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Determination of Protein Content in Pea by Near Infrared Spectroscopy

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Abstract: Near infrared reflectance spectroscopy (NIRS) was used as a rapid and non-destructive method to determine the protein content in intact and ground seeds of pea (*Pisum sativum* L.) germplasms grown in Korea. A total of 115 samples were scanned in the reflectance mode of a scanning monochromator at intact seed and flour condition, and the reference values for the protein content was measured by auto-Kjeldahl system. In the developed ground and intact NIRS equations for analysis of protein, the most accurate equation were obtained at 2, 8, 6, 1 math treatment conditions with standard normal variate and detrend scatter correction method and entire spectrum (400-2,500 nm) by using modified partial least squares regression (n=78). External validation (n=34) of these NIRS equations showed significant correlation between reference values and NIRS estimated values based on the standard error of prediction (SEP), R^2, and the ratio of standard deviation of reference data to SEP. Therefore, these ground and intact NIRS equations can be applicable and reliable for determination of protein content in pea seeds, and non-destructive NIRS method could be used as a mass analysis technique for selection of high protein pea in breeding program and for quality control in food industry.

Keywords: pea, near infrared reflectance spectroscopy (NIRS), protein, non-destructive analysis, modified partial least squares

Introduction

Peas (*Pisum sativum* L.) are cultivated for the fresh green seeds, tender green pods, dried seeds, and foliage (1). Green peas are eaten cooked as a vegetable and are marketed fresh, canned, or frozen while ripe dried peas are used whole, split, or made into flour (2). In some parts of the world, dried peas are consumed split as roasted, parched, or boiled. Green peas are the number one processed vegetable specifically in UK and USA. Green foliage of garden pea is also used as vegetable in parts of Asia and Africa. Leaves are used as a pot herb in Myanmar and parts of Africa (3).

Peas are of great nutritional importance due to their high content of protein, complex carbohydrates, dietary fiber, minerals, vitamins, and antioxidant compounds. Although peas are widely used in animal nutrition (4), human consumption of peas is lower than that of other traditionally more accepted pulses (4,5). Nevertheless, in recent years, the wealth of nutrients available from the pea and its beneficial functional properties have prompted increasing interest and demand for this legume for the food preparation oriented to geriatric and infant nutrition (6).

Legume seeds are of prime importance in human and animal nutrition due to their high protein content (20-50%). Their protein content is twice the level found in cereal grains and significantly more than the level in conventional root crops. Dry legumes are important ingredient of diet in many parts of the world and have been considered as the most significant food sources for people of low incomes (7). Legumes have historically been utilized mainly as whole seeds. However, in recent years, interest has grown in the utilization of legumes in other forms (e.g., like flour, concentrate, isolate) rather than the whole seeds (8). The use of plant protein products in food as functional ingredients to improve the stability and texture as well as the nutritional quality of the product or for economic reasons is much extended.

The protein concentration of peas ranged from 15.5-39.7% (2,9). Fresh green peas contain 6.2 g protein/100 g, while dried peas and flours contain 22.9 g protein (1). Pea seed storage proteins are mainly composed of globulins and albumins. The commercial processes that are employed to isolate pea proteins tend to form a product rich in globulins, which is heterogenic and composed of legumins (11S) and vicilins (7S) (10).

Recently, more emphasis has been placed on breeding for improving the pea protein content, but the lake of a fast, efficient mass screening method to determine protein content has slowed breeding progress. For measuring protein content in pea seeds, the Kjeldahl method is widely utilized. However, this method and related methodologies are relatively complicated and time consuming and involved corrosive chemicals and required elaborate laboratory facilities, which has deterred use in many breeding programs (11,12). Due to these difficulties, rapid and less hazardous methods, such as the use of near infrared reflectance spectroscopy (NIRS), needed to be developed to estimate protein contents in pea seeds.

The NIRS is a multi-trait technique that fulfills most of the requirements for rapid, accurate, and cost-effective mass screening for several seed quality traits in many crops (13-15). NIRS was first used to measure moisture content

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in soybean and NIRS has been used to measure moisture, protein, oil, and starch contents in many cereals, legumes, forages, and other food commodities over the past 20 years (16-18).

NIRS is known to play a fundamental role in the simplification of the analysis of chemical and physical properties without sample preparation. NIRS was used to analyze quality characteristics in food and agricultural commodities (19,20). NIRS has also been used for the determination of diverse compounds and classifications of numerous foods and industrial crops, such as sesame (21,22), soybean (23), peanut (15), sunflower (24), rice (25,26), maize (27,28), and sweet potato (29). However, application of the NIRS to determine protein content in pea seeds has not been previously reported.

