Master's Thesis of Science in Agriculture

Development of Calibration for Predicting Feed Value of Imported Forage using Near Infrared Reflectance Spectroscopy (NIRS)

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Development of Calibration for Predicting Feed Value of Imported Forage using Near Infrared Reflectance Spectroscopy (NIRS)

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Abstract

Currently, Korea is heavily reliant on imports for forages. However, quality analysis is not performed while forage is imported and distributed to the final purchaser. The reason is that the quality analysis of forage is not legally compulsory, and the analysis procedure is complex, time-consuming, and expensive. Therefore, this study was conducted to develop the calibration equation for a rapid and inexpensive quality analysis for imported hays using near infrared reflectance spectroscopy (NIRS). The representative samples of alfalfa hay (227) and timothy hay (360) were collected nationwide. Furthermore, this study compared sample of intact form (cut into 10 cm) with that of ground form to check the possibility of omitting the drying and grinding process. The partial least squares (PLS) was used as a regression method and several mathematical treatments were conducted to develop more accurate calibration equations. The standard error of cross validation (SECV) was used as an evaluation index of the calibration equation, and the coefficient of determination (R^2) was used as an index of the correlation between reference values and NIRS prediction values.

The R^2 values of moisture content, acid detergent fiber (ADF), neutral detergent fiber (NDF), crude protein (CP), *in vitro* dry matter digestibility (IVDMD), total digestible nutrients (TDN) and relative feed value (RFV) on intact alfalfa hay were 0.74, 0.42, 0.68, 0.89, 0.52, 0.68 and 0.54, respectively. The R^2 values of moisture content, ADF, NDF, CP, IVDMD, TDN and RFV on ground alfalfa hay were 0.84, 0.90, 0.97, 0.92, 0.95, 0.97 and 0.93, respectively. The R^2 values of moisture content, ADF, NDF, CP, IVDMD, TDN and RFV on intact timothy hay were 0.48, 0.95, 0.75, 0.38, 0.61, 0.75 and 0.61, respectively. The R^2 values of moisture content, ADF, NDF, CP, IVDMD, TDN and RFV on ground timothy hay were 0.95, 0.98, 0.94, 0.94, 0.84, 0.94 and 0.96, respectively. Intact alfalfa hay had no trend on derivative. However, ground alfalfa hay had the best results on the fourth derivative with the exception of moisture and NDF contents which had the best results on the second and first derivative, respectively. Intact timothy hay had the best results of calibration on the first derivative with the exception of moisture and CP contents which had the best results in the third derivative. However, ground timothy hay had no trend on derivative.

The results of the development of the calibration equations were good and can be used in field. The results of intact form were worse than those of ground form. However, intact form scanning method has a merit which drying and grinding processes can be omitted. Therefore, intact form scanning method can be used in field for quick and rough prediction rather than exact prediction. The calibration results of intact alfalfa hay in this study were poorer when compared to previous reports, most likely because the leaf part of the alfalfa hay was powdered and covered in the scanning cup. This problem could be overcome if the hay is firmly fixed and scanned.

Keyword : NIRS, alfalfa, timothy, imported hay, calibration
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List of Abbreviations

ADF: Acid detergent fiber
CP: Crude protein
DDM: Digestible dry matter
DM: Dry matter
DMI: Dry matter intake
IVDMD: *In vitro* dry matter digestibility
ND: Neighborhood distance
NDF: Neutral detergent fiber
NFTA: National forage testing association
NIR: Near infrared reflectance
NIRS: Near infrared reflectance spectroscopy
PCR: Principal component regression
PLS: Partial least squares
SD: Standard deviation
SE: Standard error
SEC: Standard error of calibration
SECV: Standard error of cross validation
SEL: Standard error of laboratory
SEP: Standard error of prediction
SNV: Standard normal variate
TDN: Total digestible nutrients
R^2: Coeffiecient of determination
R^2CV: Coeffiecient of determination of cross validation
RFV: Relative feed value

1. Introduction

1.1 Study Background

Forage production is limited in Korea because of the lack of arable land. In 2020, the amount of forage required was 5,010,982 t, and domestic forage cultivated was 3,923,000 t in the same year (MAFRA, 2021). This shortage is met by imported hay. The amount of hay imported in 2020 was 1,087,982 t, accounting for 21.71 % of the total forage requirement. Hay was mainly imported from the United States, Australia, Canada and Spain, accounting for 74.17 %, 17.64 %, 3.96 % and 2.18 % of total imported hay, respectively. Alfalfa, timothy and tall fescue are the most commonly imported forages. Importation quantity of these forages is estimated at 226,243 t for tall fescue straw, 208,134 t for alfalfa hay and 183,845 t for timothy hay, accounting for 20.79, 19.13 and 16.90 % of the total import volume, respectively (APQA, 2021).

Imported hay is mainly consumed for dairy farming, which requires high quality forage. This is because dairy farmers were willing to purchase relatively expensive imported forage for high-quality milk production. Currently, however, both dairy and beef farms in Korea rely heavily on imported forage. That is why the straws from tall fescue, bluegrass, annual ryegrass, wheat and barley are imported in huge quantities. Imported straw totaled 455,785 t in 2020, accounting for 41.89 % of total imported forage (APQA, 2021). This means that livestock farmers in Korea do not just want high-quality forage. Unlike forage cultivated in Korea, which is mainly stored in the form of silage with high moisture content, the imported forage is supplied in standardized bales with low moisture content. For livestock farms in Korea, which have recently become large-scale, such imported forage is a great attraction. Furthermore, imported forage maintains a constant quality and is convenient to use. Therefore, it is a preferred option for both livestock farmers and total mixed ration (TMR) factories.

Problems arise in the distribution process of imported hay. The nutritional analysis of imported hay is not enacted by law; therefore, some sellers and customers trade without knowing the exact nutritional value of imported hay. Others trade the imported hay relying on reports issued by producers. Imported forage is traded from the ship to the final consumer without any nutritional evaluation. For this reason, the quality of imported hay tends to be evaluated by its appearance such as its color, odor, and toughness. There should be a price that everyone in the market can agree on for proper trade and the development of the livestock industry. Therefore, the forage quality analysis should be systematized to form a reasonable and reliable price according to its nutritional value.

This problem was raised 50 years ago in USA. The potential of using near infrared reflectance spectroscopy (NIRS) to estimate forage quality was first suggested in 1975 at a workshop, cosponsored by United States Department of Agriculture – the Agricultural Research Service (USDA-ARS) and the American Forage and Grassland Council (AFGC). Then the National NIRS Forage Research Project Network was approved for funding in 1979. As a result of 10 years of research, researchers introduced the method of estimating forage quality using the NIRS (Marten et al., 1989). Currently, forage producers in US request NIRS analysis to laboratory certified by the National Forage Testing Association (NFTA), and trade forage based on the results.

Most of the quality evaluation of forage in Korea is performed by wet analysis in laboratories. However, this technique is both expensive and time consuming, which make it unsuitable for the routine evaluation of forage quality. Therefore, both livestock farmers and the livestock market are demanding a way to quickly reveal the nutritional value of forage. Currently, in the United States, hay producers are providing consumers with analysis results from NFTA certified laboratories. Although the analysis requester has the option of choosing between wet analysis and NIRS analysis, NIRS analysis is more commonly used due to its low cost and quick analysis time. Forage quality analysis through NIRS is periodically verified by the NFTA for the accuracy of the analysis, and the forage quality report becomes effective only after verification by the NFTA.

Livestock farming in Korea is becoming larger and the demand for imported forage is growing. If the size of imported forage increases and the price rises, importers must negotiate the price based on accurate quality, and smart consumers must make better choices for their livestock. For these reasons, technology must be developed and universalized, and NIRS is at the forefront of this effort.

1.2 Aims of research

The purpose of this study is to obtain calibrations to predict quality of imported legume and grass. The constituents to develop calibration equations are moisture content, crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), *in vitro* dry matter digestibility (IVDMD), total digestible nutrients (TDN) and relative feed value (RFV). The difference in the calibration equation according to pretreatments (intact and ground) of sample will be evaluated. Various mathematical treatments will be performed to develop the best calibration equation on each constituent. Finally, the study will determine the possibility of calibration of intact and ground forage for field use.

2. Literature review

2.1 The definition of Near-Infrared Reflectance Spectroscopy (NIRS)

Near-infrared ray is the ray between visible and mid-infrared ray, meaning a wavelength between 700 nm and 2,500 nm. In the case of near-infrared rays, as the wavelength decreases, the pathlength and penetration depth increase, and the absorptivity decreases. The absorption of near infrared rays is higher than that of visible light and lower than that of infrared rays because absorption of visible rays and ultraviolet rays is mainly caused by outer electrons, whereas absorption of infrared rays is caused by molecular vibrations. Absorption of the near-infrared spectrum appears mainly as a combination of vibration and overtone vibration of molecular vibration energy due to fundamental absorption of functional groups such as C-H, N-H, O-H and C=O occurring in the mid-infrared region (Williams and Norris, 1987).

Spectroscopy is a field of optics that studies the structure, qualitative and quantitative analysis of the material by measuring and analyzing the spectrum of light absorption and radiation by a material using a spectrometer (Burns and Ciurczak, 2007).

NIRS is a relative evaluation method that measures with the calibration curve obtained using the standard sample like general spectroscopy. The characteristic of NIRS is that it uses a continuous spectrum based on the change in inter-molecular vibration energy according to the absorbance of light energy (Williams and Norris, 1987). As a result, unlike general spectroscopy, this spectrum may be used to quickly study organic molecules with multiple components at the same time.

2.2 The history of NIRS

The discovery of near-infrared energy is ascribed to William Herschel in the 19th century, and the first reports of NIRS appeared in the literature in 1939 by Gordy and Martin. Ben-Gera and Norris (1968) applied NIRS to the analysis of agricultural products and recognized the potential to diffuse reflectance measurements in the NIR region for rapid analysis of fat and moisture of meat products. Norris et al. (1976) demonstrated new applications for NIRS. They showed that forages could be analyzed by NIRS for quality constituents, including sheep digestion and intake measurements. NIRS was mostly used to analyze the moisture, oil, and protein component of agricultural products (Watson, 1977). The National NIRS Forage Research Project Network has been accelerating the use of NIRS in the field of forage research since 1978. The scope of the NIRS grew even larger when its potential in the field of forage became acknowledged. Clark et al. (1987) have studied about mineral analysis using NIRS and Park et al. (1983) used NIRS technique for predicting carotene content in baled alfalfa hay. NIRS is now utilized to assess not just the forage quality, but also the quality of fermentation (Park et al., 2015a) and whether it is infected with bacteria (Tallada et al., 2011). Furthermore, the portable NIRS has been developed so that its usability is further expanded.

The study of NIRS started on 1990 in Korea. Kim et al. (1990) studied about the hydrogen bonding using NIRS. Thereafter, Oh and Grossklaus (1992) studied about food using NIRS. The use of NIRS in agricultural studies was for the first time by Kim et al. (1995). The authors determined components of barley grain using NIRS. In the field of grassland and forage, Lee et al. (2001) analyzed the feed value of corn silage using NIRS for the first time in Korea. The authors reported that the coefficient of determination (R^2) of calibration on dry matter (DM), NDF, ADF, ADF, CP, hemicellulose and TDN were 0.84, 0.94, 0.91, 0.87, 0.95, 0.86 and 0.92, respectively. The results were similar with those of former researches. Following their study, studies were performed to measure feed value of domestic silage forage, and to confirm the distribution of forage in grassland using NIRS (Park et al., 2006; Park et al., 2015b).

2.3 The process of obtaining calibration

2.3.1 Sample collection and analysis

Proper sampling is essential for any chemical technique in order to achieve an aliquot for analysis that properly represents the composition of the larger sample of interest. Dried samples require tumbling or thorough mixing to minimize the effects of repack error. Repack error is measured as the differences between sample spectral measurements of several aliquots of a sample (Abrams, 1989). The samples used to develop the calibration equations should be representative of the group and have a wide and even distribution.

To determine the protein content of forage, Kjeldahl N determination (AOAC, 1995) is the most practiced. But Shenk and Westerhaus (1994) recommended the combustion method of Dumas (1831) rather than the Kjeldahl N determination because the multiple step of analyzing in Kjeldahl N procedure can cause errors. Therefore, they concluded that the combustion method is a more reliable and suitable method for development of the calibration for NIRS.

2.3.2 Measurement of spectra and mathematical treatment

The same device used to develop the calibration must be used on the host instrument and different instrument have different sample viewing devices. The measurement method differs depending on the type (solid or liquid) and size of the sample. The small cup is used for finely ground materials and large cup is used for unground or high-moisture materials (Shenk and Westerhaus, 1994).

The characteristics of the absorbance spectrum of near-infrared rays are that the peaks are wide and overlapping, and the baseline is changed due to the scattering difference. These features contribute to a large number of analytical errors, which can be corrected and stabilized through mathematical treatment. Barnes et al. (1989) introduced standard normal variate transfromation (SNV) and detrending for chemometrics to normalize a spectra collection and remove the baseline effect. The standard normal variate approach effectively removes the multiplicative interferences of scatter and particle size. De-trending accounts for the variation in baseline shift and curvilinearity, generally found in the reflectance spectra of powdered or densely packed samples, with the use of a second-degree polynomial regression. NIR diffuse reflectance spectra transposed by these methods are free from multi-collinearity and are not confused by the complexity of shape encountered with the use of derivative spectroscopy.

Derivative method can remove the baseline change by differentiating the spectrum to emphasize the change in the absorbance spectrum. Norris et al. (1976) developed calibrations for digestibility, fiber, and crude protein using the derivative method and reported that the second derivative of the spectra was the best for analyzing the fibrous components and minerals. Hruschka (1987) also reported the effect of derivative method in mathematical treatment to develop NIRS calibration. The gap of the derivative of the spectrum can be set in nm, and the derivative is applied as the average of the gap values. The larger the particle size of samples, the more extraneous signals can be seen in the spectrum. If the gap value of the derivative is set higher, the effect of removing extraneous signals can be seen in the spectrum (Davies, 2007). In the NIRS, the smoothing function for derivative exists. The smoothing method fits a curve through a small section of the spectrum and then finds the slope of the tangent to this curve at the central point (Savitzky and Golay, 1964). The number of smoothing points can be chosen and it can be helpful for removing the multiplicative effects of light scattering. This derivative method is expressed as derivative order, derivative gap, derivative smooth such as (1,8,8).

2.3.3 Calibration development

The calibration measures the absorbance for each wavelength of near-infrared light in the sample, calculates the correlation between the absorbance and the lab data as a linear equation using regression analysis, and predicts the lab data value using this equation from the absorbance of an unknown sample. The spectral data produced by an NIR instrument represent the total chemical and physical properties of a sample. Calibration is the term used to describe the conversion of NIR absorption information into laboratory reference method. The accuracy of this conversion is measured as the standard error of calibration (SEC) and the standard error of prediction (SEP).

