

Analysis of Nitrogen Fractions in Silage by Near-Infrared Spectroscopy

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Total nitrogen, soluble nitrogen (SN), nonprotein nitrogen (NPN), and acid-detergent insoluble nitrogen (ADIN) were analyzed in grass silage by near-infrared (NIR) spectroscopy. A set of 144 samples was used to calibrate the instrument by modified partial least-squares regression, and the following statistical results were achieved: standard error of calibration (SEC) = 0.449 and square correlation coefficient (R^2) = 0.98 for total nitrogen \times 6.25, SEC = 0.425 and R^2 = 0.95 for SN \times 6.25, SEC = 0.414 and R^2 = 0.94 for NPN \times 6.25, and SEC = 0.139 and R^2 = 0.84 for ADIN \times 6.25. To validate the calibration performed, a set of 48 silage samples was used. Standard errors of prediction were 0.76, 0.64, 0.63, and 0.25 for total nitrogen, SN, NPN, and ADIN (all of them multiplied by 6.25), respectively, and R^2 for the regression of measurements by reference method versus NIR analysis were 0.94, 0.92, 0.90, and 0.48 for total nitrogen, SN, NPN, and ADIN, respectively. To compare the results obtained by NIR spectroscopy with those obtained by the reference methods for total nitrogen, SN, and NPN of the validation set, linear regression and paired *t* tests were applied, and the results were not significantly different ($p = 0.05$). When mean square prediction error analysis was applied, it could be concluded that for total nitrogen, SN, and NPN, a robust calibration model was obtained and that the main error was unexplained error. Statistical data for ADIN were worse than those of the other parameters; as a result NIR spectroscopy is not an effective method for quantitative analyses of ADIN in silage; nevertheless, it may be an acceptable method for semiquantitative evaluation.

KEYWORDS: NIR spectroscopy; nitrogen fractions; silage

INTRODUCTION

Classical analytical methods are slow and expensive and need highly qualified staff; therefore, chemical methods are not effective enough at present to cover the growing demands and low costs that are required for feed analysis. These problems can be solved by near-infrared (NIR) spectroscopy, which has been widely used for silage analysis since Norris et al. (1) began to apply this technique. Afterward, other authors continued to study the suitability of NIR spectroscopy for the analysis of the chemical composition and digestibility of silages (2–6).

Characterization of silage is important when optimal feeding regimens for ruminants are developed. Acid-detergent fiber and neutral-detergent fiber analysis by NIR are well studied and extensively used with success; however, the determination of the different nitrogen fractions needs more research. Silage may contain a wide range of low molecular weight nitrogenous products such as nonprotein nitrogen (NPN) and soluble nitrogen

(SN). The NPN is the nitrogen filtered after the protein has been precipitated with a specific reagent such as tungstic acid, which includes peptides in the protein fraction, because they are precipitated, or trichloroacetic acid, which includes the peptides in NPN, because they are not precipitated. The SN can be defined as the nitrogen soluble in a buffer solution with a pH similar to that of rumen; SN includes soluble true proteins and NPN. In addition to total nitrogen, the ratios NPN/total nitrogen and SN/total nitrogen are also of interest in animal nutrition.

The acid-detergent insoluble nitrogen (ADIN) is a portion of the total nitrogen that is bonded to the lignin of the plant cell wall; although this fraction is present even in fresh silages, its amount increases with Maillard browning reactions due to excessive drying or excessive heating during fermentation. Because the ADIN has a low biological availability (7), its content is a measurement of interest when silage quality is assessed; despite that interest, ADIN is not frequently analyzed routinely, perhaps due to the cumbersome nature of the analysis method.

The aim of our work was to study the suitability of NIR spectroscopy to analyze total nitrogen, NPN, SN, and ADIN in silages.

