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Verification of Silage Type Using Near-Infrared Spectroscopy Combined with Multivariate Analysis

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The ability to authenticate the feed given to animals has become a major challenge in animal production, where the diet fed to the animal is one of the most important production factors affecting the composition of milk and meat from cattle, sheep, and goats. Hence, there is currently an increased consumer demand for information on herbivore production factors and particularly the animal diet. The aim of this study was to evaluate the reliability and accuracy of near-infrared (NIR) reflectance spectroscopy as a tool to verify and authenticate the type of silage used as fed for ruminants. Grain silage (GrS, $n = 94$), grass and legume silage (GLegS, $n = 121$), and sunflower silage (SunS, $n = 50$) samples were collected from commercial farms and analyzed in the visible and NIR regions (400−2500 nm) in a monochromator instrument in reflectance. Principal component analysis (PCA), partial least-squares discriminant analysis (PLS1-DA), and linear discriminant analysis (LDA) models were used as methods to verify the different silage types. The classification models based on the NIR data correctly classified more than 90% of the silage samples according to their type. The results from this study showed that NIR spectra combined with multivariate analysis could be used as a tool to objectively authenticate silage samples used as a feed for ruminants.

KEYWORDS: Near-infrared spectroscopy; silage; identification; principal component analysis; partial least-squares discriminant analysis; linear discriminant analysis

INTRODUCTION

The new global market asks agriculture for a deep technical and operational revision aiming to improve its competitiveness (*1, 2*). Product safety, transparency of production and processing methods, and geographical characterization of typical products as well as organoleptic characteristics constitute an important added value on the market enhancing competitiveness of food products (*1, 2*). However, the industrial food production has created a distance between the consumer and food producers, resulting in the consumer-reduced confidence on the food production system and inducing an increased demand for an accurately documented history of any product in the food chain. For that reason, several producers and associations together with national and international institutions, taking into account this new expectation of public opinion, have recently prepared voluntary traceability systems for their products (*1, 2*).

The diet fed to the animal is one of the most important production factors affecting the composition of milk and meat from cattle, sheep, and goats (*3*). For example, it is well-known that sensory and nutritional properties of meat from pasture-fed lambs differ from those of concentrate-fed lambs (*4, 5*). Hence, there is currently an increased consumer demand for information on herbivore production factors and particularly the animal diet (*3*). The ability to authenticate the feed given to animals has therefore become a major challenge for the scientist, regulatory bodies, both commercial and farmer organizations, and consumers (*3*). Several methods have been proposed on the basis of chemical, physical, and DNA analysis. However, all of these methods are expensive, require sophisticated equipment, and are time-consuming when a large number of samples are analyzed. Furthermore, plant DNA could be degraded during ensiling, feed processing (e.g., heating, extrusion) (*6*).

Monitoring the quality of agro-food products is very important for economic and sanitary reasons. Depending on the particular quality characteristic being monitored and the nature of the agro-food product, this might be done either on-line, at-line, or off-line in the quality control laboratory, using a wide range of rapid instrumental techniques. Among these techniques, near-infrared (NIR) reflectance spectroscopy has emerged in the last 30 years as a rapid method for testing the quality of agricultural products and foods produced (*7, 8*). One of the advantages of NIR spectroscopy is not only to assess chemical structures through the analysis of the molecular bonds (e.g., C−H, N−H, O−H) in the near-infrared spectrum but also to build a characteristic spectrum that represents the "fingerprint" of the sample. Multivariate data analysis methods are also applied to the

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Table 1. Mean and Standard Deviation of the Chemical Composition of Silage Samples (% Dry Matter Basis)[a]

	GRS	GrLegS	SunS
DM	73.7[a] (7.93)	21.9[b] (8.2)	38.9[c] (13.6)
IVOMD	84.6[a] (4.7)	64.5[b] (7.2)	61.0[b] (6.0)
CP	7.5[a] (1.1)	12.4[b] (2.3)	13.8[b] (4.2)
ADF	9.5[a] (5.5)	39.12[b] (4.9)	37.8[b] (8.1)
pH	4.6[a] (0.6)	5.3[b] (1.1)	4.9[c] (0.9)

[a] Abbreviations: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; IVOMD, in vitro organic matter digestibility (OMD); GRS, grain silage; GrLegS, grass and legume silage; SunS, sunflower silage. Different superscript letters in the row indicate statistically significant differences ($p > 0.05$); standard deviation in parentheses.