Error terms used in NIR analysis (Marten et al., 1984):

The standard error of laboratory (SEL) can be estimated from the standard error of difference between blind duplicate measurements L_1 and L_2 using the reference method:

$$SEL = \sqrt{\frac{\sum(L_1 - L_2)}{n}}$$

n = number of samples

The standard error of calibration (SEC), which is one of calibration results is defined as:

$$SEC = \sqrt{\frac{\sum(L - M)^2}{n - 1 - p}}$$

L = laboratory reference

M = NIR measured value

n = number of samples

p = number of terms in the calibration equation

One of the most difficult decisions in calibration is determining how many terms are required. The few terms cause the accuracy to decrease while too many terms are associated with occurance of overfitting. The calibration is specific to the spectra in the calibration file and performs poorly on other spectra. A good number of terms can be determined in 4 ways (Shenk and Westerhaus, 1994):

1. A general guideline is to fit one term for every 10 samples.
2. Cross validation estimates prediction error by splitting the calibration samples into groups. One group is reserved for validation and the remaining groups are used for calibration. The process is repeated until all groups have been used for validation once.

3. External validation measures the accuracy of the calibration equation on samples not used in the calibration.
4. Equation repeatability measures the consistency of repeated NIRS analyses on the same sample.

And the coefficient of determination (R^2) is used as a index for calibration result as well as SEC. R^2 is defined by Wright (1921) as:

$$R^2 = 1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i (y_i - \bar{y})^2}$$

$y_i =$ reference value

$f_i =$ NIRS measured value

$\bar{y} =$ overall mean of reference values

The statistical model that relates NIR to laboratory data can be highly complex. Several regression methods are used in the statistical model to overcome the complexity and achieve better results. The first one is principal component regression (PCR), which is basically a data reduction method. It is designed to reduce the intercorrelated wavelengths to a smaller set of independent variables called principal components. By using several principal components, spectra information can be condensed into few scores. Principle components are constructed to give the maximum variability in the scores. PCR is multiple regression on the scores (Shenk and Westerhaus, 1994).

Partial least squares (PLS) is similar to PCR, but differs in that the loading calculations include lab data values. PLS is usually better than PCR as a method for predicting lab data values because the lab data information helps from relevant factors. PLS informs the minimum factors required to develop a calibration equation using regression analysis. R^2 and the standard error of cross validation (SECV) are different for each number of factors, and the user can select the optimal value. However, the number of factors is set to be less than 10% of the total number of samples, because the error becomes too large if the number of factors increases too much (Shenk and Westerhaus, 1994).

Calibrations for NIRS are often limited by use of linear mathematical models. Neural networks have been suggested as a calibration technique. In many cases, neural networks were more accurate than traditional calibration method. Since 2000s, the studies for neural networks in NIRS calibration have been conducted actively and more research is needed for general use.

2.3.4 Validation of calibration

The validation of the calibration equation is to evaluate the predictive ability of the selected calibration equation. There are two representative methods to validate the accuracy of calibration equation. The first one is to evaluate the accuracy of the already prepared calibration equation using independent samples that are not used to develop the calibration equation (Williams, 1987). In this case, SEP is used to evaluate the predictive ability of the calibration equation. SEP is the standard error of samples only used for validation.

The second one is the cross validation. The cross validation is a procedure for obtaining validation error by partitioning the calibration set into several groups. A calibration is performed for each group, reserving that group for validation and calibrating on the remaining group until every sample has been predicted once (Shenk and Westerhaus, 1994). The validation errors are combined into a standard error of cross validation (SECV) and the coefficient of determination are combined into a coefficient of determination of cross validation (R^2CV).

2.4 Evaluation of feed value of forage using NIRS

2.4.1 Crude protein

Norris et al. (1976) were the first to use NIRS for evaluation of crude protein (CP) content of forages. After the authors confirmed the possibility of prediction using NIRS, Shenk et al. (1981) reported that log 1/R (R = reflectance) was the best mathematical treatment of the spectra for CP. In addition, Shenk and Westerhaus (1994) recommended the combustion method of Dumas (1831) rather than the Kjeldahl N determination (AOAC, 1995) because the multiple steps of analyzing in Kjeldahl N procedure can cause errors. CP tends to have good result of calibration (R^2>0.95), and Roberts et al. (2003) explained this through: (1) N-H bond shows the strongest absorbance in the near infrared region, and (2) CP is contained in high concentration on the range of 30 to 500 g/kg in forage.

2.4.2 Fiber

Forage fiber is generally analyzed as acid detergent fiber (ADF) and neutral detergent fiber (NDF) (Goering and Van Soest, 1970). Both ADF and NDF were evaluated using NIRS for the first time by Norris et al. (1976). Thereafter, Fales and Cummins (1982) examined the effects of storing forage-type sorghum samples under different humidities on NIRS analysis of ADF, and reported that the standard errors of estimate (SEE) for the highly humid samples were as much as two times the SEE for the low humid samples. NDF is measured by NIRS from the difference between C-H and O-H bonds in the range of 300 to 800 g/kg (Roberts et al., 2003).

2.4.3 Digestibility

In vitro dry matter digestibility (IVDMD) was also evaluated using NIRS for the first time by Norris et al. (1976), and Shenk et al. (1981) reported that log 1/R (R = reflectance) was the best mathematical treatment of the spectra for IVDMD like CP. Winch and Major (1981) compared the results of calibration between *in vitro* and in *vivo dry* matter digestibility; 0.91 and 0.60 of R^2, 2.57 and 4.83 of SEP, respectively. In addition, the authors reported that the better results were achieved when moisture content was below 8 %.

Total digestible nutrients (TDN) was evaluated for the first time by Amri and Abe (1997). The authors compared various regression equations including Adams method, Martin method, Enzymatic method, Donker method, Summation method and ADF method with *in vivo* TDN obtained from digestion trials carried out in sheep, then they developed the calibration of NIRS. The R^2 and SEP for hay, grass silage, corn silage and mixed hay-grass silage were 0.86 and 2.76, 0.94 and 2.76, 0.81 and 2.31, and 0.85 and 2.31, respectively.

3. Materials and methods

3.1 Collection of samples

Alfalfa (*Medicago sativa* L.) hay and timothy (*Phleum pratense* L.) hay imported from the United States were collected from importers, livestock farms, and total mixed ration factories nationwide to represent the population. About 800 g of alfalfa hay (227 samples) and timothy hay (360 samples) were collected. Samples were sealed immediately after collection and stored in a shaded place at room temperature.

3.2 Spectrum acquisition using NIRS

The NIRS instrument “SpectraStar XT (Unity Scientific of KPM Analytics, U.S.A.)” was used to scan samples and develop the calibration equations. The instrument specifications are provided in Table 1.

Table 1. The specification of NIRS instrument

Class	Specifications
Name	SpectraStar XT-R of Unity Scientific
Light source	Tungsten halogen lamp
Measurement mode	Reflectance of transflectance
Detector	High performance ultra-cooled InGaAs extended range detector
Optical bandwidth	10.0 ± 0.3 nm actual full width at half maximum
Absorbance range	Up to 3 Absorbance Unit (AU)
Analysis time	10 – 60 sec
Wavelength accuracy	< 0.02 nm to traceable standard reference material
Wavelength precision	< 0.005 nm
Number of data points	1,920

3.2.1 Intact sample

The samples in intact state were cut into about 10 cm and scanned by filling the large cup (diameter 11.5 cm) with about 2 cm. After the scan, the samples were put into an air-forced drying oven at 65°C for 72 hours for the moisture content determination. The scanning surface is shown Figure 1a.

3.2.2 Ground sample

After the samples were dried to a constant weight, they were ground through a Wiley mill with 1 mm screen. The ground samples were scanned by small cup (diameter 8.5 cm) with about 1 cm. Then, the ground samples were not discarded, but used for measurement of moisture, neutral detergent fiber (NDF), acid detergent fiber (ADF), crude protein (CP) and *in vitro* dry matter digestibility (IVDMD) by wet chemistry. The scanning surface is shown in Figure 1b.

(a) (b)

Figure 1. The scanning surfaces of intact sample(a) and ground sample(b)

3.3 Chemical analysis

3.3.1 Preparation of chemical analysis

After the scans of both intact and ground samples were completed, the samples were packed into zip lock bags and stored at dark and dry storage room.

3.3.2 Moisture content

Moisture content was measured in duplicate to compare with spectrum of both intact and ground samples. To measure the moisture content of intact samples (IS), the samples were put an into air-forced oven at 65°C for 72 hours after scanning with intact form. Moisture content was calculated by the following formula:

$$\text{Moisture content for IS (\%)} = \frac{\text{Forage weight - dried forage weight}}{\text{Forage weight}} \times 100$$

To measure the moisture content of ground samples (GS), the samples were put into air-forced oven at 65°C for 72 hours after sealed in nylon filter bags (50 mm x 55 mm, ANKOM F57, ANKOM Tech., Fairport, NY). The moisture content was calculated by the following formula:

$$\text{Moisture content for GS (\%)} = \frac{\text{Forage weight - }\{W_2 - (W_1 \times B)\}}{\text{Forage weight}} \times 100$$

W_1 = Bag weight

W_2 = Final bag weight

B = Blank bag correction = Final weight of blank bag / Initial bag weight

3.3.3 Crude protein content

Crude protein (CP) was measured by combustion method of Dumas (1831) using an Automatic Elemental Analyzer "Euro Vector EA3000 (EVISA Co., Ltd, Milan, Italy)". All samples were mixed well so that only the specific parts such as leaves and stems were not analyzed. About 9 to 11 mg of the sample was weighed, wrapped in a foil capsule, and combusted to measure nitrogen content.

3.3.4 Fiber content

A sample uniformly ground to 1 mm was weighed (0.5 to 0.6 g), placed in a nylon filter bags (50 mm x 55 mm, ANKOM F57, ANKOM Tech., Fairport, NY) and sealed. NDF was determined inclusive of heat-stable amylase (Ankom Technology, 2005b). Acid detergent fiber (ADF) was also determined sequentially in samples previously subjected to NDF analysis, using the procedure specified by Ankom Technology (2005a). Both NDF and

ADF were determined using ANKOM 2000 Automated Fiber Analyzer (ANKOM Tech., Fairport, NY) following the method of Goering and Van Soest (1970).

3.4 Digestion analysis

3.4.1 *In vitro* dry matter digestibility

The two-stage technique of Tilley and Terry (1963) was used to determine the IVDMD. The nylon filter bags (50 mm x 55 mm, ANKOM F57, ANKOM Tech., Fairport, NY) were soaked in 99% acetone for 5 minutes and then air dried for 20 minutes. Thereafter, the samples were put into an air-forced oven at 105°C for 4 hours. Approximately, 0.5 to 0.6 g of each sample was placed in a dried nylon filter bag and sealed. Twenty-four nylon filter bags (2 blank bags + 22 sample bags) were put in a bottle containing 1,330 mL of the solution A and 266 mL of the solution B, and kept at 39°C in the incubator (ANKOM Daisy II, ANKOM Tech., Fairport, NY). The solution A and B was made by the following combination:

$$\text{Solution A (g/L)} = KH_2PO_4\ (10.0) + MgSO_4 \cdot 7H_2O\ (0.5) + NaCl\ (0.5) + CaCl_2 \cdot 2H_2O\ (0.1) + \text{Urea}\ (0.5)$$

$$\text{Solution B (g/L)} = Na_2CO_3\ (15.0) + Na_2S \cdot 9H_2O\ (1.0)$$

Two ruminally cannulated Holstein steers were used for rumen fluid collection. Rumen fluid was harvested before morning feeding and rumen fluid was filtered with gauze and placed in a warmed thermos for preservation during transport. Rumen fluid (400 mL) was poured into the prepared bottle, the headspace was flushed with carbon dioxide for 30 seconds, and then the lid was closed. The bottles were rotated while maintaining 39 °C for 48 hours. After 48 hours, the nylon filter bags were rinsed thoroughly with water. Finally, the NDF analysis procedure was performed with those nylon filter bags.

3.4.2 Total digestible nutrients

Total digestible nutrients (TDN) equal to the sum of digestible nutrients including nitrogen-free extract, crude fiber, CP and ether extract. In this study, TDN was calculated by the formula described by Holland et al. (1990) as follows:

TDN (%) = 0.89 - (0.79 x ADF %)

3.4.3 Relative feed value

The relative feed value (RFV) is an index that estimates digestible dry matter (DDM) from ADF, and calculates the dry matter intake (DMI) potential from NDF. The calculation of RFV was described by Rohweder et al. (1978). The formulas are as follows:

DDM = 88.9 - (0.779 x ADF %)
DMI (% of body weight) = 120 / NDF %
RFV = (DDM x DMI) / 1.29

3.5 Development of NIRS calibration and validation

After scoring the spectrum of each sample, samples with a global distance of 3.0 or more and neighbor distance (ND) of 0.6 or more were removed. The standard normal variate (SNV) and detrend were used to correct the spectral scattering effect, and various mathematical treatments were performed to correct the spectral error. The mathematical treatments used were 1,4,4 1,8,8 1,16,16 2,4,4 2,8,8 2,16,16 3,4,4 3,8,8 3,16,16 4,4,4 4,8,8 and 4,16,16, which mean derivative order, derivative gap, and derivative smooth, respectively. The calibration equation was prepared using partial least square (PLS). In proportion to the total number of samples, the maximum number of factors was set to 16. The number of factors was determined at the lowest standard error of cross validation (SECV). The coefficient of determination

(R^2) and standard error of calibration (SEC) were used as indices to evaluate the calibration equation. The closer R^2 to 1 is associated with a lower SEC, which means better results.

The validation of the developed calibration was performed using the method of cross validation. Therefore, the separated unknown samples were not needed. Instead, the samples used in the calibration equation were randomly divided into 4 groups, and a method in which 1 group validates the calibration of 3 groups in turn was used. To evaluate the validation, the coefficient of determination on cross validation (R^2CV) and SECV were used as indices. The closer R^2CV to 1 is associated with a smaller SECV, and thus better results. For all statistical processing, generating calibration, and validation, Ucal version 4.0 (Unity Scientific of KPM Analytics, USA) was utilized.

4. Results and discussion

4.1 Development of calibration for alfalfa hay

4.1.1 Chemical composition of alfalfa hay

A total of 227 samples of alfalfa hay were collected nationwide. The detailed chemical composition analysis are presented in Table 2. Moisture content ranged from 4.5 to 17.14 %, and CP ranged from 8.53 to 21.22 %. ADF ranged from 23.04 to 49.19 %, and NDF ranged from 35.12 to 61.63 %. IVDMD ranged from 52.55 to 78.42 %, TDN ranged from 50.04 to 70.70 % and RFV ranged from 76.47 to 187.96. Lee et al. (2020) also analyzed the imported alfalfa hay, and reported that moisture content, CP, ADF, NDF, TDN and RFV were 2.1 to 13.3 %, 17.8 to 18.6 %, 49.5 to 53.1 %, 56.8 to 64.9 % and 82.3 to 108.7, respectively. However, Kim et al. (2019) reported that the standard deviation (SD) of their study results was as low as 4 or less. SD in this study were also less than 4, possibly suggesting that the collected alfalfa samples had similar constituents. Table 3. shows the correlation coefficient among the chemical constituents of alfalfa hay. Mason et al. (1983) divided the range of correlation coefficient by (1) ≤ 0.35 are considered to represent low or weak correlations, (2) 0.36 to 0.67 modest to high correlations, (3) 0.68 to 0.9 strong or high correlations, and (4) > 0.90 are considered as very high correlation. Following tho authors' standard, every constituents except of moisture contents had strong correlations.