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EXPERIMENTAL PROCEDURES

Samples. A total of 192 different grass silages were sampled in different dairy farms in Galicia (northwestern Spain). The grass fields in Galicia are mixtures in differing proportions of Italian ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), hybrid ryegrass (*Lolium hybridum*), cocksfoot (*Dactylis glomerata*), and meadow fescue (*Festuca pratensis*) and sometimes white clover (*Trifolium repens*) or red clover (*Trifolium pratense*). The grass was harvested at various stages of maturity between flowering and fruiting; the silage was prepared in bunkers or in ~1000 kg plastic bags. The samples were divided into two groups: 144 were used for calibration, and another 48 were used to validate the performed calibration.

A 2.5 cm diameter and 1 m long probe was used to sample the silage at three different points, and ~1 kg of sample was taken. The samples were packed in plastic bags, sealed, and sent to the laboratory under refrigeration within 24 h. Samples were dried at 60–65 °C for 24–48 h in a forced-air oven, passed through a centrifugal mill (Retsch ZM 100, Haan, Germany) fitted with a 1 mm screen, and stored in polyethylene containers until analyses were carried out.

Reference Analysis. All measurements were made in duplicate.

Total nitrogen was determined according to the Kjeldahl method in accordance with the AOAC (8).

NPN, SN, and ADIN were analyzed according to the methods of Licitra et al. (9): NPN was analyzed by precipitation of true protein with tungstic acid, filtration, and determination of the insoluble nitrogen in the residue by the Kjeldahl method; NPN was calculated by subtracting tungstic acid insoluble nitrogen from total nitrogen. SN was determined by subtracting borate–phosphate buffer (pH 6.7–6.8) insoluble nitrogen from total nitrogen, both analyzed according to the Kjeldahl method. ADIN was analyzed according to the Kjeldahl method in the acid detergent fiber previously extracted with cetyl trimethylammonium bromide in sulfuric acid solution.

NIR Analysis. A wavelength scanning instrument, NIRSystems 6500 (NIRSystems, Silver Spring, MD), with a scanning range from 400 to 2500 nm and wavelength increments of 2 nm was used. Instrument checks recommended by the manufacturer were performed daily prior to use. Samples were analyzed at room temperature (~20 °C) in a small ring cup cell. Reflectance measurements of monochromatic light were made from 400 to 2498 nm. An average of 25 spectral scans was taken for each sample; data were recorded as $\log 1/R$, where R is the reflectance energy. The part of the spectra between 1108 and 2492 nm was used for calibration.

NIR Calibration. ISI software, version WINISI II, was used (10). Scatter correction was performed by standard normal variate transformation (SNV) and detrend method (11) and by multiplicative scatter correction (MSC) (12).

A general Mahalanobis distance (H statistic) was calculated from principal component analysis (PCA) scores. The H values were standardized by dividing them by the average H value of the calibration file. If a new spectrum sample differed by >3.0 standardized units from the mean spectrum of the calibration file, the sample was defined as a global H outlier, liable to give inaccurate predictions.

The calibrations were performed by modified partial least-squares (MPLS) regression (13) using first and second derivatives of the spectra (14): The first derivative of the spectra was calculated using a subtraction gap and smoothing segment of four data points. The second derivative of the spectra was calculated using a subtraction gap and smoothing segment of six data points.

The optimal number of terms for the calibration, minimizing overfitting, was based on the standard error of cross validation (SECV). The approach used was as follows: 80% of the samples from the calibration set were used for calibration, and for the remaining 20% NIR values were obtained. This operation was carried out a total of five times, each time using a different group for calibration and prediction; the SECV is the standard deviation of the differences between NIR-predicted values and the reference results. The final calibration equation was developed with the total samples of the calibration set using the number of factors with the lowest SECV.