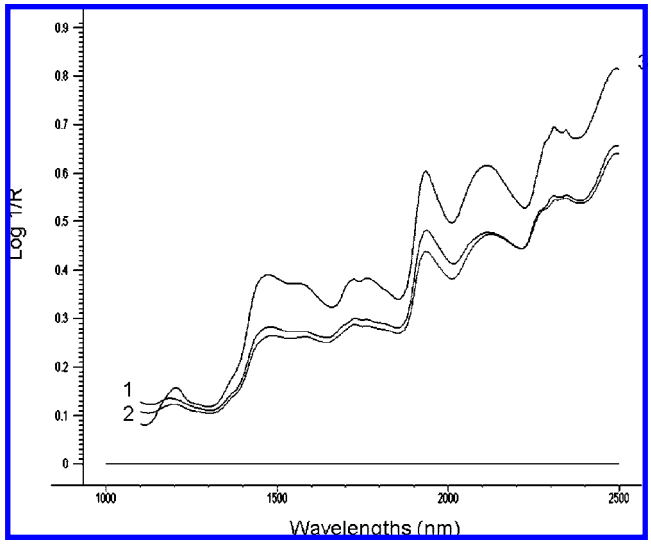

Figure 1. Near-infrared raw spectra of silage samples (1, grass and legumes; 2, sunflower; 3, grain).

acquired signals recorded from such instruments in order to detect and interpret spectra or drifts in the spectral properties of the samples related with chemical or physical characteristics. The application of mathematical operations such as principal component (PCA) or discriminant analysis (DA) provides the possibility to understand the spectral properties of the sample and classify them without the need for further chemical information (*7, 8*). The use of NIR spectroscopy has been examined to assess its suitability for this application by different authors in different types of agricultural products (*9–17*). NIR spectroscopy has been assessed for its suitability as a rapid tool to measure chemical composition and nutritive value for a broad range of silage materials (*18–24*). No reports have been found in relation to the use of NIR spectroscopy as a tool to verify or authenticate the type of silage.

The aim of this study was to evaluate the reliability and accuracy of near-infrared reflectance spectroscopy as a tool to verify and authenticate the type of silage as fed for ruminants in typical silage samples used for farmers in Uruguay (South America).

MATERIALS AND METHODS

Samples and Reference Analysis. Silage samples ($n = 268$), comprised of grain silage (GRS, $n = 94$), grass and legume silage (GrLegS, $n = 121$), and sunflower silage (SunS, $n = 50$), were collected from commercial farms during 1998−2002, representing a wide range of agronomic and soil characteristics across Uruguay and silo structures as well as different varieties and hybrids. Samples were collected directly from the farms, placed in plastic bags, frozen (−20 °C), and delivered immediately to the laboratory for further chemical and NIR analysis. Upon arrival to the laboratory, silage samples were dried in an oven at 60 °C for 48 h and ground in a Wiley forage mill to pass a 1 mm screen (Arthur H. Thomas, Philadelphia, PA). Nitrogen (N) was determined on the dried samples using a semi-micro automated Kjeldhal method (Tecator, Sweden) and converted to crude protein (CP = N × 6.25) (*25*). Acid detergent fiber (ADF) was estimated using the procedures reported elsewhere (*26*). Organic matter digestibility (OMD) was estimated using the in vitro two-stage rumen−pepsin technique with rumen fluid (48 h) followed by HCl−pepsin digestion (48 h) (*27*). Ash was determined by incinerating the dry sample at 500 °C for 4 h (*25*). Sample pH was determined on the liquid phase using a glass electrode pH meter (Orion Model 230 A). All chemical analysis was expressed on a dry weight basis (%) and analyzed in duplicate.

