and 22 (52% of responses) sowed more than one variety of clover within a single ley. Sowing for increased varietal richness helps mitigate the risk of any one variety performing poorly (Surault et al., 2010). In total 38 different grass varieties including varieties of italian ryegrass, ryegrass hybrids, timothy, cocksfoot, fescues, and festuloliums were sown, but the predominant species was perennial ryegrass (23 of the 38 varieties). In comparison, only 19 different varieties of clover were represented within the sample set which was likely reflective of the wider range of grass varieties available on the market. The two most frequent grass varieties sown were ‘Solid’ (34% of responses) and ‘Tetragraze’ (26% of responses) which are both hybrids of Italian and perennial ryegrass. ‘High sugar’ grass varieties ‘AberDart’ and ‘AberStar’ were the joint 5th most frequently sown grasses (each sown in 12% of reported swards) indicating good uptake, possibly as a result of research showing the use of high-sugar grasses in combination with red clover produces a favourable balance of metabolisable protein and fermentable energy in the ruminant diet (Merry et al., 2006). Of the four most frequently sown clover varieties, all were red clovers with the varieties ‘Merviot’ and ‘Milvus’ being the most popular (sown in 33% and 21% of reported swards). These are both older varieties (for example, Merviot was first introduced to the UK recommended list of varieties in 1980) perhaps indicating a need for greater adoption of newer clover varieties to take advantage of genetic gains (Frick et al., 2008, Capstaff and Miller, 2018). A recent study showed that, out of 12 clover varieties sown, 10 new varieties showed increased persistency within a 3-year ley when compared to Merviot or Milvus (Marshall et al., 2012).

Information relating to applications that occurred in the year prior to harvesting was given for 46 of the silages. Of the 46 silages, 23 silages (50%) had slurry applied and 12 (26%) had been fertilised using an inorganic fertiliser containing nitrogen (N). A further 5 (11%) had an application of farm yard manure. Only 5 silages (11%) had been reported as having no fertiliser applied. Where excess applications of N are applied, clover adapts by reducing the rate of fixation, and is more likely to be outcompeted by grass (Nyfeler et al., 2011). Farmers were not required to state the timing of the application; therefore, the applications may have been early in the year or during establishment for first year silages, however, even out-of-season N applications have been shown to reduce CC over the summer months (Laidlaw et al., 1992). Those applying slurry may benefit from the application of potassium and phosphorous for soil fertility, however, even slurry may contain enough N to adversely affect N fixation efficiency by clover and sward CC if applied in excess of 50 T/ha (Nesheim et al., 1990). This evidence suggests that few farmers are maximising the N-fixation potential of clover and possibly seeing reduced economic performance as a result, particularly where expensive inorganic N fertiliser is applied to clover-containing swards.

The most common months for taking silage cuts were May, July and September for 1^{st}, 2^{nd}, and 3^{rd} cuts respectively. Stepwise increases in CC were seen in 1^{st} to 3^{rd} cuts ($P < 0.001$) as shown in Figure 1b. The use of silage additives was relatively common with 13 different brands of additive reported. Of these 13 additives used, 10 were bacterial innoculants and the remainder contained either enzymes or salt as the active ingredient. Of 29 total responses to the question on the use of additives, 5 specified that no additive had been used.

Farmers were retrospectively asked to estimate the CC of the sample based on their memory of the sward at harvest. Fifty-five estimations were received and compared against reference values obtained by hand separation (Figure 2). The majority of farmers estimated a value between 300 to 700 g/kg DM clover. As a result samples containing a measured CC of less than 400 g/kg DM were generally over-estimated while samples containing greater than 400 g/kg DM CC were often under-estimated. The number of farmers that successfully predicted CC to within ±100 and ±200 g/kg DM was low at 15 and 31 out of 55 respectively. One possible explanation for this would be poor uniformity within the sward meaning that the sample taken was not representative of the general crop (Marriott et al., 1997), although this explanation is more valid for baled samples as opposed to those ensiled in a clamp where mixing is performed in the forage harvester. The inability of many farmers to recall an accurate estimate of the CC of their forage highlights the need for tools to be developed which automate this process and provide lasting records; one option being the use of NIRS on resulting silage which has been explored in the present study. Another option which has been investigated previously is to determine n-alkane concentration which differs distinctively between species (Jurado et al., 2015), however, the use of a laboratory assay of this kind is expensive and time consuming.