The standard error of calibration (SEC), which is the standard deviation of the residual of the calibration set, was calculated, and the

Table 1. Mean and Range of Chemical Composition (Percent w/w) and SD of Silages in the Calibration and Validation Sets

| parameter | calibration set | | | validation set | | |
|----------------|-----------------|------------|-------|----------------|------------|-------|
| | mean | range | SD | mean | range | SD |
| total N × 6.25 | 13.05 | 6.42–21.30 | 2.924 | 13.47 | 7.60–19.83 | 3.075 |
| SN × 6.25 | 7.04 | 2.69–12.90 | 1.988 | 7.23 | 3.08–11.11 | 2.111 |
| NPN × 6.25 | 5.00 | 2.28–10.72 | 1.628 | 6.32 | 2.33–9.65 | 1.867 |
| ADIN × 6.25 | 1.26 | 0.66–2.94 | 0.389 | 1.24 | 0.66–2.13 | 0.368 |

Table 2. Repeatability Standard Deviation (Percent w/w) by Reference and NIR Methods

| method | total N × 6.25 | SN × 6.25 | NPN × 6.25 | ADIN × 6.25 |
|-----------|----------------|-----------|------------|-------------|
| reference | 0.061 | 0.054 | 0.066 | 0.034 |
| NIR | 0.141 | 0.080 | 0.078 | 0.013 |

critical T value for eliminating outliers was fixed at 2.5 ($T = \text{residual}/\text{SEC}$).

To check the calibration performed, the validation set was used. The standard error of prediction (SEP), which is the standard deviation of the residuals of the validation set, and the square correlation coefficient (R^2) of reference analyses versus NIR values were calculated. Statistical errors were calculated in accordance with Workman (15).

RESULTS AND DISCUSSION

Chemical Composition of Silages. Table 1 shows the average, standard deviation, minimum, and maximum values of silage samples of the calibration and validation groups.

The calibration set was selected with the aim of achieving a robust calibration by maximizing variability in the composition of samples and obtaining a wide range of spectra to avoid H outliers in the validation set. As a consequence, the mean value for the H statistic for the validation set of samples was 0.96 with respect to the mean value of the calibration set, and no H outliers were found. Therefore, no statistical differences were found between the spectra of the validation and calibration sets, indicating that the calibration set was wide enough. Each of the nitrogen fractions varied over a wide enough range to perform a satisfactory calibration. Moreover, calibration and validation sets cover similar ranges, and mean value and standard deviation were very close.

Repeatability. Repeatability standard deviation (Table 2) of the reference methods and of NIR spectroscopy was calculated over 20 duplicated silage samples (16). With the aim of considering the errors of sampling packaging, for NIR determination, samples were analyzed after repackaging of each duplicate.

For total nitrogen determination, a better value of repeatability was found with the Kjeldahl method; however, the value shown by NIR spectroscopy was good enough for a rapid method. NIR spectroscopy obtained satisfactory and very similar values of repeatability for SN and NPN; these values were scarcely higher than those found using the reference methods. As far as ADIN is concerned, a very low value of repeatability was found by NIR spectroscopy, almost a third of that of the reference method.

Calibration. To evaluate the different calibrations, the SEC, SECV, and R^2 of the calibration set, and the bias, SEP, R^2 , and mean square prediction error (MSPE), which is the square of the standard deviation of the residuals, of the validation set were evaluated.

In agreement with Park et al. (5) relatively small differences were found for the statistical results of calibrations, when comparing the use of SNV and detrend method with MSC for