Near-Infrared Reflectance Analysis. Spectra were collected in the visible (vis) and near-infrared (NIR) regions in reflectance (400−2500 nm) at 2 nm intervals using a scanning monochromator, NIRSystems 6500 (NIRSystems, Silver Spring, MD). Dry samples were scanned in a small circular quartz cup (50 mm diameter) back-sealed with disposal paper. Reflectance data were stored as the reciprocal of the logarithm of reflectance, log(1/*R*). Samples were not rotated when spectra collection was made, where the spectrum of each sample was the average of 32 successive scans (1050 data points per scan). Two pairs of lead sulfide detectors collected the reflectance spectra, and the readings were referenced using a ceramic disk. Spectral data collection and manipulation were performed using NIRS 2 software, version 3.01, from Infrasoft International (ISI, Port Matilda, PA). The performance of the instrument was checked weekly using the diagnostic options provided by the instrument manufacturer. For the purpose of this study only the NIR region was used as input to develop the multivariate models.

Multivariate Analysis. Spectra were exported from the NIRS 2 software as a NSAS file for multivariate analysis. Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS1 and PLS2-DA) were performed using The Unscrambler, version 9.2 (CAMO ASA, Oslo, Norway). Principal component analysis (PCA) is a mathematical procedure for resolving sets of data into orthogonal components whose linear combinations approximate the original data to any desired degree of accuracy (*28*). PCA was used to derive the first principal components from the condensed spectral data in order to examine the natural groupings of the samples. Discrimination models were developed using partial least-squares discriminant (PLS1 and PLS2-DA) regression techniques described elsewhere (*28*). In this technique, each sample in the calibration set is assigned a dummy variable as a reference value. The PLS-DA models were developed using a nonmetric dummy variable (set to 1 = grain silage, 2 = grass silage, and 3 = sunflower silage). The criteria for classification of the samples accordingly to silage type were on the basis of the 0.5 cutoff.

Linear discriminant analysis (LDA) is a supervised classification technique where the number of categories and the samples that belong to each category are previously defined (*28*). The criterion of LDA for selection of latent variables is a maximum differentiation between the categories and minimizes the variance within categories (*28*). LDA was carried out using JMP software (version 5.01; SAS Institute Inc., Cary, NC) on the PCA sample scores on principal components (PCs) 1 to 3 which gave the highest level of separation (98% of the variance) in the PCA models developed.

Full cross-validation (CV) (*leave one out method*) was used when PCA, LDA, and PLS-DA calibration models were developed (*28*).

Statistical analysis of the chemical composition was performed by JMP statistical software (version 5.01; SAS Institute Inc., Cary, NC) with a general linear model (GLM) procedure.

Samples used for the NIR analysis were selected to represent the whole spectral and chemical variability in the target population in the calibration and validation groups, respectively. The Mahalanobis distance (*H*) was used as a criterion for selecting those samples in the population as being more variable on the basis of spectral features. The ISI (Infrasoft International, Port Matilda, PA) algorithm CENTER was used to establish population boundaries with a maximum standardized *H* distance of 3.0. Then, the algorithm SELECT was used for efficient selection, by choosing samples with a minimum standardized

