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Nutritive evaluation of herbage from permanent meadows by near-infrared reflectance spectroscopy: 1. Prediction of chemical composition and *in vitro* digestibility

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Abstract: This study was implemented to evaluate the potential of near-infrared reflectance spectroscopy (NIRS) technology to estimate the chemical composition and *in vitro* digestibility of botanically complex herbage mixtures characterised, moreover, by a noteworthy variation among samples in the maturity of the forage plants. A total of 107 herbage samples harvested from permanent meadows located in the uplands of León (northwestern Spain) were analysed to determine their chemical composition. In addition, the *in vitro* digestibility of each herbage sample was measured by two different *in vitro* procedures using buffered rumen fluid. A Bran + Luebbe InfraAlyzer 500 spectrophotometer was used to obtain the near-infrared spectra corresponding to each herbage sample. Prediction equations developed for the estimation of the chemical components showed that NIRS technology could predict these parameters accurately, especially the crude protein and neutral detergent fibre contents ($R^2_{adj} > 0.95$ in both cases). *In vitro* digestibility parameters could also be predicted with an acceptable degree of accuracy using NIRS technology, particularly the *in vitro* Tilley and Terry organic matter digestibility ($R^2_{adj} = 0.925$, standard error of prediction (SEP) = 2.165% organic matter) and the *in vitro* dry matter true digestibility measured according to the Goering and Van Soest procedure ($R^2_{adj} = 0.891$, SEP = 2.208% dry matter).
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Keywords: chemical composition; *in vitro* digestibility; forages; NIRS; ruminant

INTRODUCTION

Herbage hay harvested from permanent meadows, together with silage, represents the principal preserved forage for feeding ruminant animals in many parts of southern Europe, in particular the uplands of Spain. The permanent mountain meadows of these areas are characterised by a highly diverse botanical composition, so it is possible to find, in each meadow, more than 50 plant species corresponding to different botanical families, mainly Graminae and Leguminosae. On the other hand, these natural resources are harvested several times per year, usually after the spring primary growth (between late May and mid July) and then after the summer/autumn secondary regrowth. Thus the plants may be at different stages of maturity at harvest time. Furthermore, there are other factors related to the management of the meadows (possibility of irrigation, fertiliser use, etc) and to the environmental conditions that have a certain influence on the chemical composition and nutritive value of these forages.[1,2] In this last sense the digestibility of the forages is an essential attribute of their nutritive value, as it greatly influences not only the voluntary intake of dry matter but also the efficiency of utilisation of the available nutrients.[3] This is the reason why its accurate estimation is required by most feeding systems to calculate the metabolisable energy[4,5] or net energy[6–8] of the feedstuffs.

Chemical composition parameters have been used to estimate the digestibility of forages, since it is well known that the structure and thus the components of the plant vary as the stage of maturity advances.[2] However, the relationship between digestibility and chemical composition is very complex and depends

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on the botanical species.[9] Currently, several *in vitro* techniques[10–12] are commonly used for routine analyses in commercial laboratories to estimate the digestibility. *In vitro* procedures are less expensive and time-consuming than the *in vivo* method requiring total faecal collection. Digestibility coefficients obtained *in vitro* are highly repeatable and closely correlated with those obtained *in vivo*, although the relationship between *in vitro* and *in vivo* digestibility is not so simple for legume-rich forages.[9] Nevertheless, when a large-scale testing of feedstuffs is required, *in vitro* techniques may also be expensive and time-consuming. Therefore a faster and less laborious, and at the same time feasible, repeatable and reliable, alternative procedure for nutritive evaluation is desirable.

Near-infrared reflectance spectroscopy (NIRS) has been successful in predicting, rapidly and accurately, the chemical composition and digestibility of very different forages.[13] NIRS technology is also a non-destructive method requiring small amounts of sample. Nevertheless, there is little information about the potential of this analytical procedure to estimate parameters related to the nutritive value of botanically complex resources[14–17] such as those harvested from permanent mountain meadows situated in the uplands of León (northwestern Spain). This study is the first of a series concerned with the ability of NIRS technology to estimate the nutritive value of these kinds of forages. Thus, in the present study, NIRS technology was used to estimate the chemical composition and *in vitro* digestibility. The second study will examine the ability of NIRS technology to predict the kinetic parameters and extent of degradation of these herbage samples in the rumen.[18]