Table 2. The chemical composition of collected alfalfa hays (n=227)

Items	Mean	Range	SD	SE
Moisture (as received, %)	8.22	4.50 ~ 17.14	1.242	0.08
Moisture (after ground, %)	5.62	2.66 ~ 10.12	0.778	0.05
Crude Protein (% of DM)	15.53	8.53 ~ 21.22	2.630	0.17
Acid Detergent Fiber (% of DM)	34.96	23.04 ~ 49.19	3.583	0.24
Neutral Detergent Fiber (% of DM)	50.18	35.12 ~ 61.63	3.714	0.25
In vitro Dry Matter Digestibility (% of DM)	69.24	52.55 ~ 78.42	4.213	0.28
Total Digestible Nutrients (% of DM)	61.28	50.04 ~ 70.70	2.831	0.19
Relative Feed Value (RFV)	115.27	76.47 ~ 187.96	13.361	0.88

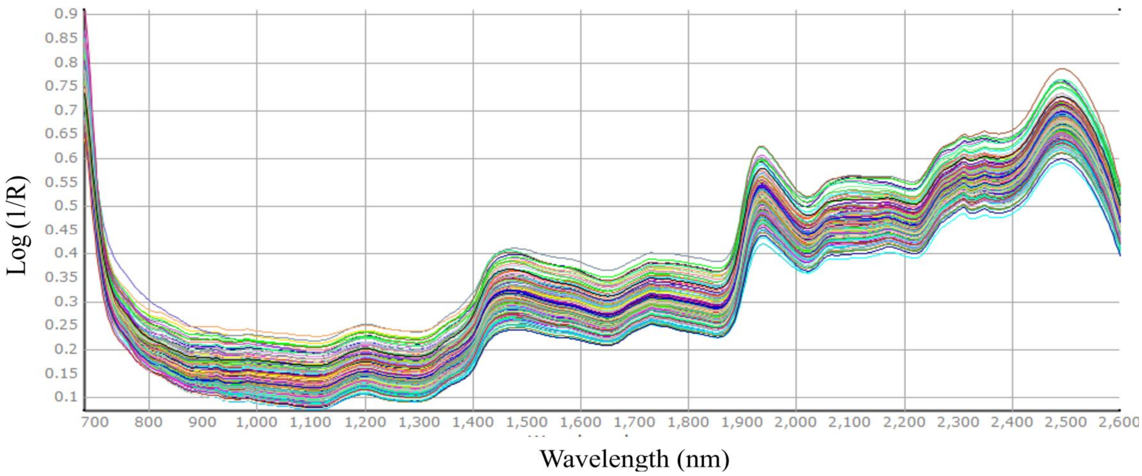
Table 3. Pearson correlation coefficient among the constituents of alfalfa hay

	Moisture	gMoisture	ADF	NDF	CP	IVDMD	TDN	RFV
Moisture	1							
gMoisture	-0.134*	1						
ADF	0.056	0.111	1					
NDF	0.005	0.068	0.846**	1				
CP	0.060	-0.081	-0.705**	-0.684**	1			
IVDMD	-0.098	-0.049	-0.730**	-0.697**	0.681**	1		
TDN	-0.056	-0.111	-1**	-0.846**	0.705**	0.730**	1	
RFV	-0.034	-0.078	-0.922**	-0.971**	0.699**	0.714**	0.922**	1

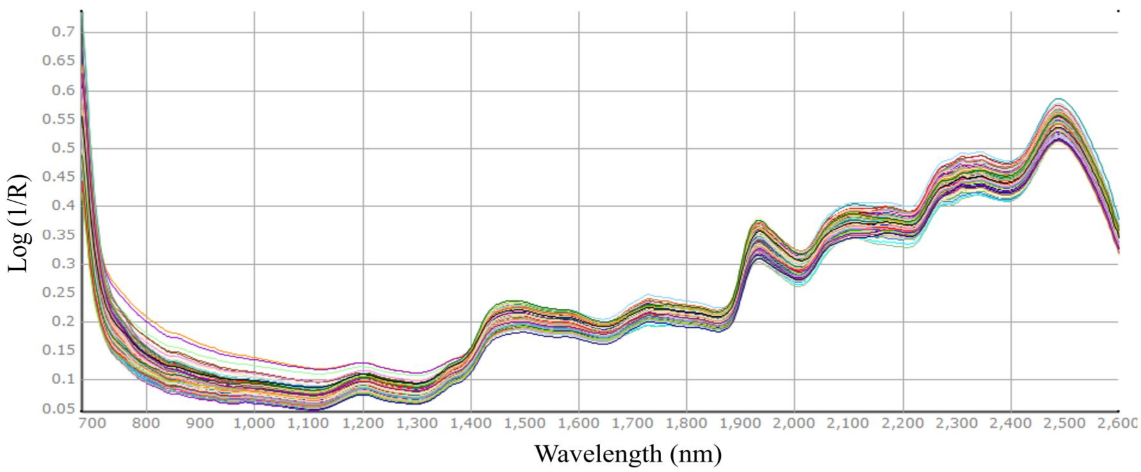
*: $p<0.05$
**: $p<0.0001$
gMoisture: moisture content for ground sample
ADF: acid detergent fiber
NDF: neutral detergent fiber
CP: crude protein
IVDMD: *in vitro* dry matter digestibility
TDN: total digestible nutrients
RFV: relative feed value

4.1.2 The characteristics of NIR spectra of alfalfa hay

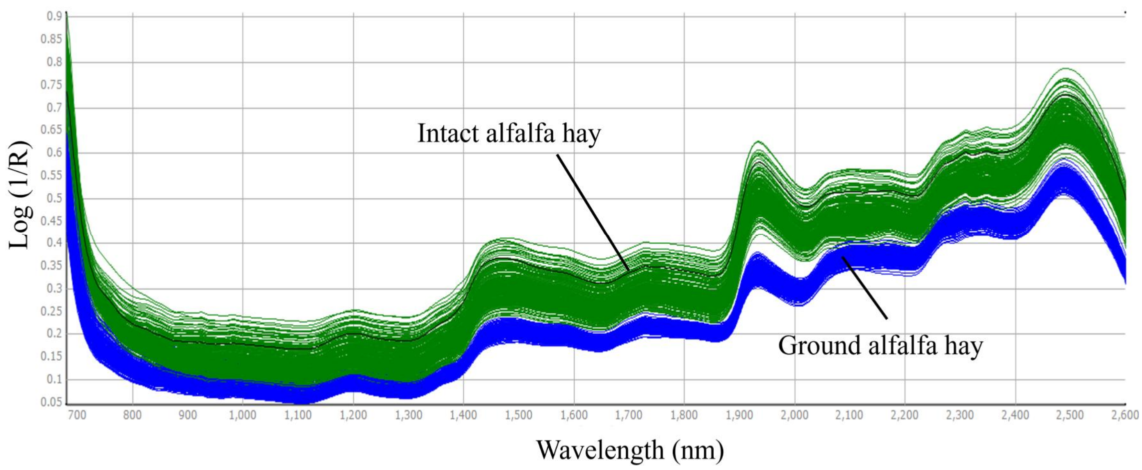
Near-infrared reflectance spectroscopy (NIRS) is a method that analyzes the wavelength and absorption intensity of the absorbed ray. NIRS measured spectra up to a wavelength range of 650 to 2,500 nm. Figure 2 shows the spectra of both intact and ground samples and their comparison. Alfalfa in both intact and ground form had peaks on a wavelength of around 1,200 nm 1,400 to 1,500 nm and 1,900 to 2,000 nm. Moron and Cozzolino (2002) identified a high correlation between wavelength and macro-element content around 1,200, 1,400 and 2,200 nm in alfalfa and white clover, indicating that these regions had considerable influence in the spectra due to the strong relationship between minerals and other constituents, principally water. It was confirmed that the spectra of intact alfalfa hay were more dispersed than that of ground alfalfa hay, and that the absorbance of intact alfalfa hay was higher.



(a) The spectra of intact alfalfa hay samples



(b) The spectra of ground alfalfa hay samples



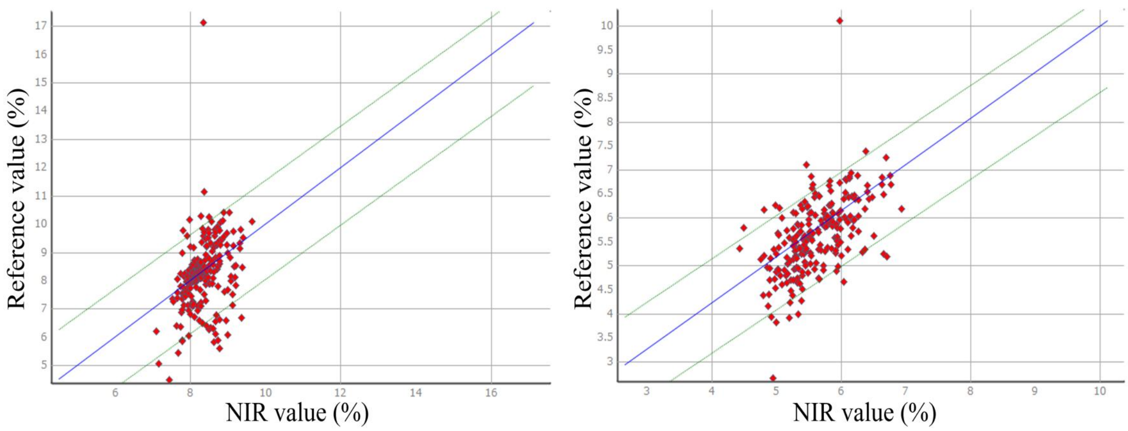
(c) The overplot of the spectra of intact one and ground one

Figure 2. The spectra characteristics of alfalfa hay

4.1.3 Development of calibration and validation for alfalfa hay

4.1.3.1 Moisture

Among the various mathematical treatment results, intact alfalfa hay had the lowest standard error of cross validation (SECV) of 3,16,16 and ground alfalfa hay had the lowest SECV of 2,16,16. The comparison of mathematical treatment results is shown in Table 4. The coefficient of determination (R^2) and SECV were 0.74 and 0.51 on intact alfalfa hay, respectively. On the other side, R^2 was 0.84 and SECV was 0.34 on ground alfalfa hay. Although the values in the table look the same, but the lowest value was selected by comparing up to three or four decimal places. Figure 3 shows the correlation between NIR value and reference value of moisture content of alfalfa hay. Figure 3a shows that the prediction values were concentrated 8 to 9 % despite various reference values, suggesting that intact alfalfa hay had poor results of correlation between prediction values and reference values. However, Figure 3b shows that the correlation between prediction values and reference values was improved after the samples were dried and ground.



(a) intact alfalfa hay (b) ground alfalfa hay

Figure 3. The correlation between NIR value and reference value of alfalfa hay moisture content in intact and ground form

Table 4. The results of mathematical treatment on moisture content of alfalfa hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	5	102	0.50	0.27	0.30	0.57
	1,8,8	3	107	0.42	0.30	0.22	0.60
	1,16,16	2	101	0.44	0.29	0.29	0.58
	2,4,4	3	127	0.65	0.35	0.34	0.62
	2,8,8	6	107	0.64	0.25	0.32	0.58
	2,16,16	15	109	0.76	0.20	0.42	0.54
	3,4,4	3	130	0.72	0.31	0.27	0.70
	3,8,8	8	121	0.76	0.23	0.31	0.60
	3,16,16	12	106	**0.74**	0.22	0.42	**0.51**
	4,4,4	5	151	0.85	0.28	0.25	0.70
	4,8,8	9	128	0.81	0.24	0.29	0.64
	4,16,16	9	109	0.67	0.24	0.38	0.52
	Mean	6.7	116.5	0.66	0.27	0.32	0.60
Ground	1,4,4	5	108	0.87	0.19	0.67	0.34
	1,8,8	5	113	0.86	0.20	0.62	0.36
	1,16,16	5	105	0.86	0.18	0.64	0.34
	2,4,4	8	164	0.95	0.15	0.68	0.37
	2,8,8	2	102	0.81	0.20	0.57	0.34
	2,16,16	4	107	**0.84**	0.19	0.61	**0.34**
	3,4,4	4	171	0.88	0.23	0.54	0.45
	3,8,8	9	134	0.94	0.14	0.70	0.35
	3,16,16	10	104	0.88	0.16	0.51	0.37
	4,4,4	4	134	0.90	0.17	0.40	0.49
	4,8,8	6	139	0.90	0.19	0.67	0.37
	4,16,16	12	99	0.89	0.13	0.58	0.35
	Mean	6.2	123.3	0.88	0.18	0.60	0.37

R^2: the coefficient of determination

SEC: the standard error of calibration

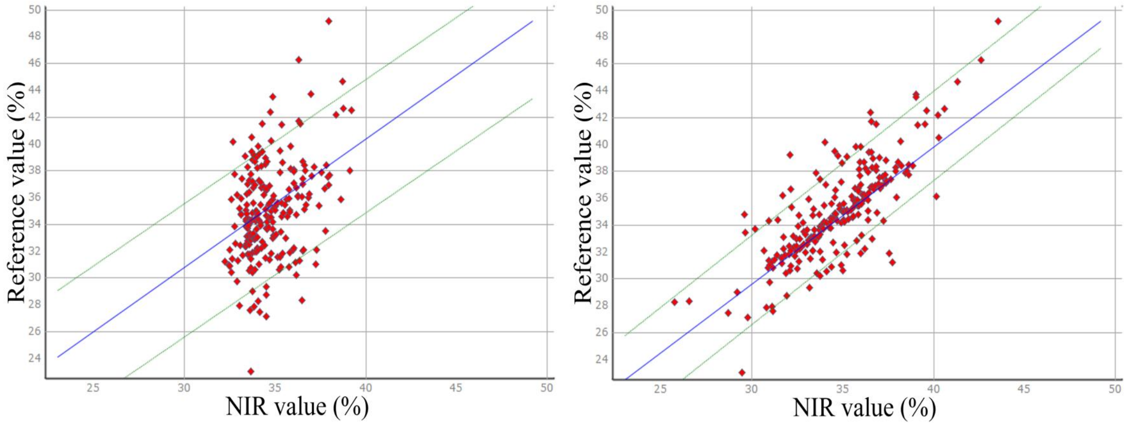
R^2CV: the coefficient of determination of cross validation