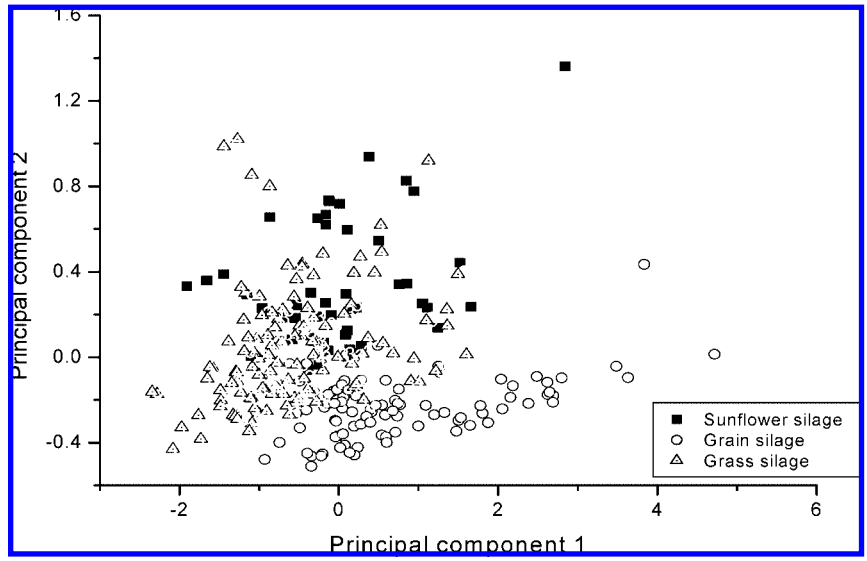

Figure 2. Score plot of the first two principal components of silage samples analyzed by NIR spectroscopy.

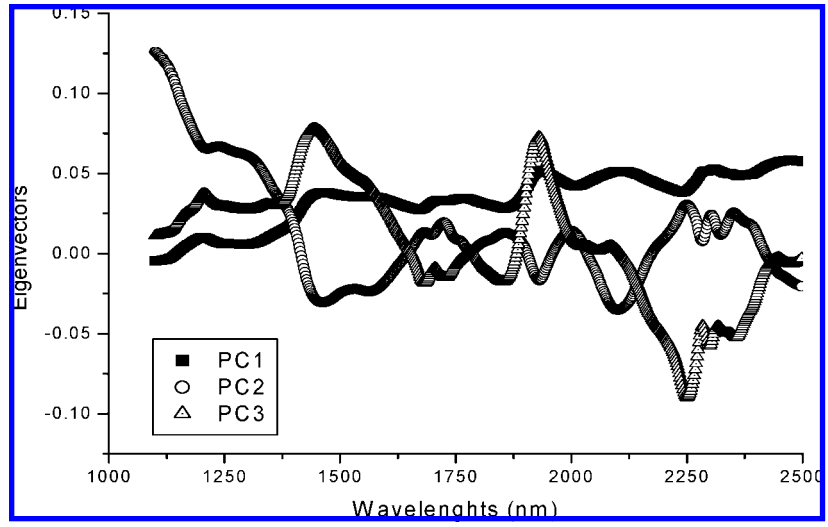

Figure 3. Eigenvectors for the first three principal components of silage samples analyzed by NIR spectroscopy.

Table 2. Partial Least-Squares Discriminant (PLS1-DA) Classification Results of Silage Samples Analyzed by NIR Spectroscopy[a]

	no. of samples correctly classified			
actual model	GRS	GrLegS	SunS	total
GRS ($n = 47$)	**42 (90%)**	0	5 (10%)	
GrLegS ($n = 60$)	0	**60 (100%)**	0	
SunS ($n = 25$)	0	2 (8%)	**23 (92%)**	
total				**125 (95%)**

[a] Abbreviations: GRS, grain silage; GrLegS, grass and legume silage; SunS, sunflower silage. Percent of correct classification in parentheses.

Table 3. Linear Discriminant Analysis (LDA) Classification Results of Silage Samples Analyzed by NIR Spectroscopy[a]

	no. of samples correctly classified			
actual model	GRS	GrLegS	SunS	total
GRS ($n = 47$)	**45 (95%)**	0	2 (4%)	
GrLegS ($n = 60$)	0	**48 (80%)**	12 (20%)	
SunS ($n = 25$)	1 (4%)	4 (16%)	**25 (80%)**	
total				**110 (89%)**

[a] Abbreviations: GRS, grain silage; GrLegS, grass and legume silage; SunS, sunflower silage. Percent of correct classification in parentheses.

H distance of 0.6 from their nearest neighbors (*29*). Neither scatter correction nor mathematical treatments were used to perform the classification models.