EXPERIMENTAL

Forage samples

This work was carried out with 107 herbage samples harvested after the spring primary growth and again after the summer/autumn secondary regrowth of permanent meadows located in the uplands of León (northwestern Spain) at an altitude of 900–1450 m. These meadows are plant communities classified within the vegetation type *Arrhenatheretalia*, ie pastures and meadows on well-drained, relatively fertile mineral soils.[19] Predominant forage species were *Alopecurus pratensis* L, *Anthoxanthum odoratum* L, *Arrhenatherum elatius* (L) Beauv ex J & K Presl, *Bromus hordeaceus* L, *Cynosurus cristatus* L, *Dactylis glomerata* L, *Festuca rubra* L, *Holcus lanatus* L, *Lolium perenne* L, *Poa pratensis* L, *Poa trivialis* L, *Trisetum flavescens* (L) Beauv, *Trifolium pratense* L, *Trifolium repens* L, *Bellis perennis* L, *Carum carvi* L, *Centaurea nigra* L, *Cerastium fontanum* Baumg, *Plantago lanceolata* L, *Ranunculus bulbosus* L, *Rumex acetosa* L, *Taraxacum officinale* GH Weber ex Wiggers and *Veronica arvensis* L. The proportion of each species in the herbage samples was highly variable. Owing to the different harvest seasons (spring, summer and autumn) and the various cutting dates within each season, plants were at significantly different stages of maturity. Samples were oven dried at 60 °C and then ground to pass a 1 mm screen, to be used subsequently for chemical analyses, *in vitro* digestibility studies and NIRS.

Chemical composition

Dry matter (DM), ash and crude protein (CP = N × 6.25, N being the nitrogen content) were determined by the proximate procedures outlined by the AOAC.[20] The procedure described by Van Soest *et al*[21] was used for the analysis of neutral detergent fibre (NDF), whereas acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to the procedures proposed by Goering and Van Soest.[10] Acid detergent-insoluble nitrogen (ADIN) was determined by measuring the N content of the ADF residue by the macro-Kjeldahl technique.[10]

In vitro digestibility

The method of Tilley and Terry[12] was used to determine *in vitro* DM digestibility (DMD_{TT}) and organic matter (OM) digestibility (OMD_{TT}). *In vitro* DM digestibility was also determined according to the Goering and Van Soest[10] procedure (DMD_{GV}). All methods were performed with the modifications proposed by the ANKOM-DAISY procedure.[22]

Three mature Merino ewes fitted with permanent rumen cannulae and fed alfalfa hay were used to obtain the rumen liquor needed for the incubations just before the morning feeding. The rumen liquor was filtered through four layers of gauze in the laboratory. Previously, a culture medium was prepared with macro- and micro-mineral solutions, bicarbonate buffer and resazurin as described by Goering and Van Soest.[10] The medium was kept at 39 °C, saturated with CO_2 and reduced by the addition of a solution containing cysteine-HCl and Na_2S. Rumen fluid was diluted into the medium in the proportion 1:4 (v/v). Samples (250 mg) were weighed out into filter bags (size 5 cm × 5 cm, pore size 20 μm; ANKOM Technology, Macedon, NY, USA), which were sealed with a heater and placed in 5 l incubation jars. Each incubation jar was filled with 2 l of buffered rumen fluid dispensed anaerobically and closed with a plastic lid provided with a single-way valve which avoids the accumulation of fermentation gases. Then the jars were shaken thoroughly and placed in a revolving incubator (DAISY, ANKOM Technology) at 39 °C, with continuous rotation to facilitate the effective immersion of the bags in the rumen fluid. After 48 h of incubation in buffered rumen fluid, bags were either subjected to a 48 h pepsin-HCl digestion and incineration (DMD_{TT} and OMD_{TT})[12] or gently rinsed in cold water and treated with a neutral detergent solution at 100 °C for 1 h (DMD_{GV}).[10] Measurements were made in duplicate and standards were included in each method.

Near-infrared spectroscopy

Herbage samples were scanned at 2 nm intervals over the near-infrared (NIR) spectral range (1100–2500 nm) using an InfraAlyzer 500 spectrophotometer (Bran + Luebbe GmbH, Norderstedt, Germany). Samples were scanned twice in duplicate repacking using two different cells (four spectra per sample) and the absorbance data recorded as $\log(1/R)$, R being the reflectance. The mean spectrum was used for each sample and, finally, different mathematical treatments of the spectra based on first- or second-order derivatives were used in order to optimise the extraction of useful information. The collection of the spectra and the application of derivatives were performed using SESAME software (version 2.1, Bran + Luebbe, New York, NY, USA).