SECV: the standard error of cross validation

4.1.3.2 Fiber

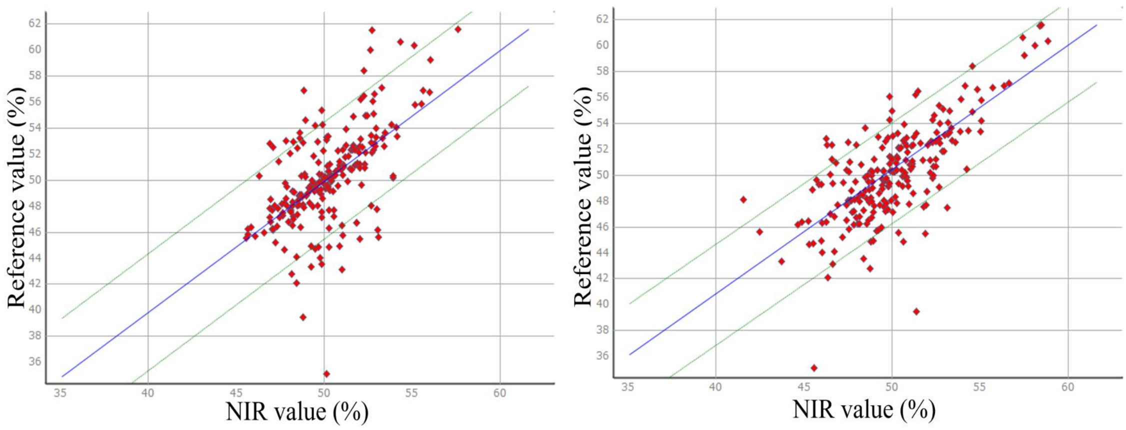
ADF and NDF were used for fiber analysis of alfalfa hay. Among the various mathematical treatment results, intact alfalfa hay had the lowest SECV of 2,4,4 and ground alfalfa hay had the lowest SECV of 4,16,16 on ADF. The mathematical treatments for ADF are shown in Table 5. Intact alfalfa hay had R^2 and SECV of 0.68 and 1.63, respectively. However, alfalfa in ground form had R^2 and SECV of 0.97 and 1.17, respectively. The reason for the low R^2 on intact alfalfa hay is that the samples previously used for NDF analysis were reused. In addition, Park et al. (1983) explained the reason of low R^2 by (1) the narrow ranges of variation among samples, (2) possible errors in laboratory procedures, and (3) possible changes in infrared spectra due to interaction or other chemical entities. Figure 4 shows the correlation between NIR value and reference value of ADF in alfalfa hay. Figure 4a shows an abnormal distribution concentrating on 35 %, which means that NIRS could predict only limited values for various values. However, the graph was uniformly distributed along the trend line (blue line) after the samples were dried and ground.

Intact alfalfa hay had the lowest SECV of 3,4,4 and ground alfalfa hay had the lowest SECV of 1,8,8 on NDF. The mathematical treatments for NDF are shown in Table 6. Alfalfa hay in intact form had R^2 and SECV of 0.89 and 1.72, respectively. However, alfalfa in ground form had R^2 and SECV of 0.92 and 1.33, respectively. The results of calibration on intact and ground alfalfa hay were similar and good. Therefore, it is thought that NDF content of alfalfa hay can be predicted using NIRS for field use regardless of the sample form (intact or ground). Figure 5 shows the correlation between NIR value and reference value of NDF of alfalfa hay. Both Figure 5a and 5b had similar graphs drawing trend lines (blue lines).



(a) intact alfalfa hay (b) ground alfalfa hay

Figure 4. The correlation between NIR value and reference value of ADF of alfalfa hay in intact and ground form



(a) intact alfalfa hay (b) ground alfalfa hay

Figure 5. The correlation between NIR value and reference value of NDF of alfalfa hay in intact and ground form

Table 5. The results of mathematical treatment on ADF of alfalfa hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	2	98	0.58	0.93	0.25	1.72
	1,8,8	3	99	0.60	0.92	0.26	1.70
	1,16,16	2	98	0.59	0.93	0.26	1.69
	2,4,4	2	97	**0.68**	0.84	0.32	**1.63**
	2,8,8	2	95	0.59	0.91	0.28	1.70
	2,16,16	2	96	0.58	0.91	0.33	1.63
	3,4,4	2	118	0.66	0.98	0.32	1.83
	3,8,8	2	100	0.65	0.90	0.28	1.77
	3,16,16	2	93	0.62	0.88	0.30	1.64
	4,4,4	2	122	0.66	1.06	0.23	1.98
	4,8,8	2	93	0.65	0.84	0.31	1.71
	4,16,16	3	94	0.65	0.89	0.27	1.73
	Mean	2.2	100.3	0.63	0.92	0.28	1.73
Ground	1,4,4	5	104	0.92	0.64	0.77	1.21
	1,8,8	6	99	0.93	0.56	0.78	1.20
	1,16,16	5	99	0.93	0.61	0.79	1.20
	2,4,4	3	106	0.92	0.68	0.75	1.35
	2,8,8	5	103	0.92	0.62	0.77	1.22
	2,16,16	6	101	0.92	0.61	0.79	1.25
	3,4,4	4	107	0.94	0.58	0.66	1.58
	3,8,8	3	114	0.91	0.71	0.78	1.27
	3,16,16	13	98	0.96	0.41	0.80	1.19
	4,4,4	3	127	0.89	0.82	0.59	1.79
	4,8,8	5	118	0.94	0.63	0.80	1.23
	4,16,16	14	99	**0.97**	0.35	0.81	**1.17**
	Mean	6	106.3	0.93	0.60	0.76	1.31

ADF: acid detergent fiber
R^2: the coefficient of determination
SEC: the standard error of calibration
R^2CV: the coefficient of determination of cross validation
SECV: the standard error of cross validation

Table 6. The results of mathematical treatment on NDF of alfalfa hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	4	91	0.47	0.94	0.25	1.81
	1,8,8	3	89	0.44	0.96	0.26	1.77
	1,16,16	3	89	0.45	0.97	0.25	1.79
	2,4,4	2	96	0.48	1.00	0.26	1.80
	2,8,8	2	88	0.51	0.98	0.25	1.82
	2,16,16	3	90	0.54	0.96	0.26	1.76
	3,4,4	5	143	**0.89**	0.72	0.43	**1.72**
	3,8,8	4	97	0.69	0.84	0.32	1.82
	3,16,16	3	98	0.49	0.99	0.31	1.78
	4,4,4	3	135	0.82	0.91	0.32	2.04
	4,8,8	2	94	0.59	0.91	0.26	1.77
	4,16,16	4	99	0.55	0.97	0.29	1.79
	Mean	3.2	100.8	0.58	0.93	0.29	1.81
Ground	1,4,4	4	103	0.92	0.71	0.76	1.35
	1,8,8	5	103	**0.92**	0.69	0.77	**1.33**
	1,16,16	5	104	0.92	0.70	0.77	1.33
	2,4,4	5	129	0.93	0.68	0.67	1.70
	2,8,8	5	113	0.92	0.72	0.74	1.40
	2,16,16	5	101	0.92	0.67	0.75	1.34
	3,4,4	3	123	0.90	0.87	0.61	1.69
	3,8,8	5	125	0.91	0.77	0.76	1.49
	3,16,16	5	112	0.90	0.75	0.75	1.40
	4,4,4	2	123	0.83	1.02	0.48	1.99
	4,8,8	6	132	0.93	0.73	0.73	1.59
	4,16,16	6	111	0.91	0.70	0.75	1.35
	Mean	4.7	114.9	0.91	0.75	0.71	1.50

NDF: neutral detergent fiber

R^2: the coefficient of determination

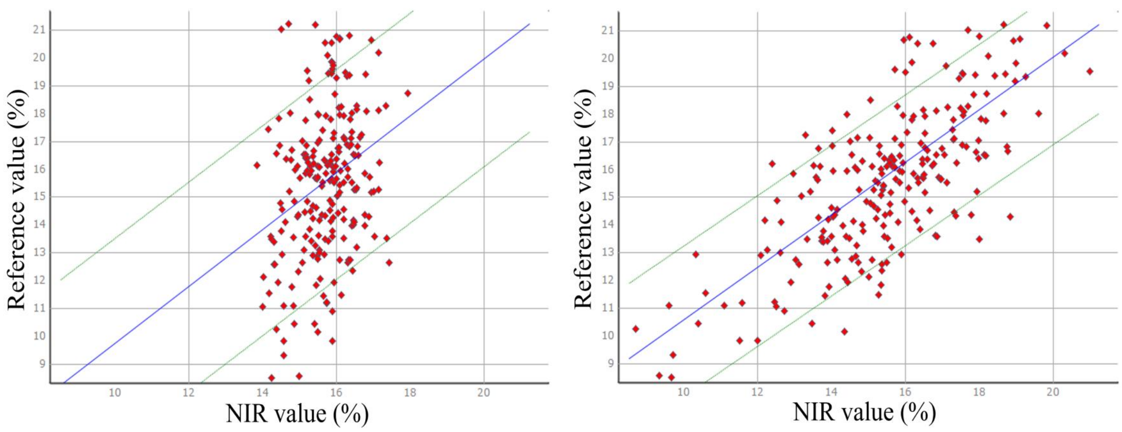
SEC: the standard error of calibration

R^2CV: the coefficient of determination of cross validation

SECV: the standard error of cross validation

4.1.3.3 Crude protein

A calibration equation for CP was derived through regression analysis using the spectra of two types of samples and analysis results by combustion method. The mathematical treatments for CP are shown in Table 7. Intact alfalfa hay had the lowest SECV of 1,16,16 and ground alfalfa hay had the lowest SECV of 4,16,16 on CP. Intact alfalfa hay had R^2 and SECV of 0.42 and 1.38, respectively. However, alfalfa in ground form had R^2 and SECV of 0.90 and 0.97, respectively. Gonzalez Martin et al. (2007) reported that R^2 and SECV in intact alfalfa were 0.83 and 1.17, respectively. However, in their experiment with alfalfa in ground form, R^2 and SECV were 0.87 and 0.82, respectively. The reason that the results of this study on intact alfalfa differ from those of their studies is that the alfalfa leaves were powdered and covered the contact surfaces of the sample cups. While they made the intact alfalfa samples compact, this study made the intact alfalfa samples cut into 10 cm pieces and placed them in a cup and scanned. Figure 6 shows the correlation between NIR value and reference value of CP of alfalfa hay. Although Figure 6a showed a scattered appearance, Figure 6b showed an aligned shape drawing a trend line.



(a) intact alfalfa hay (b) ground alfalfa hay

Figure 6. The correlation between NIR value and reference value of CP of alfalfa hay in intact and ground form

Table 7. The results of mathematical treatment on CP of alfalfa hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	2	87	0.37	0.75	0.17	1.43
	1,8,8	3	84	0.44	0.71	0.19	1.39
	1,16,16	3	90	**0.42**	0.74	0.19	**1.38**
	2,4,4	5	123	0.85	0.72	0.46	1.42
	2,8,8	2	96	0.42	0.83	0.16	1.44
	2,16,16	2	96	0.40	0.82	0.14	1.45
	3,4,4	4	114	0.86	0.61	0.45	1.45
	3,8,8	4	110	0.68	0.86	0.37	1.41
	3,16,16	2	95	0.42	0.80	0.18	1.41
	4,4,4	2	121	0.75	0.87	0.45	1.54
	4,8,8	4	92	0.62	0.66	0.19	1.46
	4,16,16	4	92	0.49	0.71	0.22	1.40
	Mean	3.1	100	0.56	0.76	0.26	1.43
Ground	1,4,4	4	86	0.89	0.53	0.71	1.00
	1,8,8	5	86	0.89	0.53	0.71	0.99
	1,16,16	3	92	0.90	0.57	0.74	1.06
	2,4,4	10	177	0.97	0.40	0.76	1.25
	2,8,8	4	94	0.89	0.56	0.72	1.02
	2,16,16	7	93	0.88	0.54	0.69	1.02
	3,4,4	7	173	0.96	0.50	0.74	1.29
	3,8,8	7	128	0.94	0.50	0.74	1.11
	3,16,16	13	101	0.94	0.44	0.76	1.01
	4,4,4	3	120	0.91	0.52	0.61	1.23
	4,8,8	12	148	0.98	0.33	0.76	1.17
	4,16,16	4	92	**0.90**	0.54	0.77	**0.97**
	Mean	6.6	115.8	0.92	0.50	0.73	1.09

CP: crude protein

R^2: the coefficient of determination

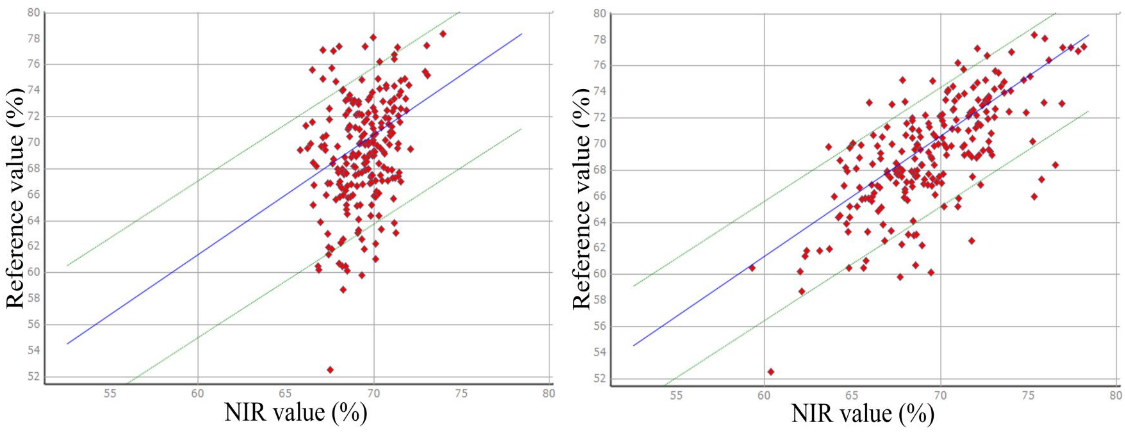
SEC: the standard error of calibration

R^2CV: the coefficient of determination of cross validation

SECV: the standard error of cross validation

4.1.3.4 *In vitro* dry matter digestibility

Intact alfalfa hays had the lowest SECV of 1,4,4 and ground alfalfa hay had the lowest SECV of 4,16,16 on IVDMD. The mathematical treatments for IVDMD are shown in Table 8. Figure 7 also shows the correlation between NIR value and reference value of IVDMD of alfalfa hay. Intact alfalfa hay had R^2 and SECV of 0.52 and 2.26, respectively. However, alfalfa in ground form had R^2 and SECV of 0.95 and 1.62, respectively. Winch and Major (1981) also reported the poor results on IVDMD, which coarse samples of legume had results of 0.26 and 5.62 on R^2 and the standard error of prediction (SEP), respectively. Fine samples of legume had results of 0.39 and 5.39 on R^2 and SEP, respectively. Norris et al. (1976) and Shenk and Barnes (1977) showed that where the 1,670 nm, 1,700 nm and 2,336 nm wavelength substituted for 1,680 nm, 2,330 nm and 2,330 nm wavelengths, the standard error and R^2 of IVDMD were improved considerably. Wavelength substitution was not performed in this study, but it is necessary to demonstrate the effectiveness of wavelength substitution in future studies.