RESULTS AND DISCUSSION

Table 1 shows the mean and standard deviation for the chemical composition of the silage samples analyzed. Statistically significant differences were observed between the silage types in dry matter (DM) content and pH. No statistically significant differences between GrLegS and SunS were observed for crude protein (CP) and acid detergent fiber (ADF) content and in vitro organic matter digestibility (OMD). These data suggested that GrLegS and SunS samples were similar in terms of chemical composition, compared with the GRS samples.

The typical spectra of GRS, GrLegS, and SunS silage samples in the NIR region are shown in **Figure 1**. Visual differences were observed between the spectra of the different silage samples analyzed in the NIR region around 1460 nm and at 1960 nm related with OH second and first OH stretch overtones (water content). Differences were also observed around 1200 nm, related with CH second overtone, at 1738 nm with CH_2 stretch first overtone related with oil (sunflower and corn grain) and at 2310 nm with CH combinations associated with oil content in the seeds (*8, 30, 31*).

Table 4. Partial Least-Squares Discriminant (PLSI-DA) Classification Results of Silage Samples Analyzed by Chemical Methods[a]

	no. of samples correctly classified			
actual model	GRS	GrLegS	SunS	total
GRS ($n = 47$)	**39 (82%)**	0	8 (17%)	
GrLegS ($n = 60$)	0	**48 (80%)**	12 (20%)	
SunS ($n = 25$)	0	4 (16%)	**21 (84%)**	
total				**108 (81%)**

[a] Abbreviations: GRS, grain silage; GrLegS, grass and legume silage; SunS, sunflower silage. Percent of correct classification in parentheses.

Figures 2 and **3** showed the score plot and eigenvectors for the PCA analysis, respectively. The score plots showed a clear separation between GRS and the other two silage types (**Figure 2**). The eigenvectors from the PCA showed a shape similar to the mean spectrum in PC1 (**Figure 3**). PC1 explains 68% of the total variance in the samples. The highest loadings on PC1 were found around 1400 and 1980 nm (water content). PC2 explains 19% while PC3 explains 8% of the total variance. The highest loadings on PC2 were found around 1500 nm (water), 1700 nm (fatty acids and oil content), 1930 nm (water), and around 2300 nm (saturated and unsaturated fatty acids). Spectral bands between 2200 and 2300 nm were related to unsaturated ═C—H and C═C groups, which suggests differences in fatty acids from corn and sunflower seeds (*8, 30, 31*).

The PLS1-DA models were developed using the NIR raw spectra. The coefficient of determination (R^2) and the root mean square of the standard error of cross-validation (RMSECV) for the PLS1-DA calibration model were 0.94 and 0.21 (nine PLS latent variables), respectively. The calibration statistics indicated that the model developed could be acceptable to classify new samples. **Table 2** shows the PLS1-DA classification rates (percent of classification) for the validation set according to silage type. The PLS1-DA models produce an overall rate of correct classification of 95%. The PLS2-DA models produce an overall rate of correct classification of 100% (data not shown).

Table 3 shows the LDA classification rates according to silage type based on the first three PCs scores from PCA, which account for 98% of the variance in the NIR spectra. An overall rate of 89% of correct classification was achieved using LDA. Overall, the two classification methods used in this study (PLS1-DA and LDA) achieved correct classification rates between 89% and 95%.

In order to compare the LDA and PLS1-DA results obtained with the NIR spectra, silage samples were also classified using the chemical data. **Table 4** shows the results of classification using PLS1-DA. The overall classification rate using the chemical data was 82% compared to 95% using NIR spectra. Although it can be argued that only DM, ADF, CP, OMD, and pH were measured in the set of silage samples analyzed, the results suggested that the NIR spectra contains relevant information to allow the discrimination between samples according to silage type.