Prediction equations

Sixty-two samples (calibration set) were selected on the basis of their chemical composition for developing the prediction models. The remaining 45 samples were used as the validation set.

Calibration equations for the prediction of the chemical composition data or the *in vitro* digestibility from NIR spectra were obtained using stepwise multiple linear regression (MLR). The prediction equations derived with the calibration set were tested subsequently using the data of the samples included in the validation set. The collinearity of the absorbance data is well known, so, in order to avoid overfitting the prediction equations, the optimum model for each variable was selected on the basis of minimising the standard error of prediction (SEP) obtained for the validation set. The calibrations were performed using SESAME software (version 2.1, Bran + Luebbe).

Moreover, the different components of the mean square prediction error (MSPE) were analysed by means of the Theil decomposition:[23,24]

$$\mathrm{SEP}^2 = \mathrm{MSPE} = \frac{\sum (A-P)^2}{n} = (\overline{A}-\overline{P})^2 + (S_P - rS_A)^2 + (1-r^2)S^2{}_A$$

where n represents the number of samples in the validation set, A denotes the reference values, P denotes the NIRS-predicted values, $\overline{A}$ and $\overline{P}$ are the means of the reference and predicted values respectively, $S^2{}_A$ and $S^2{}_P$ are the variances of the reference and predicted values respectively and r is the coefficient of correlation between the reference and predicted values. Then these three terms were standardised by dividing by the total MSPE to calculate the proportion of errors due to bias (U^{M}), regression (U^{R}) and unexplained variance (U^{D}) respectively:

$$\frac{\mathrm{MSPE}}{\mathrm{MSPE}} = \frac{(\overline{A}-\overline{P})^2}{\mathrm{MSPE}} + \frac{(S_P - rS_A)^2}{\mathrm{MSPE}} + \frac{(1-r^2)S^2{}_A}{\mathrm{MSPE}} = U^{\mathrm{M}} + U^{\mathrm{R}} + U^{\mathrm{D}} = 1$$

The concordance correlation coefficient (ρ) for the validation set was calculated as defined by Lin:[24,25]

$$\rho = \frac{S^2{}_A + S^2{}_P - S^2{}_{(A-P)}}{S^2{}_A + S^2{}_P + (\overline{A}-\overline{P})^2}$$

This statistic assesses not only the linear relationship between the reference and NIRS-predicted values but also the agreement between them, assuming that the intercept is zero and the slope is one, in contrast to the Pearson correlation coefficient which does not entail these assumptions.

On the other hand, the *in vitro* digestibility was alternatively estimated using the different chemical components as independent variables. In this last case the independent variables were selected by stepwise multiple linear regression (MLR) using the SAS program.[26] Once independent variables had been selected, the UNSCRAMBLER program (version 5.03, Camo, Trondheim, Norway) was used to estimate the standard error of prediction (SEP) for the validation set to enable comparison with the NIR equations. In fact, the optimal equation for the prediction of *in vitro* digestibility was selected on the basis of minimising the SEP for the validation set.

RESULTS AND DISCUSSION

The range, mean and standard deviation (SD) of the chemical data and *in vitro* digestibility coefficients of the samples included in the calibration and validation sets are summarised in Table 1. With the exception of the acid detergent lignin (ADL) and acid detergent-insoluble nitrogen (ADIN) contents, the differences observed between the two sets in the mean and SD of each parameter were less than 10 and 25% respectively, so it could be considered that samples used to perform the NIR equations, ie the calibration set, were similar to those included in the validation set.[27,28]

NIR spectra were used to predict the chemical composition and *in vitro* digestibility coefficients of the samples studied. Statistics corresponding to the selected equations for the prediction of each parameter are shown in Table 2.

Table 3 shows the wavelengths of the NIR spectra (1100–2500 nm) that were selected for the prediction of each chemical and biological parameter.