(a) intact alfalfa hay (b) ground alfalfa hay

Figure 7. The correlation between NIR value and reference value of IVDMD of alfalfa hay in intact and ground form

Table 8. The results of mathematical treatment on IVDMD of alfalfa hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	3	89	**0.52**	1.23	0.18	**2.26**
	1,8,8	4	91	0.50	1.24	0.15	2.27
	1,16,16	4	92	0.48	1.26	0.18	2.28
	2,4,4	7	91	0.86	0.70	0.24	2.38
	2,8,8	2	92	0.48	1.31	0.14	2.33
	2,16,16	5	93	0.55	1.26	0.19	2.27
	3,4,4	3	123	0.79	1.20	0.33	2.32
	3,8,8	4	97	0.52	1.38	0.24	2.42
	3,16,16	3	103	0.45	1.43	0.18	2.45
	4,4,4	4	134	0.80	1.15	0.30	2.88
	4,8,8	5	87	0.64	1.12	0.28	2.35
	4,16,16	3	100	0.48	1.39	0.29	2.29
	Mean	3.9	99.3	0.59	1.22	0.23	2.38
Ground	1,4,4	7	110	0.93	0.94	0.74	1.81
	1,8,8	7	123	0.93	0.90	0.73	1.82
	1,16,16	7	115	0.92	0.98	0.71	1.91
	2,4,4	5	124	0.92	0.93	0.71	1.99
	2,8,8	7	98	0.90	0.84	0.70	1.77
	2,16,16	2	97	0.90	0.88	0.71	1.74
	3,4,4	4	134	0.90	1.03	0.62	2.14
	3,8,8	6	120	0.92	0.97	0.73	1.88
	3,16,16	6	95	0.92	0.86	0.77	1.62
	4,4,4	4	126	0.92	0.90	0.59	2.19
	4,8,8	8	123	0.95	0.81	0.73	1.95
	4,16,16	6	103	**0.95**	0.81	0.79	**1.62**
	Mean	5.8	114	0.92	0.90	0.71	1.87

IVDMD: *in vitro* dry matter digestibility

R^2: the coefficient of determination

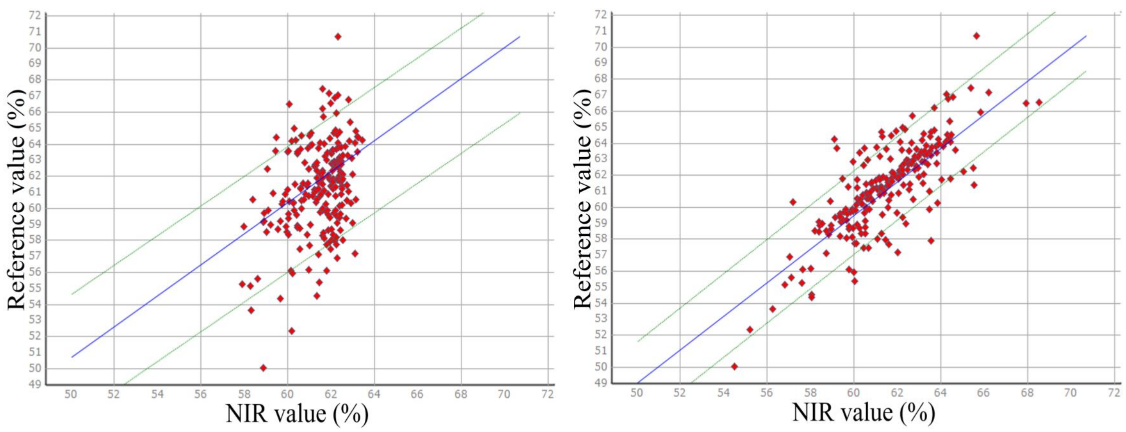
SEC: the standard error of calibration

R^2CV: the coefficient of determination of cross validation

SECV: the standard error of cross validation

4.1.3.5 Total digestible nutrients

Among the various mathematical treatment results, intact alfalfa hay had the lowest SECV of 2,4,4 and ground alfalfa hay had the lowest SECV of 4,16,16 on TDN. The mathematical treatments for TDN are shown in Table 9. Intact alfalfa hay had R^2 and SECV of 0.68 and 1.29, respectively. However, alfalfa in ground form had R^2 and SECV of 0.97 and 0.92, respectively. Amari and Abe (1997) reported that TDN which is a detergent method calculated using ADF had R^2 and SEC of 0.74 and 2.95, respectively. The authors divided the methods into two groups; indirect method and direct method. While the indirect group included Adams method, Martin method, Donker method, enzymatic method and detergent method, the direct group include *in vivo* TDN method. Indirect group had R^2 and SEC of over 0.7 and 1.5 to 3.8, respectively. However, direct group had R^2 and SEC of over 0.8 and 2.3 to 3.4, respectively. The authors reported that the detergent method calculated using ADF had the poorest value of calibration in the TDN methods. As a result, another TDN method is required for improved calibration accuracy. Figure 8 also shows the correlation between NIR value and reference value of TDN of alfalfa hay. Due to the calculation formula involving ADF, the graph of TDN also resembles the trend of ADF graph.



(a) intact alfalfa hay (b) ground alfalfa hay

Figure 8. The correlation between NIR value and reference value of TDN of alfalfa hay in intact and ground form

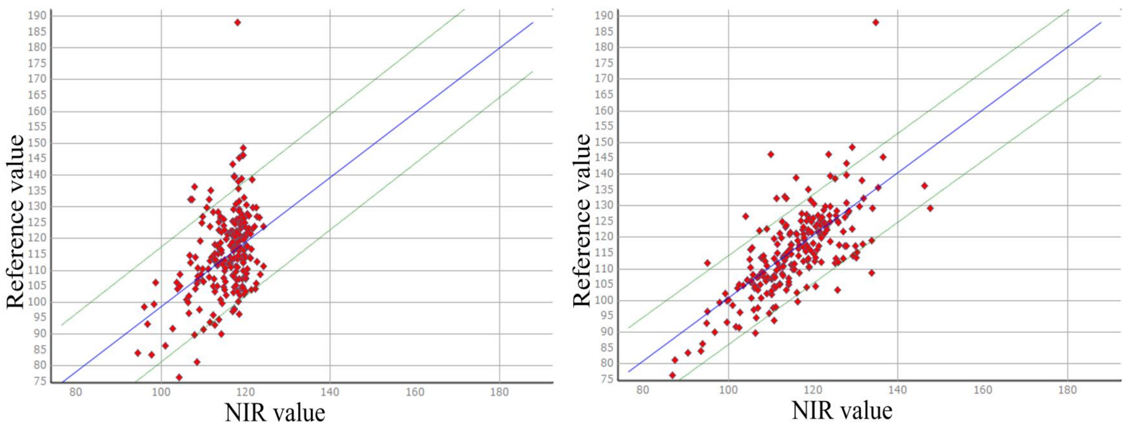
Table 9. The results of mathematical treatment on TDN of alfalfa hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	2	98	0.58	0.73	0.25	1.36
	1,8,8	3	99	0.60	0.72	0.26	1.34
	1,16,16	2	98	0.59	0.74	0.26	1.34
	2,4,4	2	97	**0.68**	0.67	0.32	**1.29**
	2,8,8	2	95	0.59	0.72	0.28	1.34
	2,16,16	2	96	0.58	0.72	0.33	1.29
	3,4,4	2	118	0.66	0.78	0.32	1.45
	3,8,8	2	100	0.65	0.71	0.28	1.40
	3,16,16	2	93	0.62	0.70	0.30	1.30
	4,4,4	2	122	0.66	0.84	0.23	1.57
	4,8,8	2	93	0.65	0.66	0.31	1.35
	4,16,16	3	94	0.65	0.70	0.27	1.36
	Mean	2.2	100.3	0.63	0.72	0.28	1.37
Ground	1,4,4	5	104	0.92	0.51	0.77	0.96
	1,8,8	6	99	0.93	0.44	0.78	0.95
	1,16,16	5	99	0.93	0.49	0.79	0.95
	2,4,4	3	106	0.92	0.53	0.75	1.07
	2,8,8	5	103	0.92	0.49	0.77	0.96
	2,16,16	6	101	0.92	0.48	0.79	0.99
	3,4,4	4	107	0.94	0.46	0.66	1.25
	3,8,8	3	114	0.91	0.56	0.78	1.01
	3,16,16	13	98	0.96	0.32	0.80	0.94
	4,4,4	3	127	0.89	0.65	0.59	1.42
	4,8,8	5	118	0.94	0.50	0.80	0.97
	4,16,16	14	99	**0.97**	0.28	0.81	**0.92**
	Mean	6	106.3	0.93	0.48	0.76	1.03

TDN: total digestible nutrients
R^2: the coefficient of determination
SEC: the standard error of calibration
R^2CV: the coefficient of determination of cross validation
SECV: the standard error of cross validation

4.1.3.6 Relative feed value

Among the various mathematical treatment results, both intact and ground alfalfa hay had the lowest SECV of 4,16,16 on RFV. The mathematical treatments for RFV are shown in Table 10. Intact alfalfa hay had R^2 and SECV of 0.54 and 6.18, respectively. However, alfalfa in ground form had R^2 and SECV of 0.93 and 4.60, respectively. Figure 9 also shows the correlation between NIR value and reference value of RFV of alfalfa hay. Figure 9a shows that the NIR prediction values of alfalfa hay in intact form were centered around 120, ignoring various reference values. However, Figure 9 shows the prediction was improved after drying and grinding samples. The reason that the predicted value of intact alfalfa hay for RFV was not good is thought to be because a calculation formula based on ADF and NDF was used rather than the value being directly analyzed.



(a) intact alfalfa hay (b) ground alfalfa hay

Figure 9. The correlation between NIR value and reference value of RFV of alfalfa hay in intact and ground form

Table 10. The results of mathematical treatment on RFV of alfalfa hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	3	93	0.53	3.43	0.26	6.34
	1,8,8	2	95	0.49	3.68	0.26	6.31
	1,16,16	2	95	0.48	3.72	0.26	6.30
	2,4,4	2	93	0.60	3.40	0.27	6.56
	2,8,8	4	91	0.59	3.21	0.24	6.39
	2,16,16	1	97	0.35	3.95	0.19	6.56
	3,4,4	2	114	0.68	3.77	0.29	7.07
	3,8,8	2	98	0.52	3.60	0.22	6.61
	3,16,16	4	100	0.55	3.71	0.23	6.76
	4,4,4	3	123	0.72	3.74	0.24	7.56
	4,8,8	5	97	0.68	2.89	0.28	6.67
	4,16,16	5	103	**0.54**	3.70	0.23	**6.18**
	Mean	2.9	99.9	0.56	3.57	0.25	6.61
Ground	1,4,4	4	101	0.92	2.48	0.74	4.80
	1,8,8	5	101	0.93	2.38	0.75	4.79
	1,16,16	4	101	0.92	2.51	0.75	4.92
	2,4,4	4	124	0.91	2.73	0.71	5.36
	2,8,8	4	108	0.91	2.66	0.76	4.70
	2,16,16	5	107	0.90	2.60	0.73	4.87
	3,4,4	3	115	0.92	2.78	0.69	5.53
	3,8,8	3	104	0.92	2.54	0.72	5.10
	3,16,16	4	102	0.91	2.49	0.73	4.83
	4,4,4	2	131	0.82	3.84	0.57	6.80
	4,8,8	9	113	0.98	1.23	0.77	4.63
	4,16,16	6	98	**0.93**	2.18	0.76	**4.60**
	Mean	4.4	108.8	0.91	2.5	0.72	5.08

RFV: relative feed value
R^2: the coefficient of determination
SEC: the standard error of calibration
R^2CV: the coefficient of determination of cross validation
SECV: the standard error of cross validation

4.2 Development of calibration for timothy hay

4.2.1 Chemical composition of timothy hay

A total of 360 pieces of timothy hay were collected nationwide. Detailed chemical analysis results are shown in Table 11. The moisture content ranged from 5.25 to 19.09 %, and CP ranged from 2.21 to 11.50 %. ADF ranged from 23.69 to 43.39 %, and NDF ranged from 61.97 to 72.45 %. IVDMD ranged from 45.52 to 70.27 %, TDN ranged from 54.62 to 70.19 % and RFV ranged from 71.57 to 94.86. Kim (2006) studied the inclusion effects of timothy hay to diet of Hanwoo steers, and reported moisture, CP, ADF, NDF and TDN contents of imported timothy hay averaged 9 %, 11.37 %, 33.97 %, 60.55 % and 61.75 %, respectively. However, Kim et al. (2020) reported that moisture, CP, ADF and NDF contents of imported timothy hay were 12.1 %, 5.6 %, 46.0 % and 75.7 %, respectively. Comparing the results of chemical analysis, timothy hay samples in this study were collected on a various range of quality. However, each of standard deviation in timothy hay was too low. Valdes et al. (1987) reported that a calibration equation with excellent predictive accuracy can be constructed only when the population has a diverse range and even distribution. Therefore, since the standard deviation was less than 5, it was considered that many samples with similar components were collected. Table 12 shows the Pearson correlation among the constituents of timothy hay. Following the standard of Mason et al. (1983), there were strong correlations among ADF, NDF, TDN and RFV.