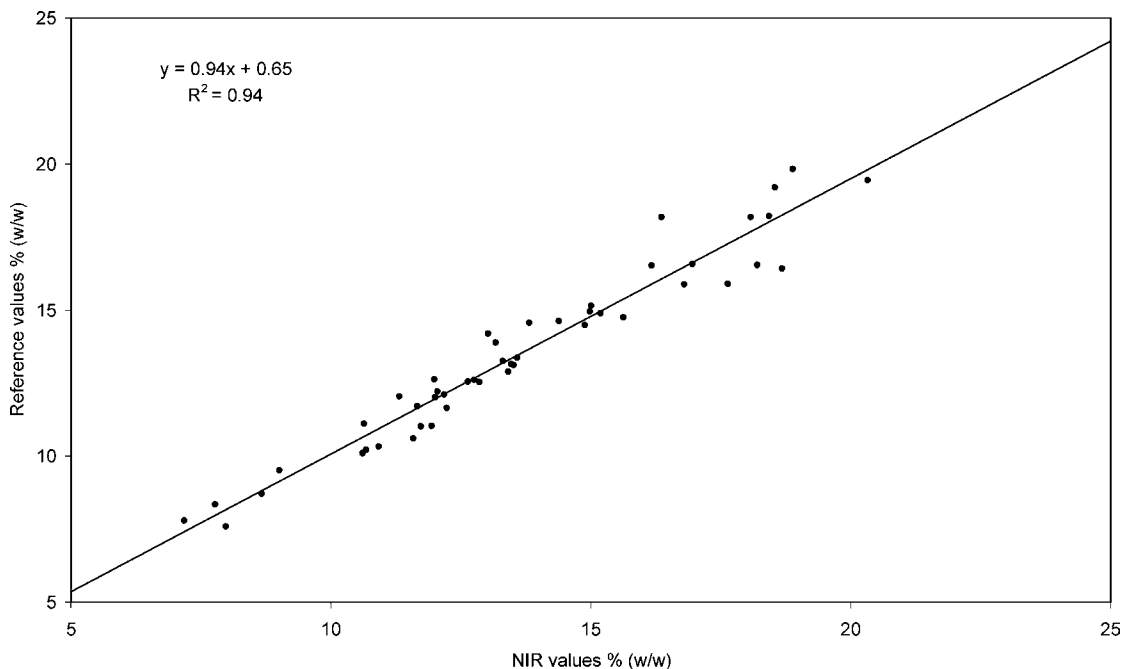


Figure 1. Validation set: total nitrogen \times 6.25; reference method versus NIR.

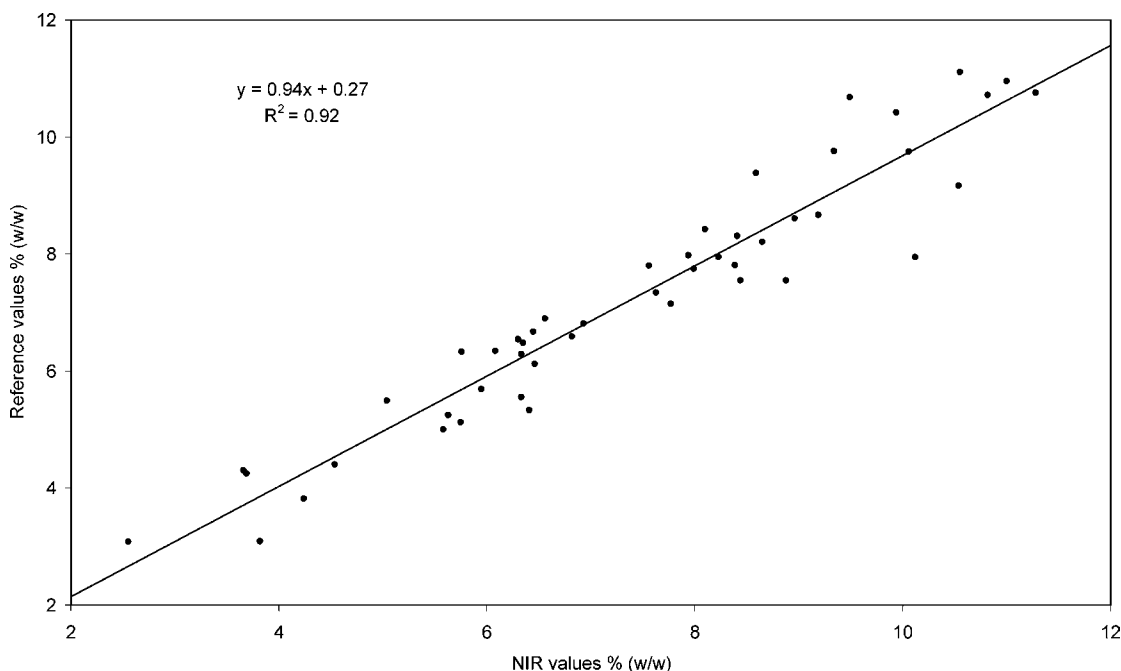


Figure 2. Validation set: SN \times 6.25; reference method versus NIR.