It is well-known that spectroscopic techniques as generally applied to authenticity issues are nonselective. In general, spectra contain information about the complete composition and physical state of the material under analysis and yield structural information that constitutes the fingerprint of a sample (*10–13*). The ability of a NIR model to discriminate or identify similar or different individuals in a population is based on the vibrational responses of chemical bonds to NIR radiation. Therefore, it is probable that the higher the variability in these chemical entities (e.g., protein, oil, dry matter), which respond to this range of electromagnetic spectrum, the better the accuracy of the model can be (*32*). Although the NIR method presented here is qualitative in nature, it avoids the need for a further quantitative method that would require the use of expensive and tedious chemical procedures. Some factors such as the number of samples used to build the calibration models and the similarities between some silage samples due to similar plant structure analyzed (stems, seeds, leaves) or chemical characteristics, however, limit the precision of the classification models. The results of this study suggest that NIR spectroscopy coupled with multivariate methods holds the necessary information for a successful classification of silage samples of different types.

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LITERATURE CITED

(1) Hobbs, J. E.; Bailey, D. V.; Dickinson, D. L.; Haghiri, M. Traceability in the Canadian red meat sector: Do consumers care? *Can. J. Agric. Econ.* **2005**, *53*, 47–65.
(2) Arhurst, P. R.; Dennis, M. J. *Food Authentication*; Chapman-Hall: London, 1996; 315 pp.
(3) Prache, S. Developing a diet authentication system from the composition of milk and meat in sheep: a review. *J. Agric. Sci.* **2007**, *145*, 435–444.
(4) Prache, S.; Cornu, A.; Berdague, J. L.; Priolo, A. Traceability of animal feeding diet in the meat and mil of small ruminants. *Small Rum. Res.* **2005**, *59*, 157–168.
(5) Priolo, A.; Lanza, M.; Barbagallo, D.; Finocchiaro, L.; Biondi, L. Can the reflectance spectrum be used to trace grass feeding in ewe milk? *Small Rum. Res.* **2003**, *48*, 103–107.
(6) Flachowsky, G. Feeds from genetically modified plants (GMP) in animal nutrition. *Tieraerztl. Umsch.* **2006**, *61*, 591–600.
(7) Deaville, E. R.; Flinn, P. C. Near infrared (NIR) spectroscopy: an alternative approach for the estimation of forage quality and voluntary intake. In *Forage evaluation in ruminant nutrition*; Givens, D. I., Owen, E., Axford, R. F. E., Omed, H. M., Eds.; CABI Publishing: Wallingford, U.K., 2000; pp 301–320.
(8) Osborne, B. G.; Fearn, T.; Hindle, P. H. *Practical NIR spectroscopy with application in food and beverage analysis*, 2nd ed.; Longman: Essex, England, 1993.
(9) Downey, G. Qualitative analysis in the near infrared region. *Analyst* **1994**, *119*, 2367–2375.
(10) Downey, G. Authentication of food and food ingredients by near infrared spectroscopy. *J. Near Infrared Spectrosc.* **1996**, *4*, 47–61.
(11) Downey, G. Food and food ingredient authentication by mid-infrared spectroscopy and chemometrics. *Trends Anal. Chem.* **1998**, *17*, 418–424.
(12) Downey, G. Discrimination strategies in food authentication: what is the correct question to ask? *NIR News* **2000**, *11*, 8–9.
(13) Cordella, Ch.; Moussa, I.; Martel, A.-C.; Sbirrazzuoli, N.; Lizzani-Cuvelier, L. Recent developments in food characterization and adulteration detection: technique-oriented perspectives. *J. Agric. Food Chem.* **2002**, *50*, 1751–1764.
(14) Cozzolino, D.; De Mattos, D.; Vaz Martins, D. Visible/near infrared reflectance spectroscopy for predicting composition and tracing system production of beef muscle. *Anim. Sci.* **2002**, *74*, 477–484.
(15) Downey, G.; McElhinney, J.; Fearn, T. Species identification in selected raw homogenised meats by reflectance spectroscopy in the mid-infrared, near-infrared and visible ranges. *Appl. Spectrosc.* **2000**, *54*, 894–899.
(16) Murray, I.; Aucott, L.; Pike, I. H. Use of discriminant analysis on visible and near infrared reflectance spectra to detect adulteration of fishmeal with meat and bone meal. *J. Near Infrared Spectrosc.* **2001**, *9*, 297–311.
(17) De la Haba, M. J.; Garrido-Varo, A.; Perez-Marin, D. C.; Guerrero, J. E. Near infrared analysis as a first line screening technique for