Creation and validation of an NIRS equation to predict clover concentration

The best-performing NIRS prediction equation for CC was produced using 182 spectra, and NMSC (2,10, 5,1) data pre-treatment which gave an SEC of 8.99 and an SECV of 36.8% of the measured mean. Using the industry standard data pre-treatment of SNVD (1,4,4,1) slightly reduced calibration performance by a small margin with an SEC of 10.0 and an SECV of 37.5% of the measured mean.

Spectra from 30 independent grass-clover silage samples of known CC were used in a blind validation test of the industry standard CC equation. The measured mean CC within this independent set of samples was 440 g/kg DM, which was greater than that of the calibration set. Using this data set the equation gave an RMSEP of 52.2% of the measured mean and an RPD value of 1.56. Plotting measured against predicted CC in this test indicated over-estimation at low CC and under-estimation at high CC with the average bias being towards under-estimation (Figure 3). For samples that contained 1000 g/kg clover by DM, the equation underpredicted CC by 300-400 g/kg DM. A group of samples with 150 g/kg DM CC showed the best prediction accuracy. Prediction for samples with the same botanical composition often differed by 150-400 g/kg DM CC indicating low repeatability. Other studies in which prediction equations for CC have been produced have shown more robust prediction accuracy than the present study, for example Cougnon *et al.* (2014) produced an equation with an RPD of 3.8 using just 42 silage samples as a calibration set. In another study Wachendorf *et al.* (1999) produced separate equations for freshly cut grass-red clover and grass-white clover mixtures with large calibration sets (*n* = 282 and 183 respectively) where the relative SECV were 29.9 and 23.5% respectively, which again shows an improvement over equations in the present study. A major difference between the calibration samples used in the present study and those used by Cougnon *et al*. (2014) and Wachendorf *et al*. (1999) is that no sample preparation such as drying or milling was used in line with UK recommendations whereas, in the previous studies samples were dried and milled. The drying and milling process reduces heterogeneity within the sample (which is particularly important to representatively subsample mixtures for analysis) and also removes peaks produced by water molecules from the resulting NIRS spectra, reducing noise, and improving interpretation and repeatability (Sorensen, 2004). The reference technique used to measure CC (manual separation prior to drying and milling) was the same in the present study and in the studies of Cougnon *et al.* (2014) and Wachendorf *et al.* (1999), however, the drawback of this technique is that results can be subjective, particularly on chopped samples as were used in the present study, due to the lack of species-defining characteristics on some particles. Using a reference technique prone to human error such as this reduces the likelihood of producing a robust prediction equation. We conclude that, even though the equation produced in the present study was less robust than in previous examples, the equation may still be of use to guide on-farm decisions, particularly for swards containing low CCs.

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Figure captions

Figure 1 The distribution of clover concentration within a set of 94 grass-clover silages sourced from working farms across the UK over various cuts (1^{st} - 4^{th}) and years (2012-2015; Figure 1a) and the effect of cut number on clover concentration (Figure 1b).

Figure 2 The relationship between actual clover concentration (●) and the grower's prediction of clover concentration (○) in a range of 54 grass-clover silage samples sourced from working UK farms over several years (2012-2015). White drop lines indicate over-prediction and dark drop lines indicate under-prediction of clover concentration.

Figure 3 The results of a blind validation of a Near Infra-Red Spectroscopy prediction equation for clover concentration calibrated using a set of 94 diverse fresh grass-clover silage samples which were manually aspeciated to produce reference values and validated using an independent set of 30 grass-red clover silage samples of 6 known clover concentrations (0, 150, 450, 600, and 1000 g/kg dry matter)