Table 3. Statistical Data for the Calibration Set (Percent w/w)

| component | <i>T</i> outliers | PLS terms | SEC | R^2 | SECV |
|-----------------------|-------------------|-----------|-------|-------|-------|
| total N \times 6.25 | 4 | 9 | 0.449 | 0.98 | 0.555 |
| SN \times 6.25 | 2 | 9 | 0.425 | 0.95 | 0.551 |
| NPN \times 6.25 | 3 | 9 | 0.414 | 0.94 | 0.493 |
| ADIN \times 6.25 | 6 | 10 | 0.139 | 0.84 | 0.180 |

Table 4. Statistical Data for the Validation Set (Percent w/w)

| component | bias | SEP | R^2 | SD/SEP |
|-----------------------|------|------|-------|--------|
| total N \times 6.25 | 0.13 | 0.76 | 0.94 | 4.05 |
| SN \times 6.25 | 0.16 | 0.64 | 0.92 | 3.03 |
| NPN \times 6.25 | 0.13 | 0.63 | 0.90 | 2.96 |
| ADIN \times 6.25 | 0.02 | 0.25 | 0.49 | 1.47 |

scatter correction of radiation and when using first or second derivatives of the spectra. Therefore, SNV and detrend and first derivative were chosen for the calibration of all components, with the aim of simplifying the discussion.

Table 3 shows the statistical data of the calibration set: the number of *T* outliers eliminated for each calibration and the number of partial least-squares (PLS) terms, SEC, R^2 , and SECV

values for total nitrogen, SN, NPN, and ADIN (all of them multiplied by 6.25). The number of PLS terms is not high, fewer than one per each 10 samples of the calibration set; therefore, no overfitting should be expected, which was confirmed by the low differences found between SEC and SECV. Good statistics were obtained for total nitrogen, SN, and NPN, relatively small SEC and SECV, and high R^2 , between 0.94 and 0.98.