identifying animal species in rendered animal by products. *J. Near Infrared Spectrosc.* **2007**, *15*, 237–245.
(18) Norris, K. H.; Barnes, R. F.; Moore, J. E.; Shenk, J. S. Predicting forage quality by infrared reflectance spectroscopy. *J. Anim. Sci.* **1976**, *43*, 889–897.
(19) Reeves, J. B.; Blosser, T. H.; Colenbrander, V. F. Near infrared reflectance spectroscopy for analysing undried silage. *J. Dairy Sci.* **1989**, *72*, 79–88.
(20) De Boever, J. L.; Cottyn, B. G.; De Brabander, D. L.; Vanacker, J. M.; Boucque, Ch. V. Prediction of the feeding value of grass silage by chemical parameters, in vitro digestibility and near infrared reflectance spectroscopy. *Anim. Feed Sci. Technol.* **1996**, *60*, 103–115.
(21) Deaville, E. R.; Givens, D. J. Regions of normalised near infrared reflectance spectra related to the rumen degradation of fresh grass, grass silage and maize silage. *Anim. Feed Sci. Technol.* **1998**, *72*, 41–51.
(22) Nousiainen, J.; Ahvenjärvi, S.; Rinne, M.; Hellämäki, M.; Huhtanen, P. Prediction of indigestible cell wall fractions of grass silage by near infrared reflectance spectroscopy. *Anim. Feed Sci. Technol.* **2004**, *115*, 295–311.
(23) Park, P. S.; Agnew, R. E.; Gordon, F. J.; Steen, R. W. J. The use of near infrared reflectance spectroscopy (NIRS) on undried samples of grass silage to predict chemical composition and digestibility parameters. *Anim. Feed Sci. Technol.* **1997**, *72*, 155–167.
(24) Givens, D. I.; Deaville, E. R. The current and future role of near infrared reflectance spectroscopy in animal nutrition: a review. *Aust. J. Agric. Res.* **1999**, *50*, 1131–1145.
(25) AOAC. *Association of Official Analytical Chemist*, Washington, DC, 1995.
(26) Robertson, J. B.; Van Soest, P. J. The detergent system of analysis and its application to human foods. In *The Analysis of Dietary Fibre Food*; James, W. P. T., Theander, O., Eds.; Marcel Dekker: Basel, 1981; pp 123–158.
(27) Tilley, J. M. A.; Terry, R. A. A two-stage technique for the in vitro digestion of forage crops. *J. Br. Grass Soc.* **1963**, *18*, 104–111.
(28) Naes, T.; Isaksson, T.; Fearn, T.; Davies, T. *A User-friendly guide to multivariate calibration and classification*; NIR Publications: Chichester, U.K., 2002; p 420.
(29) Shenk, J. S.; Westerhaus, M. O. Analysis of agriculture and food products by near infrared reflectance spectroscopy. *Monograph*; Infrasoft International: Port Matilda, PA, 1993.
(30) Murray, I. The NIR spectra of homologous series of organic compounds. In *Proceedings of the International NIR/NIT Conference*; Hollo, J., Kaffka, K. J., Gonczy, J. L., Eds.; Akademiai Kiado: Budapest, 1986; pp 13–28.
(31) Miller, Ch. E. Chemical principles of near infrared technology. In *Near infrared technology in the agricultural and food industries*, 2nd ed.; Williams, P. C., Norris, K. H., Eds.; American Association of Cereal Chemist: USA, 2001; pp 19–39.
(32) Alomar, D.; Gallo, C.; Castaneda, M.; Fuchslocher, R. Chemical and discriminant analysis of bovine meat by near infrared reflectance spectroscopy (NIRS). *Meat Sci.* **2003**, *63*, 441–450.

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