Protein analysis

Prediction of CP and ADIN contents by NIRS technology

The coefficient of determination adjusted for the number of degrees of freedom (R^2_{adj}) corresponding to the equation selected for the estimation of the CP content was satisfactory ($R^2_{\mathrm{adj}} = 0.969$) (Table 2). Moreover, the difference between the standard error of prediction (SEP = 5.10 g kg^{-1}DM) and the standard error of calibration (SEC = 4.91 g kg^{-1}DM) was 3.9%, significantly smaller than the acceptable limit of 20% proposed by Moya.[28] In addition, the greatest

Table 1. Range, mean and standard deviation (SD) of calibration and validation sets for chemical and digestibility parameters

	Calibration set ($n = 62$)			Validation set ($n = 45$)		
	Range	Mean	SD	Range	Mean	SD
Chemical data (g kg^{-1} DM)						
Ash	61–201	93	20.5	65–167	96	22.4
CP	52–179	118	28.1	47–168	112	35.2
NDF	359–684	518	77.9	392–673	503	92.4
ADF	210–383	290	40.6	222–423	287	53.7
ADL	11–55	32	10.2	16–64	42	13.2
ADIN	0.5–4.4	2.2	1.11	0.4–5.5	2.8	1.59
Digestibility data (%)						
DMD_{TT}	63.1–88.0	79.2	7.62	63.7–87.6	79.4	7.29
OMD_{TT}	62.2–88.9	79.6	6.87	63.3–88.1	79.2	7.61
DMD_{GV}	63.7–88.1	78.7	6.24	65.0–88.7	78.6	7.53

DM = dry matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; ADIN = acid detergent-insoluble nitrogen; DMD_{TT} = *in vitro* DM digestibility according to Tilley and Terry; OMD_{TT} = *in vitro* organic matter digestibility according to Tilley and Terry; DMD_{GV} = *in vitro* DM digestibility according to Goering and Van Soest.

Table 2. NIR calibration and validation statistics for chemical and digestibility parameters

						Theil decomposition				
	Treatment	p	R^2_{adj}	SEC	SEP	U^M	U^R	U^D	RPD	ρ
Chemical data										
CP	log(1/*R*)	8	0.969	4.91	5.10	0.180	0.053	0.767	6.90	0.99
ADIN	2,2,20	4	0.861	0.41	0.59	0.043	0.103	0.855	2.70	0.92
NDF	2,6,4	7	0.975	12.32	12.75	0.034	0.059	0.907	7.24	0.99
ADF	2,6,4	5	0.957	8.46	13.62	0.106	0.003	0.890	3.94	0.97
ADL	2,2,20	4	0.655	5.97	7.18	0.298	0.027	0.676	1.84	0.83
Digestibility data										
DMD_{TT}	2,15,5	2	0.819	2.818	2.395	0.005	0.049	0.946	3.04	0.95
OMD_{TT}	log(1/*R*)	5	0.925	1.882	2.165	0.012	0.205	0.782	3.17	0.96
DMD_{GV}	log(1/*R*)	5	0.891	2.057	2.208	0.003	0.000	0.997	3.41	0.95

Treatment = mathematical transformation applied to the spectra, where the first number is the derivative order, the second number is the gap between points used to calculate the difference, and the last one is the number of data points used to smooth the data; log(1/*R*) = absorbance data without mathematical transformation; p = number of terms in the equation; R^2_{adj} = coefficient of determination adjusted for the degrees of freedom; SEC = standard error of calibration; SEP = standard error of prediction; U_M, U_R and U_D = proportion of mean square prediction error corresponding to the bias, regression and unexplained variance respectively; RPD = ratio performance deviation (standard deviation/SEP); ρ = concordance correlation coefficient; CP = crude protein; ADIN = acid detergent-insoluble nitrogen; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; DMD_{TT} = *in vitro* dry matter digestibility according to Tilley and Terry; OMD_{TT} = *in vitro* organic matter digestibility according to Tilley and Terry; DMD_{GV} = *in vitro* DM digestibility according to Goering and Van Soest.

proportion of the mean square prediction error (MSPE) was due neither to the bias nor to the regression, but to the unexplained variance (U^D). The concordance correlation coefficient ($\rho = 0.99$) is further evidence of the close similarity between the reference data and the NIRS-predicted values for the validation set. Furthermore, Williams and Sobering[29] suggested that the statistic RPD (ratio performance deviation), which is the ratio of the SD of the reference values of the validation set to the SEP, should be larger than 2.5. Lower RPD values can be attributed either to a narrow range of the reference values (giving a small SD) or to a large error in the estimation (SEP) compared with the variability of the reference values (SD). In both cases it is more difficult to attain accurate estimates of the parameters using NIR equations.[30] In this sense the equation derived in our study showed a very high ratio performance deviation (RPD = 6.90). All these statistics seemed to indicate the outstanding ability of prediction of the equation selected to estimate the CP content of botanically complex herbage samples. These results are in agreement with those reported for similar forages[14] and also for other kinds of feeds.[13,31–37] The reason for the accurate estimation of the CP content by NIRS technology is the highly significant correlation between the absorbance of the amide bonds involving nitrogen (N) and the N content measured by the Kjeldahl method.[38,39]