Table 11. The chemical composition of collected timothy hays (n=360)

Items	Mean	Range	SD	SE
Moisture (as received, %)	10.21	5.25 ~ 19.09	1.798	0.09
Moisture (after ground, %)	7.77	5.02 ~ 12.97	1.462	0.08
Crude Protein (% of DM)	5.38	2.21 ~ 11.50	1.658	0.09
Acid Detergent Fiber (% of DM)	38.29	23.69 ~ 43.39	2.145	0.11
Neutral Detergent Fiber (% of DM)	67.35	61.97 ~ 72.45	1.759	0.09
In vitro Dry Matter Digestibility (% of DM)	59.84	45.52 ~ 70.27	4.480	0.23
Total Digestible Nutrients (% of DM)	58.65	54.62 ~ 70.19	1.695	0.09
Relative Feed Value (RFV)	81.69	71.57 ~ 94.86	4.084	0.21

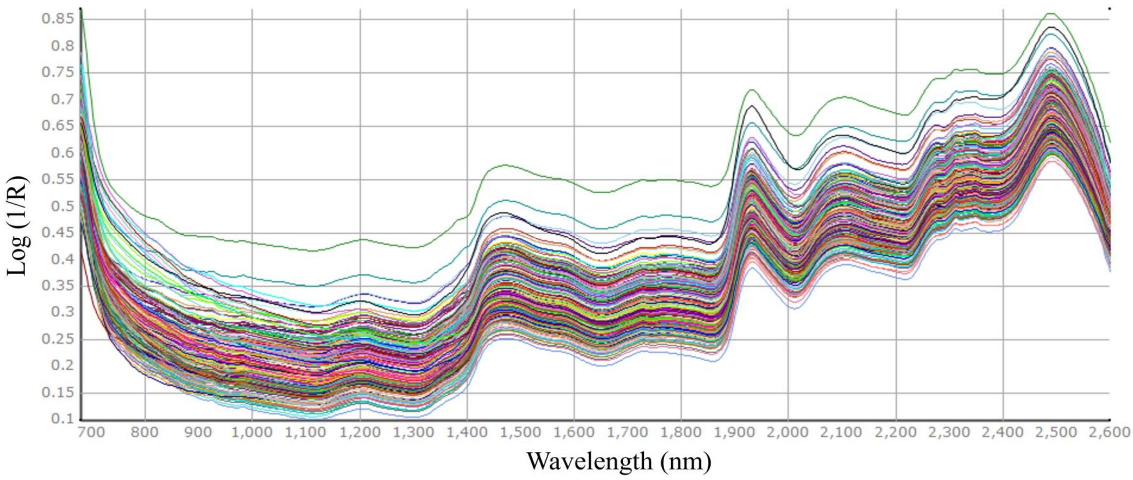
Table 12. Pearson correlation coefficient among the constituents of timothy hay

	Moisture	gMoisture	ADF	NDF	CP	IVDMD	TDN	RFV
Moisture	1							
gMoisture	-0.006	1						
ADF	0.074	-0.381**	1					
NDF	0.155*	-0.420**	0.682**	1				
CP	-0.085	0.029	-0.478**	-0.269**	1			
IVDMD	-0.080	0.139	-0.448**	-0.283**	0.511**	1		
TDN	-0.074	0.381**	-1**	-0.682**	0.478**	0.448**	1	
RFV	-0.119	0.437**	-0.921**	-0.911**	0.411**	0.399**	0.922**	1

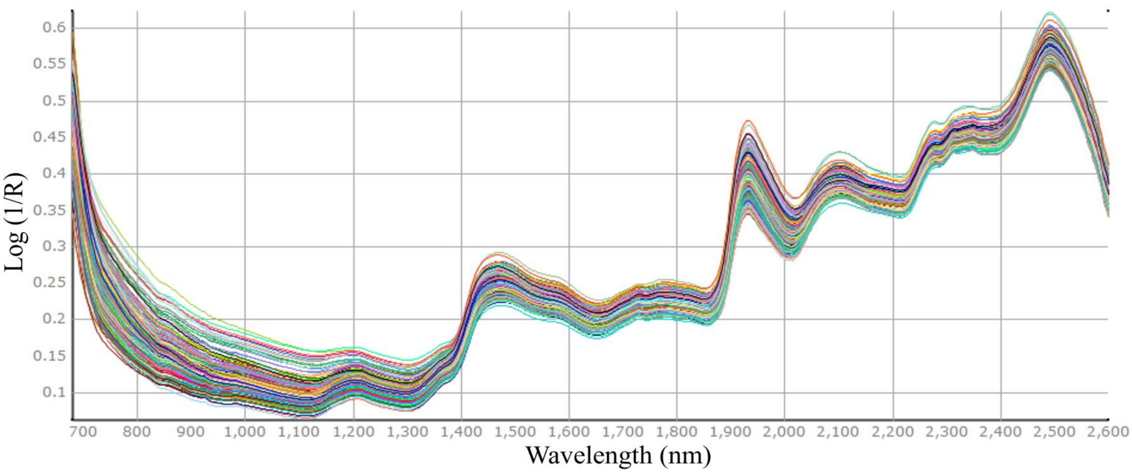
*: $p<0.05$
**: $p<0.0001$
gMoisture: moisture content for ground sample
ADF: acid detergent fiber
NDF: neutral detergent fiber
CP: crude protein
IVDMD: *in vitro* dry matter digestibility
TDN: total digestible nutrients
RFV: relative feed value

4.2.2 The characteristics of NIR spectra of timothy hay

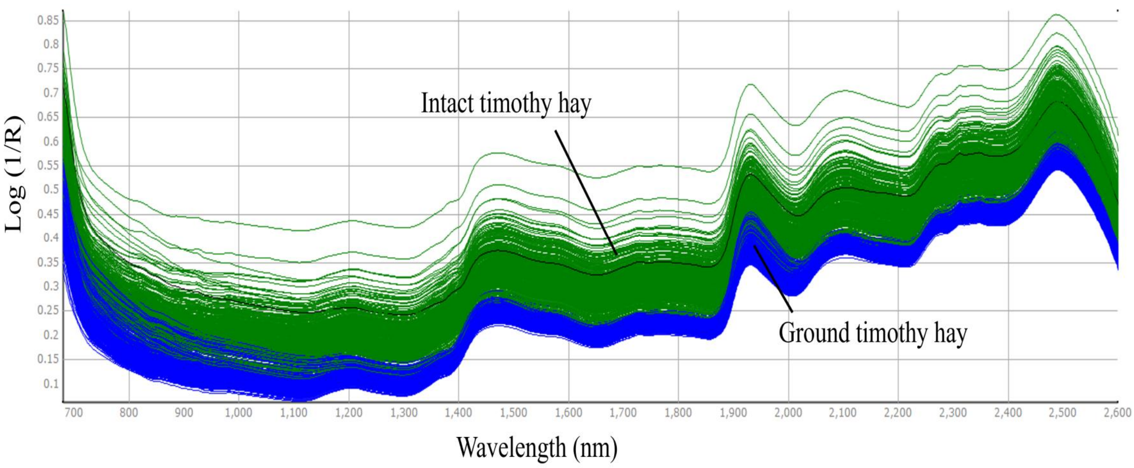
NIRS measured spectra up to a wavelength range of 650 to 2,500 nm. The spectra of both intact and ground form of timothy hay are shown in Figure 10. Both intact and ground timothy hays had peaks on a wavelength of around 1,200 nm 1,400 to 1,500 nm and 1,900 to 2,000 nm. Murray (1986) reported that absorbance bands at 1,194 nm and 1,730 nm were related with C-H stretch overtone and absorbance bands at 1,498 nm and 1,940 nm were related with O-H stretch overtone. Absorbance bands at 1,940 nm were strongly related with water. The author also reported absorbance bands at 2,164, 2,310 and 2,348 nm were related with C-H combination tones. Williams (1987) reported that ADF and NDF of forage are absorbed in 1,555 to 1,674 nm and 2,294 nm. The spectra of two pre-treatment type timothy hay showed difference at the points he reported. Therefore, it is necessary to pay attention to the values of ADF and NDF.



(a) The spectra of intact timothy hay samples



(b) The spectra of ground timothy hay samples



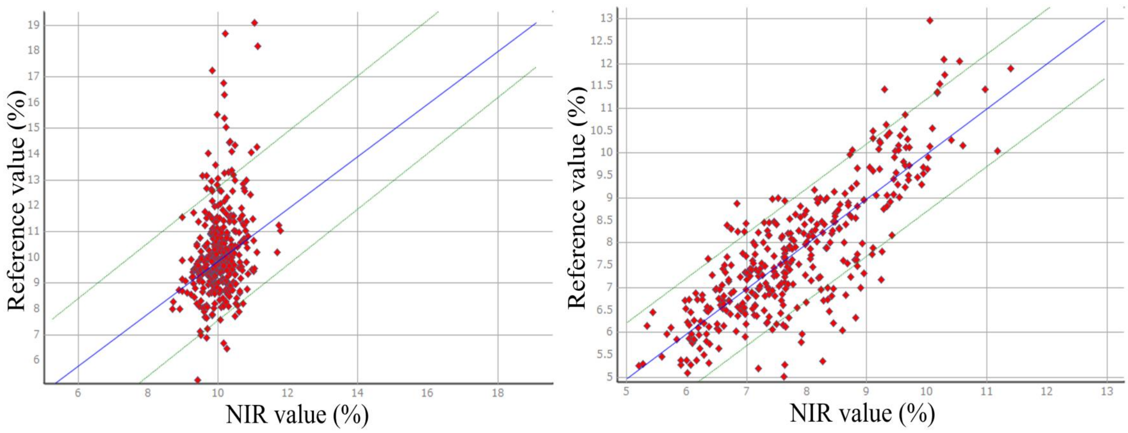
(c) The overplot of the spectra of intact one and ground one

Figure 10. The spectra characteristics of timothy hay

4.2.3 Development of calibration and validation for timothy hay

4.2.3.1 Moisture

On moisture content, intact timothy hay had the lowest SECV in mathematical treatment of 3,8,8 and ground timothy hay had the lowest SECV in mathematical treatment of 3,16,16. The comparison of mathematical treatment results is shown in Table 13. R^2 was 0.48 and SECV was 0.82 on intact timothy hay. However, R^2 and SECV were 0.95 and 0.45 on ground timothy hay, respectively. The development of calibration of forage moisture content had different results according to previous reports because the moisture content changes during pre-treatments, which causes errors. Alomar et al. (2003) compared the drying methods including liquid nitrogen drying, forced-air drying and freeze drying. The authors reported that the calibrations were different according to the drying methods and concluded that every constituents were affected by moisture level. Figure 11 shows the correlation between NIR value and reference value of moisture content of timothy hay. While Figure 11a shows NIR values predicted moisture around 10 %, Figure 11b shows the values of reference and NIR evenly distributed along the trend line (blue line).



(a) intact timothy hay (b) ground timothy hay

Figure 11. The correlation between NIR value and reference value of moisture content of timothy hay in intact and ground form

Table 13. The results of mathematical treatment on moisture content of timothy hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	6	177	0.42	0.44	0.17	0.87
	1,8,8	4	176	0.36	0.46	0.15	0.88
	1,16,16	5	178	0.36	0.47	0.13	0.87
	2,4,4	8	182	0.75	0.32	0.24	0.85
	2,8,8	5	166	0.41	0.43	0.11	0.87
	2,16,16	2	161	0.25	0.47	0.10	0.89
	3,4,4	5	215	0.77	0.40	0.24	1.00
	3,8,8	4	163	**0.48**	0.42	0.18	**0.82**
	3,16,16	6	171	0.35	0.48	0.13	0.91
	4,4,4	7	228	0.85	0.33	0.27	1.07
	4,8,8	5	168	0.57	0.41	0.20	0.83
	4,16,16	6	170	0.36	0.44	0.14	0.89
	Mean	5.3	179.6	0.49	0.42	0.17	0.90
Ground	1,4,4	16	240	0.99	0.16	0.87	0.49
	1,8,8	14	157	0.97	0.20	0.87	0.47
	1,16,16	6	153	0.96	0.26	0.86	0.48
	2,4,4	9	278	0.96	0.27	0.87	0.51
	2,8,8	14	236	0.97	0.22	0.88	0.49
	2,16,16	7	159	0.96	0.26	0.87	0.47
	3,4,4	8	292	0.96	0.29	0.82	0.59
	3,8,8	10	248	0.96	0.26	0.86	0.52
	3,16,16	6	166	**0.95**	0.26	0.88	**0.45**
	4,4,4	10	286	0.97	0.24	0.77	0.65
	4,8,8	7	203	0.96	0.24	0.88	0.45
	4,16,16	6	160	0.94	0.28	0.86	0.48
	Mean	9.4	214.8	0.96	0.25	0.86	0.50

R^2: the coefficient of determination

SEC: the standard error of calibration

R^2CV: the coefficient of determination of cross validation

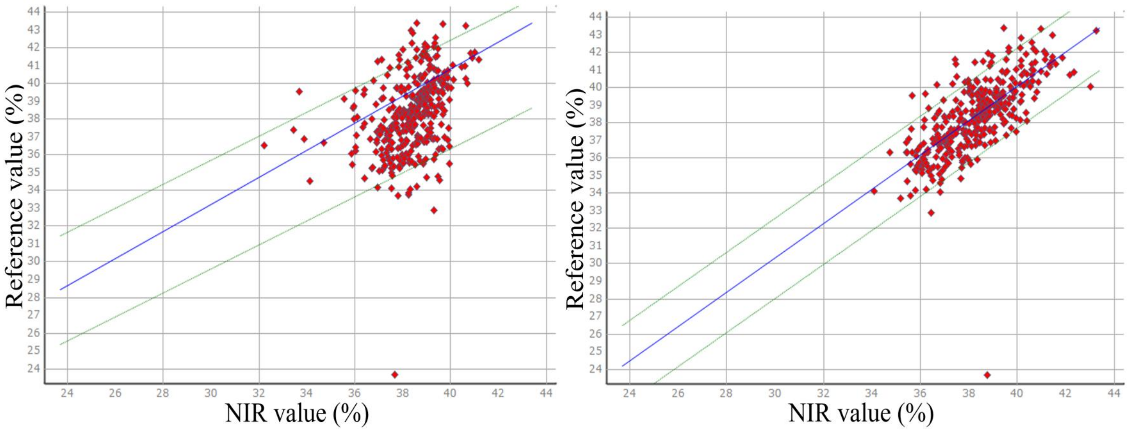
SECV: the standard error of cross validation

4.2.3.2 Fiber

Among the various mathematical treatment results, intact timothy hay had the lowest SECV of 1,16,16 and ground timothy hay had the lowest SECV of 4,16,16 on ADF. The mathematical treatments for ADF are shown in Table 14. Timothy hay in intact form had R^2 and SECV of 0.75 and 1.09, respectively. However, timothy hay in ground form had R^2 and SECV of 0.94 and 0.82, respectively. Figure 12 also shows the correlation between NIR value and reference value of ADF in timothy hay. Figure 12a shows that the prediction values were concentrated at 36 to 40 %. However, the prediction values shown in Figure 12b were changed to vary widely from 34 to 44 % after drying and grinding samples.

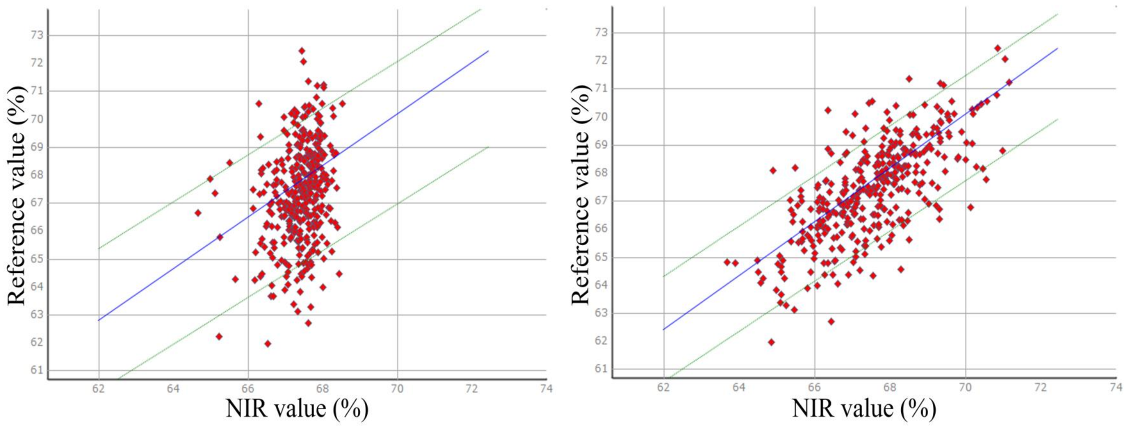
Intact timothy hay had the lowest SECV of 1,8,8 and ground timothy hay had the lowest SECV of 1,4,4 on NDF. The mathematical treatments for NDF are shown in Table 15. Timothy in intact form had R^2 and SECV of 0.38 and 0.95, respectively. However, timothy in ground form had R^2 and SECV of 0.94 and 0.73, respectively. Figure 13 shows the correlation between NIR value and reference value of NDF of timothy hay. Figure 13a shows that the prediction values focused on 66 to 68 %, which this graph shape is consistent with the low value of the correlation coefficient.