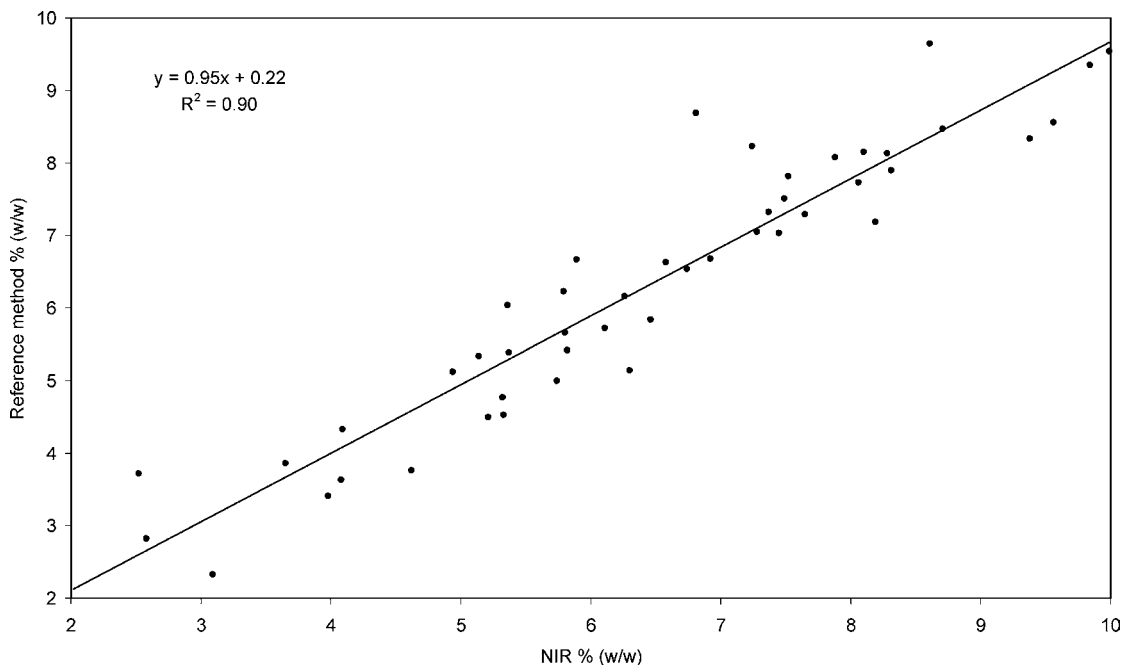


Figure 3. Validation set: NPN \times 6.25; reference method versus NIR.

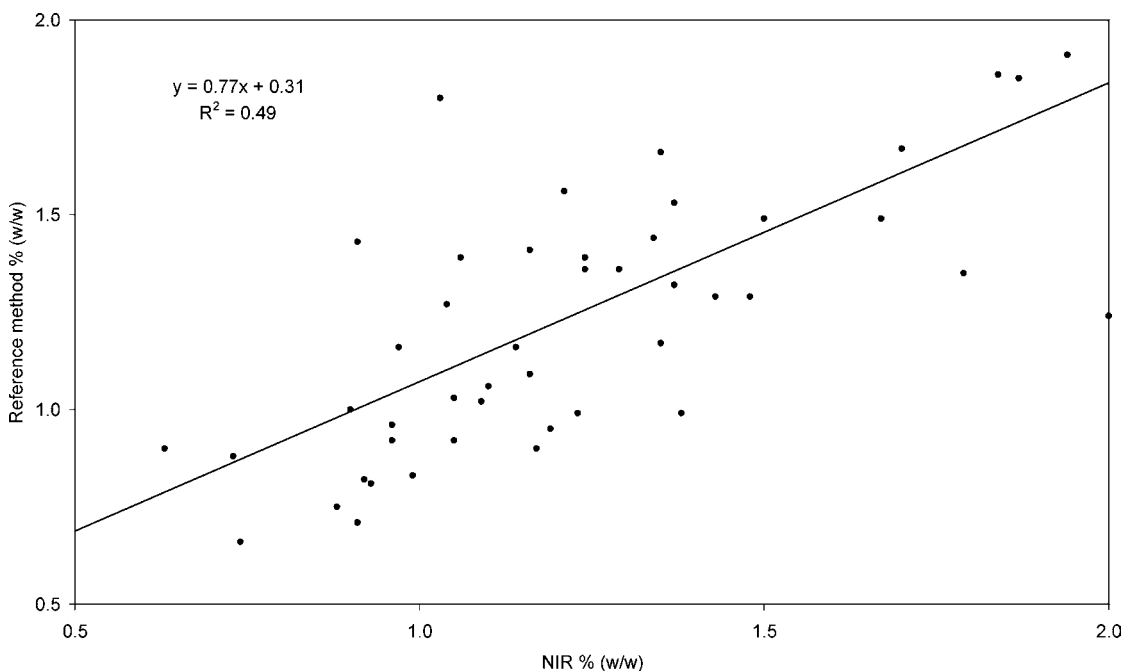


Figure 4. Validation set: ADIN \times 6.25; reference method versus NIR.

No outliers were removed from the results obtained from the validation set. Bias, SEP, R^2 , and the ratio SD/SEP of the validation set are shown in **Table 4**; acceptable values were obtained for total nitrogen, SN, and NPN.

To compare the results obtained by NIR spectroscopy for total nitrogen, SN, and NPN in the validation set with those obtained by the reference methods, the paired t test and the linear regression test were applied (16):

(i) With the paired t test one can detect if the mean of the differences of each pair of samples analyzed by the reference methods and by NIR spectroscopy differs significantly from zero. For all three components the calculated t values were lower than the theoretical t values ($p = 0.05$). Therefore, the null hypothesis was retained: the two methods did not give significantly different results.