The acid detergent-insoluble nitrogen (ADIN) content is a parameter of considerable interest in ruminant feeding systems, because it represents the indigestible fraction of the feed protein. Likewise, the ADIN content is an indicator of the heat-damaged

Table 3. Wavelengths selected from near-infrared spectra for prediction of different chemical and *in vitro* digestibility parameters

Parameter	Selected wavelengths (nm)							
CP	1466	1678	1706	1714	1740	1786	1804	2012
ADIN	1522	2028	2252	2374				
NDF	1500	1612	1726	1756	2238	2320	2350	
ADF	1196	1512	2172	2290	2458			
ADL	1256	1396	1554	2352				
DMD_{TT}	1828	2300						
OMD_{TT}	1556	1564	1638	1672	1702			
DMD_{GV}	1254	1428	1688	1752	1924			

CP = crude protein; ADIN = acid detergent-insoluble nitrogen; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; DMD_{TT} = *in vitro* dry matter digestibility according to Tilley and Terry; OMD_{TT} = *in vitro* organic matter digestibility according to Tilley and Terry; DMD_{GV} = *in vitro* DM digestibility according to Goering and Van Soest.

protein.[40] Nevertheless, it was not so accurately predicted by NIRS technology as the CP content ($R^2_{adj} = 0.861$, SEP = 0.59 g kg^{-1} DM, RPD = 2.70, $\rho = 0.92$), probably because it would be necessary to differentiate between different types of bonds in which the N is implicated,[41] as ADIN is only a specific fraction of the total nitrogen compounds. These results were not as good as those indicated by De la Roza *et al*[42] for grass and maize silages ($R^2 = 0.954$, RPD = 3.19), probably because in that study the number of samples was larger ($n = 130$) and the forages studied were botanically more uniform.

NIR spectra interpretation

In reference to the CP and ADIN contents, the wavelengths selected at 1466 and 1522 nm (Table 3) were inside the band 1463–1570 nm, which corresponds to the N–H stretch first-overtone region.[43] On the other hand, the 2012 and 2028 nm wavelengths could be related to the amides, since these nitrogen compounds have two distinctive absorption peaks at 2050 and 2180 nm.[43,44] Moreover, the equation developed to estimate the CP content showed a lot of wavelengths selected in the band corresponding to the C–H stretch first-overtone region (1600–1800 nm), which may be related to the structural carbohydrates (NDF) present in the forages,[43] which were negatively correlated with the CP content ($r = -0.779$, $P < 0.0001$). The wavelengths selected at 2252 and 2374 nm in the equation performed to estimate the ADIN content could be related to the combination of different forms of vibration of C–H features included in the structural carbohydrates as well.[43] Anyway, all these wavelengths were very close to those previously reported for the estimation of the CP content in botanically complex samples[14,15] and diverse forages[13] and for the prediction of the ADIN content in diverse forages.[35,40]

Cell wall analysis

Prediction of NDF, ADF and ADL contents by NIRS technology

The neutral detergent fibre (NDF) content of the forages represents the non-soluble fraction of the cell wall, so it greatly influences the voluntary intake of DM.[2] In the present study the NDF content could be predicted more accurately by NIRS technology ($R^2_{adj} = 0.975$, SEP = 12.75 g kg^{-1} DM, RPD = 7.24, $\rho = 0.99$) than the CP content, despite being a very complex chemical fraction composed of different structural carbohydrates and lignin. The wide range of values shown by the population of samples (Table 1) could explain this circumstance.[30] These results are similar to those reported for maize stover,[45] *Cajanus cajan*[46] and different grasses,[47] and our prediction of NDF content from NIR spectra seemed to be slightly better than those previously outlined for herbage pastures.[14,48]