The values of calibration for ADF and NDF were generally reported to be poorer than that of CP. Martin and Linn (1989) reported validation data for NIRS equations, which R^2 were 0.89, 0.91 and 0.98 for ADF, NDF and CP, respectively. And Park et al. (2017) also reported poorer values of calibration for ADF and NDF (0.76 and 0.88 of R^2; 2.65 and 2.90 of SECV, respectively) than CP (0.91 of R^2; 1.46 of SECV) from hilly pasture forage.



(a) intact timothy hay (b) ground timothy hay

Figure 12. The correlation between NIR value and reference value of ADF of timothy hay in intact and ground form



(a) intact timothy hay (b) ground timothy hay

Figure 13. The correlation between NIR value and reference value of NDF of timothy hay in intact and ground form

Table 14. The results of mathematical treatment on ADF of timothy hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	5	155	0.67	0.57	0.34	1.10
	1,8,8	7	160	0.66	0.58	0.33	1.11
	1,16,16	16	159	**0.75**	0.51	0.42	**1.09**
	2,4,4	3	172	0.74	0.64	0.43	1.13
	2,8,8	3	154	0.69	0.60	0.36	1.12
	2,16,16	6	155	0.74	0.57	0.40	1.10
	3,4,4	2	178	0.75	0.67	0.42	1.22
	3,8,8	3	173	0.73	0.67	0.45	1.12
	3,16,16	4	162	0.74	0.63	0.45	1.10
	4,4,4	2	185	0.69	0.75	0.28	1.35
	4,8,8	5	167	0.78	0.59	0.45	1.11
	4,16,16	7	159	0.71	0.64	0.43	1.13
	Mean	5.3	164.9	0.72	0.62	0.40	1.14
Ground	1,4,4	13	164	0.97	0.27	0.79	0.83
	1,8,8	10	162	0.94	0.43	0.78	0.84
	1,16,16	12	167	0.94	0.43	0.78	0.84
	2,4,4	8	217	0.95	0.41	0.76	0.91
	2,8,8	8	175	0.93	0.45	0.77	0.84
	2,16,16	15	164	0.95	0.36	0.76	0.87
	3,4,4	5	238	0.92	0.53	0.72	0.98
	3,8,8	7	181	0.93	0.45	0.76	0.88
	3,16,16	8	176	0.91	0.48	0.75	0.87
	4,4,4	6	255	0.92	0.51	0.69	1.00
	4,8,8	16	161	0.99	0.16	0.73	0.90
	4,16,16	10	169	**0.94**	0.41	0.77	**0.82**
	Mean	9.8	185.8	0.94	0.41	0.76	0.88

ADF: acid detergent fiber

R^2: the coefficient of determination

SEC: the standard error of calibration

R^2CV: the coefficient of determination of cross validation

SECV: the standard error of cross validation

Table 15. The results of mathematical treatment on NDF of timothy hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	2	145	0.33	0.56	0.12	0.96
	1,8,8	7	143	**0.38**	0.52	0.14	**0.95**
	1,16,16	3	144	0.34	0.54	0.10	0.95
	2,4,4	2	161	0.56	0.55	0.21	1.01
	2,8,8	2	144	0.49	0.51	0.15	0.97
	2,16,16	2	149	0.44	0.53	0.15	0.96
	3,4,4	2	179	0.61	0.60	0.30	1.04
	3,8,8	3	147	0.48	0.54	0.17	0.99
	3,16,16	2	137	0.38	0.52	0.13	0.96
	4,4,4	3	183	0.78	0.51	0.32	1.05
	4,8,8	4	143	0.57	0.50	0.21	0.97
	4,16,16	9	139	0.47	0.50	0.14	0.96
	Mean	3.4	151.2	0.49	0.53	0.18	0.98
Ground	1,4,4	10	163	**0.94**	0.33	0.75	**0.73**
	1,8,8	7	158	0.89	0.40	0.71	0.74
	1,16,16	6	157	0.89	0.42	0.72	0.73
	2,4,4	7	196	0.90	0.43	0.70	0.82
	2,8,8	7	162	0.92	0.39	0.74	0.73
	2,16,16	5	163	0.88	0.44	0.70	0.75
	3,4,4	3	186	0.83	0.46	0.52	0.91
	3,8,8	8	177	0.93	0.37	0.75	0.73
	3,16,16	7	161	0.90	0.40	0.68	0.77
	4,4,4	3	202	0.84	0.47	0.47	0.93
	4,8,8	7	185	0.93	0.40	0.71	0.76
	4,16,16	7	155	0.86	0.41	0.64	0.78
	Mean	6.4	172.1	0.89	0.41	0.67	0.78

NDF: neutral detergent fiber

R^2: the coefficient of determination

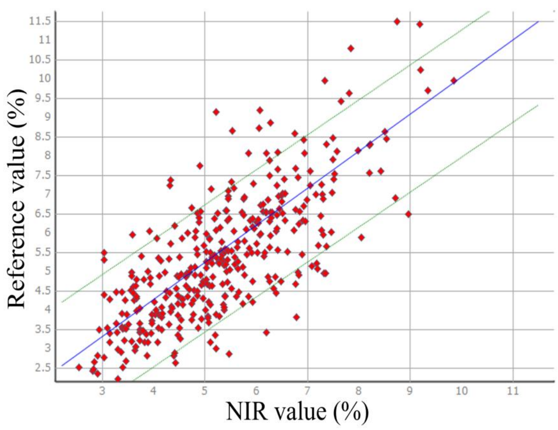
SEC: the standard error of calibration

R^2CV: the coefficient of determination of cross validation

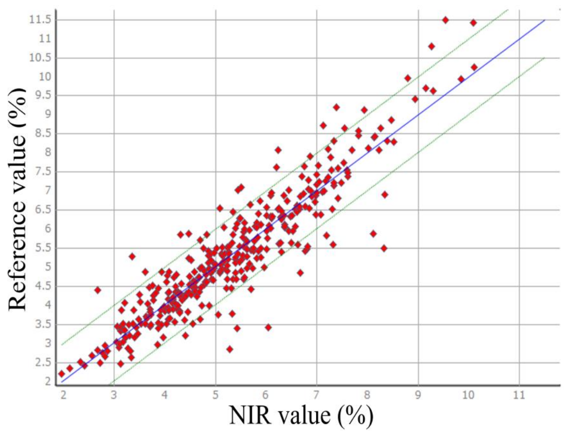
SECV: the standard error of cross validation

4.2.3.3 Crude protein

A calibration equation for CP was derived through regression analysis using the spectra of two types of samples and analysis results by combustion method. Intact alfalfa hay had the lowest SECV of 3,8,8 and ground alfalfa hay had the lowest SECV of 1,16,16 on CP. The mathematical treatments for CP are shown in Table 16. Timothy in intact form had R^2 and SECV of 0.95 and 0.58, respectively. However, timothy in ground form had R^2 and SECV of 0.98 and 0.34, respectively. Although the values in the table look the same, but the lowest value was selected by comparing up to three or four decimal places. The result of R^2 on ground timothy hay is similar with R^2 of 0.95 reported on grass forage by Winch and Major (1981). The high correlations with protein values were reported by several researchers (Norris et al., 1976; Shenk and Westerhaus, 1994; Cozzolino et al., 2000). Roberts et al. (2003) reported that these excellent results of correlation on CP are due to the strong absorption of N-H bonds in the NIR region, and the relatively high concentration of CP (30~500 g/kg, DM) in feed and forage. Figure 14 shows the correlation between NIR value and reference value of CP of timothy hay. Comparing 14a and 14b, it can be seen that the predictive ability is significantly improved after drying and grinding samples.



(a) intact timothy hay



(b) ground timothy hay

Figure 14. The correlation between NIR value and reference value of CP of timothy hay in intact and ground form

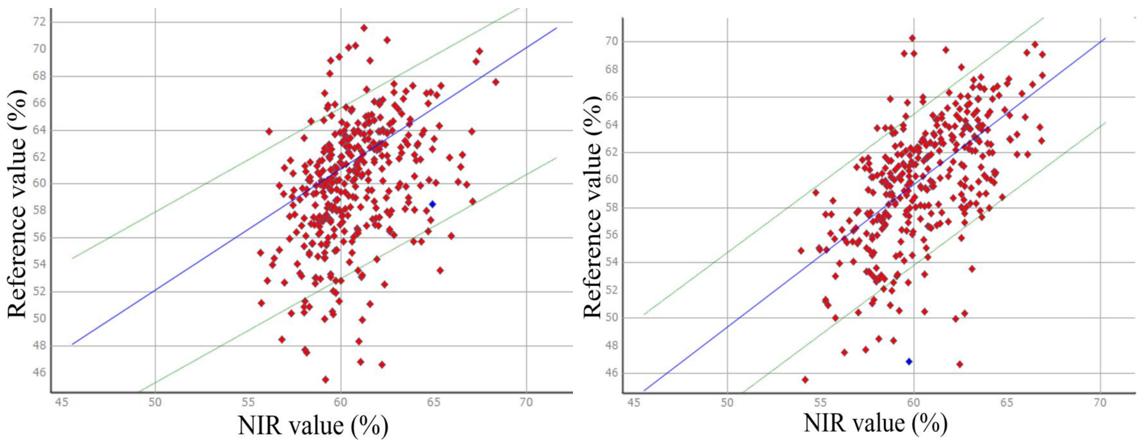
Table 16. The results of mathematical treatment on CP of timothy hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	6	175	0.92	0.36	0.76	0.65
	1,8,8	7	164	0.92	0.34	0.76	0.64
	1,16,16	7	169	0.92	0.35	0.73	0.66
	2,4,4	5	182	0.91	0.37	0.74	0.67
	2,8,8	7	175	0.94	0.34	0.78	0.63
	2,16,16	7	170	0.93	0.34	0.77	0.64
	3,4,4	6	234	0.94	0.34	0.75	0.73
	3,8,8	6	174	**0.95**	0.31	0.83	**0.58**
	3,16,16	8	168	0.93	0.34	0.80	0.60
	4,4,4	5	234	0.86	0.47	0.51	0.99
	4,8,8	6	181	0.93	0.35	0.78	0.65
	4,16,16	9	167	0.94	0.32	0.81	0.60
	Mean	6.6	182.8	0.92	0.35	0.75	0.67
Ground	1,4,4	6	174	0.98	0.19	0.95	0.35
	1,8,8	8	180	0.99	0.18	0.95	0.34
	1,16,16	9	195	**0.98**	0.19	0.95	**0.34**
	2,4,4	8	215	0.99	0.17	0.92	0.42
	2,8,8	11	207	0.99	0.17	0.94	0.37
	2,16,16	11	185	0.99	0.17	0.95	0.34
	3,4,4	6	239	0.98	0.22	0.89	0.48
	3,8,8	8	194	0.98	0.18	0.93	0.38
	3,16,16	12	206	0.99	0.18	0.95	0.34
	4,4,4	12	318	0.97	0.27	0.80	0.71
	4,8,8	6	194	0.98	0.20	0.93	0.39
	4,16,16	10	190	0.99	0.17	0.95	0.34
	Mean	8.9	208.1	0.98	0.19	0.93	0.40

CP: crude protein
R^2: the coefficient of determination
SEC: the standard error of calibration
R^2CV: the coefficient of determination of cross validation
SECV: the standard error of cross validation

4.2.3.4 *In vitro* dry matter digestibility

Intact and ground samples of timothy hay had the lowest SECV of 1,16,16 and 1,8,8, repectively, on mathematical treatment for IVDMD. The mathematical treatments for IVDMD are shown in Table 17. Timothy in intact form had R^2 and SECV of 0.61 and 2.54, respectively. However, timothy in ground form had R^2 and SECV of 0.84 and 2.07, respectively. Winch and Major (1981) reported that R^2 of finely ground sample of grass for IVDMD was 0.73, which was lower than that of legume sample (R^2 = 0.81). In this study, the R^2 of timothy samples for IVDMD was also lower than that of alfalfa samples. In addition, they also reported that the more accurate predictions were obtained in forages with higher DM (920 to 950 g/kg) than with those with lower DM (870 to 910 g/kg). After excluding 64 timothy ground samples with a water content of 9 or higher following their study, a calibration equation was developed under the same conditions. As a result, R2 was 0.87 and SECV was 2.00, which the results were better than before the 64 samples were excluded. Figure 15 shows the correlation between NIR value and reference value of IVDMD in timothy hay. Both Figure 15a and Figure 15b show the value of prediction gathered at around 60 % ignoring various values of reference data.