(ii) However, when the range of concentrations is large, the linear regression test is preferred for comparing methods; therefore, we also applied this test, which is based on the regression of the results of NIR spectroscopy versus reference methods. When both methods give the same results, the theoretical equation is $y = x$. In this study, when the slope and intercept of the regression of NIR values versus reference values for total nitrogen, SN and NPN were calculated, no statistical differences ($p = 0.05$) were found from the theoretical values of 1.00 and 0.00, respectively. Therefore, for this test the two methods did not give significantly different results either. Graphic comparisons between reference values and NIR-predicted values of the validation set are shown in **Figures 1–3**.

The MSPE (**Table 5**) is the sum of three types of errors (17): errors in central tendency, errors due to regression, and

Table 5. Mean Square Prediction Error and Its Components (Percentage in Parentheses) Calculated in Accordance with Dhanoa et al. (17)

| component | MSPE | error in central tendency | error due to regression | unexplained error |
|-----------------------|--------|---------------------------|-------------------------|-------------------|
| total N \times 6.25 | 0.5742 | 0.0174 (3.0) | 0.0328 (5.7) | 0.5244 (91.3) |
| SN \times 6.25 | 0.4053 | 0.0271 (6.7) | 0.0155 (3.8) | 0.3646 (90.0) |
| NPN \times 6.25 | 0.4025 | 0.0171 (4.2) | 0.0101 (2.5) | 0.3754 (93.3) |

errors due to uncontrolled disturbance or unexplained errors. Errors in central tendency are also known as mean bias; this error is small in percentage terms for all of the components analyzed; the highest value was for SN, where the bias value was also the highest; similar values were found for total nitrogen and for NPN. The errors linked to regression will be equal to zero when the slope of regression is unity; this error also accounts for a small proportion of the MSPE for all three parameters. The low values of these two components of MSPE indicate that the calibration achieved is acceptable.

Unexplained error accounts for a high percentage of the MSPE, >90% for all three components.

In the case of ADIN, the statistical data for validation set (Table 4) were worse than those of the other parameters. The ratio of SD/SEP was 1.47, a very low value for a quantitative determination. Therefore, R^2 was also very low, and when the linear regression test was applied (16), the slope and intercept of NIR values versus reference values showed statistical differences ($p = 0.05$) from the theoretical values of 1.00 and 0.00, respectively (Figure 4). Consequently, from the results of this study, it can be stated that NIR reflectance spectroscopy is not an effective method for quantitative analysis of ADIN in silage; this fact may be due to a low sensitivity of NIR spectroscopy to the bond of protein with acid-detergent fiber and to the poor repeatability of the reference method (Table 2). However, NIR spectroscopy may be used for semiquantitative evaluation of ADIN, with a confidence interval of $\pm 0.50\%$ (w/w).

The interest in ADIN percentage in silage is not as important as the ratio (ADIN/total nitrogen) \times 100, which indicates if the silage has undergone overheating or not; it can be considered that if this value is $> 12\%$, overheating of silage has occurred, and this fact should be taken into account for feed formulation.

With the aim of verifying if the different nitrogen fractions could be predicted one from another, the correlation between each pair of parameters was calculated, and all correlations (R^2) were found to be below 0.10 with the exception of the correlation between total nitrogen and SN, for which a value of $R^2 = 0.52$ was obtained. As a result, it can be stated that NIR spectroscopy measures the different parameters investigated and does not merely measure the parameters indirectly through the correlations between total nitrogen and the other nitrogen fractions.

ABBREVIATIONS USED

NIR, near-infrared; SN, soluble nitrogen; NPN, nonprotein nitrogen; ADIN, acid-detergent insoluble nitrogen; SNV, standard normal variate transformation; MSC, multiplicative scatter correction; PCA, principal component analysis; MPLS, modified partial least-squares; SEC, standard error of calibration; SECV,

standard error of cross-validation; SEP, standard error of prediction; PLS, partial least-squares; MSPE, mean square prediction error.

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