The calibration and validation statistics for the estimation of the acid detergent fibre (ADF) content ($R^2_{adj} = 0.957$, SEP = 13.62 g kg^{-1} DM, RPD = 3.94, $\rho = 0.97$) and acid detergent lignin (ADL) content ($R^2_{adj} = 0.655$, SEP = 7.18 g kg^{-1} DM, RPD = 1.84, $\rho = 0.83$) were poorer than those observed for the NDF fraction. It is well known that a low repeatability of the reference method negatively influences the prediction by NIRS technology.[49] The higher coefficient of variation (CV) between replicates obtained in the determination of the ADL ($CV_{ADL} = 7.47\%$), and to a lesser extent of the ADF ($CV_{ADF} = 1.31\%$), in comparison with that of the NDF ($CV_{NDF} = 0.86\%$) could be, in part, the reason for the lower accuracy of prediction of these chemical data by NIRS. Moreover, it must be taken into account that the lignin is not a homogeneous chemical fraction, and its chemical composition varies with the plant species and stage of maturity.[2] Anyway, the statistics obtained for the prediction of ADF and ADL contents were similar to those observed for other forages.[13,14,35,36,45–48,50,51]

NIR spectra interpretation

As can be observed (Table 3), most of the wavelengths selected for the estimation of the NDF, ADF and ADL contents were in the same regions indicated for the CP and ADIN contents, probably because of the negative correlation existing between the structural carbohydrates and the CP content. Thus it could be expected that wavelengths in the ranges

1463–1570 and 1600–1800 nm appeared in the prediction equations, as these bands seem to be related to the N–H stretch first overtone and the C–H stretch first overtone respectively.[43] Moreover, there were also wavelengths located at 1190–1260 nm (second overtone of the C–H stretch vibration) and 2200–2400 nm (C–H combination bands), all of which are most likely related to the structural carbohydrates present in the samples.[43] Most of the wavelengths selected for the estimation of the NDF, ADF and ADL contents were similar to those described previously by other authors.[13,14,35,37,40,45,51]

In vitro digestibility

Prediction of DMD_{TT}, OMD_{TT} and DMD_{GV}

As shown in Table 2, all the *in vitro* digestibility parameters (DMD_{TT}, OMD_{TT} and DMD_{GV}) could be successfully predicted by NIRS technology. Nevertheless, the R^2_{adj} and SEC were poorer for the model estimating DMD_{TT} ($R^2_{adj} = 0.819$, SEP = 2.395% DM) than for that corresponding to DMD_{GV} ($R^2_{adj} = 0.891$, SEP = 2.208% DM). Moreover, the predictive ability of the equation developed to estimate DMD_{GV} seemed to be better, judging by the higher RPD value (3.04 vs 3.41). The extraction with the neutral detergent removes bacterial cell walls and other endogenous products, so the Goering and Van Soest procedure has been considered a measure of the true digestibility, whereas the original method of Tilley and Terry is a measurement of the apparent *in vitro* digestibility.[2] In this last sense it is possible that the microbial contamination of the incubation residues influenced negatively the prediction of DMD_{TT} by NIRS technology. This fact, together with the shorter time required for the Goering and Van Soest procedure (48 vs 96 h), indicates the convenience of the utilisation of this method as reference procedure to perform the NIR equations to predict the *in vitro* DM digestibility of these kinds of forages. The results obtained in the present study were comparable to those indicated by Smith *et al*[52] to estimate DMD_{GV} of *Lolium rigidum* by NIRS technology ($R^2 = 0.94$, SEC = 3.4% DM, RPD = 3.68).

The *in vitro* organic matter digestibility (OMD_{TT}) could also be accurately predicted by NIRS ($R^2_{adj} = 0.925$, SEP = 2.165 g kg^{-1} DM, RPD = 3.17). The calibration and validation statistics for the estimation of OMD_{TT} were in agreement with those reported by Van Waes *et al*[53] ($R^2 = 0.85$, SEP = 2.05% DM) for grass and maize samples.

NIR spectra interpretation

The wavelengths selected for the estimation of *in vitro* digestibility (Table 3) were located in the same bands previously described for the chemical data. Thus most of the wavelengths were localised in the C–H stretch second-overtone band (1190–1260 nm), the N–H stretch first-overtone band (1463–1570 nm) or in that corresponding to the C–H stretch first overtone (1600–1800 nm). This is in agreement with those described previously by other authors.[35,54] Nevertheless, the wavelengths selected at 1672 and 1688 nm for the estimation of OMD_{TT} and DMD_{GV} could be related specifically to the aromatic region of the lignin.[34,43]

Relationship between chemical composition and in vitro digestibility of herbage

The NIR spectra interpretation would indicate a clear relation between the wavelengths selected for the estimation of chemical data and those concerned with the *in vitro* digestibility. Thus it seems that the *in vitro* digestibility of these forages is predicted from wavelengths of the NIR spectra related to some specific chemical components. This is in agreement with the well-known significant ($P < 0.0001$) correlation between digestibility and chemical composition (Table 4), showing positive correlation coefficients with the CP content and negative with the NDF and ADF contents.