(a) intact timothy hay (b) ground timothy hay

Figure 15. The correlation between NIR value and reference value of IVDMD of timothy hay in intact and ground form

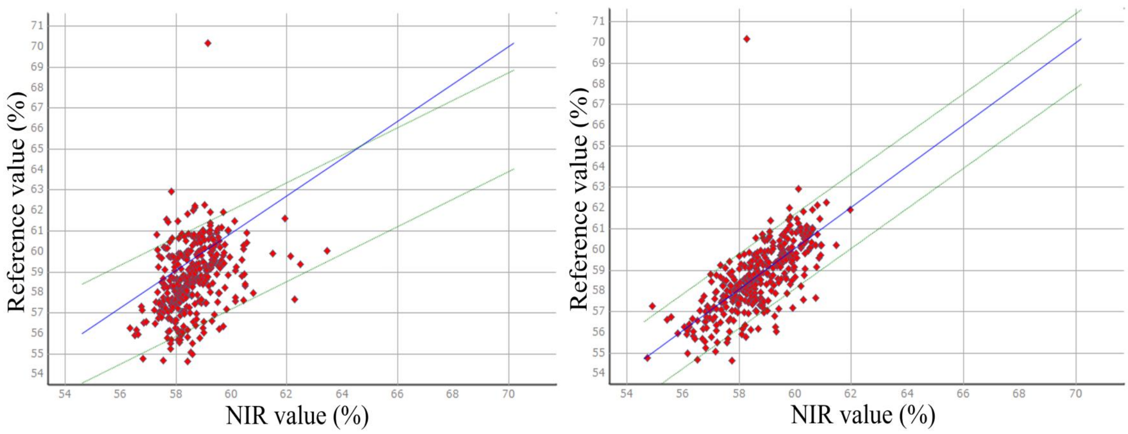
Table 17. The results of mathematical treatment on IVDMD of timothy hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	3	157	0.58	1.44	0.26	2.56
	1,8,8	3	157	0.55	1.46	0.27	2.56
	1,16,16	4	158	**0.61**	1.44	0.32	**2.54**
	2,4,4	2	166	0.60	1.55	0.31	2.65
	2,8,8	3	156	0.54	1.53	0.30	2.62
	2,16,16	7	159	0.53	1.47	0.25	2.60
	3,4,4	3	176	0.73	1.36	0.32	2.72
	3,8,8	3	173	0.56	1.62	0.26	2.70
	3,16,16	4	163	0.53	1.49	0.25	2.59
	4,4,4	3	201	0.69	1.52	0.25	2.98
	4,8,8	3	157	0.47	1.55	0.22	2.64
	4,16,16	3	156	0.50	1.45	0.21	2.66
	Mean	3.4	164.9	0.57	1.49	0.27	2.65
Ground	1,4,4	5	168	0.83	1.21	0.59	2.17
	1,8,8	8	180	**0.84**	1.15	0.62	**2.07**
	1,16,16	5	162	0.84	1.15	0.62	2.10
	2,4,4	4	181	0.86	1.15	0.61	2.12
	2,8,8	3	157	0.79	1.23	0.56	2.11
	2,16,16	3	157	0.79	0.22	0.54	2.14
	3,4,4	4	174	0.89	0.96	0.53	2.29
	3,8,8	3	153	0.82	1.21	0.63	2.08
	3,16,16	2	157	0.80	1.22	0.61	2.09
	4,4,4	2	194	0.73	1.55	0.44	2.64
	4,8,8	3	159	0.82	1.25	0.61	2.13
	4,16,16	3	156	0.81	1.22	0.61	2.08
	Mean	3.8	166.5	0.82	1.13	0.58	2.17

IVDMD: *in vitro* dry matter digestibility
R^2: the coefficient of determination
SEC: the standard error of calibration
R^2CV: the coefficient of determination of cross validation
SECV: the standard error of cross validation

4.2.3.5 Total digestible nutrients

Intact timothy hay had the lowest SECV of 1,16,16 mathematical treatment and ground timothy hay had the lowest SECV of 4,16,16 mathematical treatment on TDN. The mathematical treatments for TDN are shown in Table 18. Timothy in intact form had R^2 and SECV of 0.75 and 0.86, respectively. However, timothy in ground form had R^2 and SECV of 0.94 and 0.65, respectively. Because TDN was calculated using ADF concentration, the R^2 values and mathematical treatment of TDN and ADF in ground timothy hay were similar. Figure 16 presents the correlation between NIR value and reference value of TDN of timothy hay. Figure 16a shows that the prediction value is poor because the predicted values are concentrated at 57 to 61 %, ignoring various reference values. However, the predicted values of Figure 16b formed a trend line and showed better prediction results.



(a) intact timothy hay   (b) ground timothy hay

Figure 16. The correlation between NIR value and reference value of TDN of timothy hay in intact and ground form

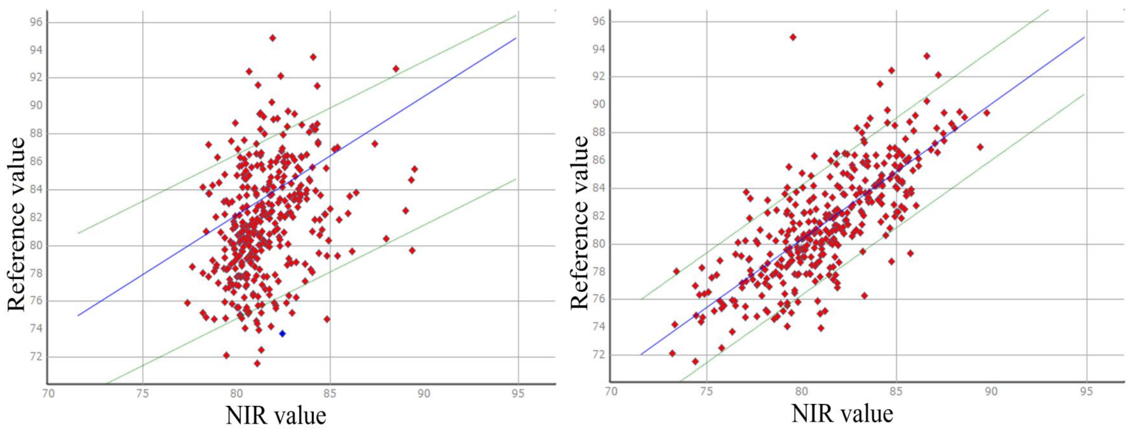
Table 18. The results of mathematical treatment on TDN of timothy hay in intact and ground form

Pretreatment	Mathematical treatment	No. of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	5	155	0.67	0.45	0.34	0.87
	1,8,8	7	160	0.66	0.46	0.33	0.88
	1,16,16	16	159	**0.75**	0.40	0.42	**0.86**
	2,4,4	3	172	0.74	0.50	0.43	0.89
	2,8,8	3	154	0.69	0.48	0.36	0.88
	2,16,16	6	155	0.74	0.45	0.40	0.87
	3,4,4	2	178	0.75	0.53	0.42	0.96
	3,8,8	3	173	0.73	0.53	0.45	0.88
	3,16,16	4	162	0.74	0.49	0.45	0.87
	4,4,4	2	185	0.69	0.59	0.28	1.07
	4,8,8	5	167	0.78	0.47	0.45	0.88
	4,16,16	7	159	0.71	0.51	0.43	0.89
	Mean	5.3	164.9	0.72	0.49	0.40	0.90
Ground	1,4,4	13	164	0.97	0.22	0.79	0.66
	1,8,8	10	162	0.94	0.34	0.78	0.67
	1,16,16	12	167	0.94	0.34	0.78	0.66
	2,4,4	8	217	0.95	0.32	0.76	0.72
	2,8,8	8	175	0.93	0.35	0.77	0.67
	2,16,16	15	164	0.95	0.28	0.76	0.69
	3,4,4	5	238	0.92	0.42	0.72	0.77
	3,8,8	10	181	0.96	0.28	0.76	0.69
	3,16,16	8	176	0.91	0.38	0.75	0.68
	4,4,4	6	255	0.92	0.40	0.69	0.79
	4,8,8	16	161	0.99	0.12	0.73	0.71
	4,16,16	10	169	**0.94**	0.33	0.77	**0.65**
	Mean	10.1	185.8	0.94	0.32	0.76	0.70

TDN: total digestible nutrients
R^2: the coefficient of determination
SEC: the standard error of calibration
R^2CV: the coefficient of determination of cross validation
SECV: the standard error of cross validation

4.2.3.6 Relative feed value

Among the various mathematical treatment results, both intact and ground timothy hay had the lowest SECV of 1,8,8 on RFV. The mathematical treatments for RFV are shown in Table 19. Timothy in intact form had R^2 and SECV of 0.61 and 2.12, respectively. However, timothy in ground form had R^2 and SECV of 0.96 and 1.52, respectively. Kim et al. (2019) reported that R^2 and SECV for whole crop rice were 0.88 and 5.21, respectively. These values on their study were poorer than other constituents including CP, ADF, NDF and ash content. Figure 17 also shows the correlation between NIR value and reference value of RFV in timothy hay. After drying and grinding samples, the graph was changed that the correlation values draw a trend line.



(a) intact timothy hay (b) ground timothy hay

Figure 17. The correlation between NIR value and reference value of RFV of timothy hay in intact and ground form

Table 19. The results of mathematical treatment on RFV of timothy hay in intact and ground form

Pretreatment	Mathematical treatment	No of factors	n	Calibration		Validation	
				R^2	SEC	R^2CV	SECV
Intact	1,4,4	3	148	0.52	1.19	0.24	2.18
	1,8,8	7	147	**0.61**	1.09	0.29	**2.12**
	1,16,16	3	145	0.49	1.17	0.23	2.17
	2,4,4	3	156	0.70	1.21	0.38	2.14
	2,8,8	2	150	0.64	1.24	0.31	2.22
	2,16,16	2	148	0.65	1.19	0.31	2.15
	3,4,4	3	168	0.79	1.11	0.43	2.32
	3,8,8	4	163	0.70	1.24	0.35	2.21
	3,16,16	5	159	0.67	1.20	0.30	2.26
	4,4,4	3	205	0.80	1.24	0.40	2.37
	4,8,8	3	146	0.72	1.17	0.36	2.18
	4,16,16	4	152	0.63	1.29	0.32	2.22
	Mean	3.5	157.3	0.66	1.20	0.33	2.21
Ground	1,4,4	7	170	0.93	0.86	0.79	1.58
	1,8,8	12	160	**0.96**	0.70	0.80	**1.52**
	1,16,16	5	163	0.92	0.92	0.78	1.57
	2,4,4	10	203	0.97	0.55	0.79	1.64
	2,8,8	7	164	0.94	0.82	0.77	1.57
	2,16,16	6	157	0.93	0.89	0.78	1.56
	3,4,4	6	213	0.96	0.70	0.75	1.73
	3,8,8	8	207	0.94	0.83	0.81	1.55
	3,16,16	6	162	0.93	0.85	0.78	1.56
	4,4,4	6	251	0.93	0.88	0.63	2.12
	4,8,8	6	162	0.92	0.82	0.72	1.70
	4,16,16	8	163	0.94	0.83	0.78	1.57
	Mean	7.3	181.3	0.94	0.80	0.77	1.64

RFV: relative feed value
R^2: the coefficient of determination
SEC: the standard error of calibration
R^2CV: the coefficient of determination of cross validation
SECV: the standard error of cross validation

5. Conclusion

The results of the development of the calibration equations were good and can be used in field. The results of intact form were worse than those of ground form. However, intact form scanning method has a merit which drying and grinding processes can be omitted. Therefore, intact form scanning method can be used in field for quick and rough prediction rather than exact prediction. The calibration results of intact alfalfa hay in this study were poorer when compared to previous reports, most likely because the leaf part of the alfalfa hay was powdered and covered in the scanning cup. This problem could be overcome if the hay is firmly fixed and scanned by portable NIRS.

The results of the development of the calibration equations for ground form were good and most of R^2 were higher than 0.9. Because of the high accuracy of ground form method, It may be used for accurate prediction in the field. The R^2 values for ground timothy were high with exception of IVDMD likely because of the high moisture content of the samples. Therefore, for more accurate prediction of IVDMD, it may be necessary to adjust the moisture content to less than 5% before scanning and to control the humidity of laboratory. Digestibility is also closely related to the condition of animals, therefore, it is necessary to control the environment, such as maintaining a diet with constant ingredients, during the experiment.

Intact alfalfa hay had no trend on derivative. However, ground alfalfa hay had the best results on the fourth derivative except of moisture content and NDF which had the best results on the second and first derivatives, respectively. Intact timothy hay had the best results of calibration on the first derivative except of moisture content and CP which had the best results in the third derivative. However, ground timothy hay had no trend on derivative.

Overall, using the samples in dry and ground forms was the optimal condition to evaluate the feed value of imported hay using NIRS. Furthermore, after improving sample storage and sorting, intact alfalfa hay calibration must be redeveloped. Further studies should be conducted to confirm the relationship between each constituents and the derivative.

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7. Abstract in Korean

현재 우리나라는 조사료 수입에 크게 의존하고 있다. 그러나 조사료를 수입하여 최종 구매자에게 도달하기까지 품질분석이 이뤄지지 않고 있는 상황이다. 그 이유로는 조사료의 품질분석이 법적으로 의무화 되어있지 않고, 분석 과정이 복잡하고, 시간이 오래 걸리며 그리고 비용이 많이 들기 때문이다. 따라서 본 연구는 근적외선분광법 (near infrared reflectance spectroscopy; NIRS)을 이용하여 수입 건초의 신속하고 저렴한 품질 분석을 위한 검량식을 개발하기 위해 수행되었다.

알팔파 건초 227 점과 티모시 건초 360 점을 전국에서 수집하였다. 건조 후 분쇄한 시료의 NIRS 검량 결과가 뛰어나지만, 본 연구에서는 건조와 분쇄의 과정을 생략하고 생 시료의 가능성을 확인하고자 두 시료를 비교하였다. 회귀 방법으로 부분 최소 제곱법(partial least squares; PLS)을 사용하였으며 정확한 검량식을 개발하기 위하여 다양한 수처리 기법을 사용하였다. 교차 검증 표준 오차(standard error of cross validation; SECV)가 검량식을 평가하는 지표로 사용되었으며, 결정계수(coefficient of determination; R^2)가 습식 분석값과 예측값의 유사도를 나타내는 지표로 사용되었다.

수분 함량, acid detergent fiber (ADF), neutral detergent fiber (NDF), crude protein (CP), *in vitro* dry matter digetibility (IVDMD), total digestible nutrients (TDN) 및 relative feed value (RFV)에 대한 알팔파 생 시료의 R^2의 값은 각각 0.74, 0.42, 0.68, 0.89, 0.52, 0.68 및 0.54 였으며, 알팔파 분쇄 시료에 대한 R^2의 값은 각각 0.84, 0.90, 0.97, 0.92, 0.95, 0.97 및 0.93 였다. 수분 함량, ADF, NDF, CP, IVDMD, TDN 및 RFV 에 대한 티모시 생 시료의 R^2의 값은 각각 0.48, 0.95, 0.75, 0.38, 0.61, 0.75 및 0.61 였으며, 티모시 분쇄 시료의 R^2의 값은 각각 0.95, 0.98, 0.94, 0.94, 0.84, 0.94 및 0.96 였다. 한편 알팔파 분쇄 시료와 티모시 생 시료는 각각 4차 도함수와 1차 도함수에서 가장 좋은 결과를 나타내었지만, 다른 시료들은 도함수의 추세를 보이지 않았다.

검량식의 개발 결과로 보아 현장에서 사용할 수 있을 것이라 판단된다. 생 시료 스캔 방법은 분쇄 시료 스캔 방법 보다 R^2의 값이 낮지만 건조 및 분쇄 과정을 생략하고 현장에서 빠르고 대략적인 수입 조사료의 성분 분석을 예측할 수 있었다. 알팔파 생 시료의 경우 잎 부분이 가루가 되어 스캔 컵을 덮는 현상이 발생하기 때문에 추후 연구에서는 알팔파 생 시료

를 단단하게 고정하여 휴대용 NIRS를 이용하여 스캔하면 더 나은 검량식 결과를 유도할 수 있을 것으로 판단된다. 한편 분쇄 시료 스캔 방법은 대부분 성분의 R^2의 값이 0.9 보다 높아 좋은 정확도를 나타내었다. 소화율에 대한 티모시 분쇄 시료의 결과가 비교적 낮았는데, 이는 샘플의 수분을 5 % 미만으로 조절하여 더 나은 검량식을 개발한 이전의 보고를 따라 추후 연구에서 더 좋은 결과를 얻을 수 있을 것이라 판단된다.

핵심 단어: 수입 조사료, 알팔파, 티모시, 근적외선 분광법
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