These correlations could be due, in part, to the different stages of maturity of the plants harvested over different seasons. A higher plant maturity determined an increased stem-to-leaf ratio and a greater development of the vascular and supporting plant tissues, resulting in a steady decline of the cell contents (sugar, fructans, amino acids, some peptides, etc), which are largely digestible.[2,55] In contrast, the cell wall content and the degree of

Table 4. Coefficients of correlation (Pearson) between chemical composition and *in vitro* digestibility parameters, and statistics of prediction equations to estimate *in vitro* digestibility using chemical composition data as independent variables

	DMD_{TT}	OMD_{TT}	DMD_{GV}
Correlation coefficients			
CP	0.884***	0.899***	0.887***
ADIN	0.747***	0.758***	0.761***
NDF	−0.892***	−0.884***	−0.899***
ADF	−0.895***	−0.883***	−0.876***
ADL	0.396***	0.385***	0.412***
Multiple regression analysis for prediction of digestibility from chemical composition			
p	ADF, CP	NDF, CP	NDF, ADL, CP, ADIN
R^2_{adj}	0.835	0.848	0.874
SEC	2.67	2.65	2.20
SEP	2.37	2.59	2.37
RPD	3.07	2.93	3.18

DMD_{TT} = *in vitro* dry matter digestibility according to Tilley and Terry; OMD_{TT} = *in vitro* organic matter digestibility according to Tilley and Terry; DMD_{GV} = *in vitro* DM digestibility according to Goering and Van Soest; CP = crude protein; ADIN = acid detergent-insoluble nitrogen; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin;

*** $P < 0.0001$; *p* = terms included in the selected multiple regression equation; R^2_{adj} = coefficient of determination adjusted for the degrees of freedom; SEC = standard error of calibration; SEP = standard error of prediction; RPD = ratio performance deviation (standard deviation/SEP).

lignification of the secondary cell wall increase as plants mature.[2] Unexpectedly, in this study the digestibility coefficients were positively correlated with the ADL content (Table 4), probably as result of the large diversity in the botanical composition and differences in plant maturity of the samples studied in this work. In this sense, herbage samples harvested in spring had a higher proportion of grasses than summer and autumn regrowths, which were richer in legumes. It is well known that the degree of lignification of the cell wall is higher in legumes, but when the plants show different stages of maturity—such as the samples of the present study—this factor is not the only one which influences the microbial cell wall degradation.[2] For example, the stem-to-leaf ratio, the cuticular layer and the silica content increase as plants mature.[2,56] These changes are more pronounced for the grasses than for the legumes and all of them seem to negatively affect the digestion of forage in the rumen. Therefore mature grasses have higher cell wall content and may be less digestible than mature legumes regardless of the higher lignin content of these latter species.

When the predictive ability of the NIRS equations (Table 2) was compared with that corresponding to the chemical data (Table 4), it could be observed that the *in vitro* digestibility parameters were predicted with similar or higher accuracy using the NIR spectra as independent variables. This could probably be due to the fact that NIR spectra contain information not only about all the chemical components but also about physical properties of the samples. In this sense, the more fibrous a sample is, the coarser are the particles recovered after grinding, and information on this physical factor, along with that on the chemical composition, can be reflected in the NIR spectra.[57]

CONCLUSIONS

The calibration and validation statistics obtained in the present study showed the potential of NIRS technology to predict accurately the chemical composition of botanically complex herbage samples, particularly the CP and NDF contents. In addition, these chemical components were highly correlated with the *in vitro* digestibility of these herbage samples. Consequently, NIR spectra resulted in satisfactory prediction equations to estimate the *in vitro* digestibility of botanically complex herbage harvested from permanent mountain meadows. Nevertheless, both the accuracy of prediction by NIRS and the relationship between *in vitro* and *in vivo* digestibility could vary depending on the botanical composition of the herbage samples.[9] This limitation should be taken into account before extrapolating the results obtained in the present study to other kinds of samples.

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