The objectives of this study were to investigate NIRS application for predicting protein content in pea flours and intact pea seeds, and to develop a massive screening technique for use with the quality test and breeding program of pea.

Materials and Methods

Pea seed samples A total of 115 seed samples of pea germplasm were used in this study. The peas were grown at the experimental field of National Yeongnam Agricultural Research Institute, Rural Development Administration (Milyang, Korea).

Seed samples were measured as intact seed by a NIRS system, and then pea seeds were ground with a micro hammer mill and sieved with a 1.0-mm screen. Then, the powdered samples were well mixed and used for scan of NIRS spectral data and analysis of protein contents by standard Kjeldahl method.

Reference analysis of protein content Auto Kjeldahl system was applied to determine the protein contents of the pea seeds. Two-hundred mg of ground sample were digested by digestion system (Buchi B-435; Buchi, Switzerland) and Buchi B-412 scrubber with 20 mL of sulfuric acid and 3 g of catalyst ($CuSO_4$:K_2SO_4=1:9). Percent nitrogen was calculated by Buchi B-339 auto Kjeldahl system and then converted to % protein by multiplying 6.25. The moisture content was analyzed by oven-dry method with 105°C for 2 hr, and then all protein content was estimated on the dry basis.

Spectra collection and pretreatment The NIR spectroscopic analysis was performed using a NIRS system (model 6500 monochromator; Foss NIRS Systems Inc., Silver Spring, MD, USA) in the reflectance mode. About 5 g of intact seed samples were placed in a reflectance vessel of horizontal module and scanned, but ground samples (about 2 g) were placed in a standard small ring cup of transport module and scanned. Reflectance energy readings were referenced to corresponding readings from an internal ceramic disk.

Each spectrum was recorded from each sample, and the average of 32 successive scans was recorded. As a control, 16 scans over the standard ceramic disk were made before and after the samples. All spectral data were recorded as the logarithm of the reciprocal of reflectance (log 1/R) in the wavelength range from 400 to 2,500 nm at 2 nm intervals to give a total of 1,050 data points/sample. Absorption of radiation in the region of 400-2,500 nm, the visible plus near infrared region, was used to develop calibration equations related to sample properties.

The scanning procedure could be completed in 1 min/sample, once the NIRS instrument was warmed up, and the stability of NIRS through photometric repeatability (noise test) and wavelength accuracy test was confirmed.

The NIRS manipulation for scanning, mathematical processing, and statistical analysis was performed with the WinISI II software (Windows version 1.50, Foss and Infrasoft International LLC, State College, PA, USA).

In WinISI software, the Score program was used to select samples for spectrum outliers and the samples to represent the entire sample set (n=115) before calibration and validation. The distance between a sample and its neighbor was measured as the H distance and was used as a criterion for selecting those samples representing the calibration and validation sets. The Score algorithm ranks spectra according to Mahalanobis distance (H distance) from the average spectrum, gives spectral boundaries to eliminate outliers with H>3.0, and eliminates samples with similar spectra with H<0.6 from their nearest neighboring samples for the development of an accurate and robust prediction equation (30). The final number of samples for calibration and validation was variable and based on the cutoff point of H distance, depending on the spectral and chemical variability of samples in the population used for NIRS estimation.

The samples (n=115) were randomly split into 2 sets using the WinISI program. The calibration set (78 samples) was used to calibrate and cross-validate the derived equation, and the other 37 samples were used as an external validation set to test the fit of the developed equations.

Data processing The equations for NIRS prediction were developed using the Global program in WinISI software with modified partial least squares (MPLS) regression using wavelengths of the entire visible (400-1,100 nm) and near infrared (1,100-2,500 nm) regions at every 2 nm. Besides MPLS, regression methods such as partial least squares (PLS), principal component regression (PCR), and multiple linear regression (MLR) were tested to develop calibration for protein content in the pea samples. Various mathematical treatments using the raw optical spectrum (log 1/R), or first or second derivatives of the 1/R data, were applied for calibration equation development. For example, in 2, 8, 6, 1, the 1st number is the second derivative of log 1/R, the 2nd number is the gap in data points over which the derivative was calculated, and the 3rd and 4th numbers represent the number of data points used in 1st and 2nd smoothing, respectively (31). The application of the second-derivative algorithm to the raw spectra (log 1/R) resulted in an increase in the complexity of spectra and a clear separation between peaks, which overlapped in the raw spectra. In addition to no scatter correction (log 1/R), scatter corrections using standard normal variate and detrending (SNVD) were evaluated for the calibration. The SNVD was designed to remove additive baseline and multiplicative signal effects resulting in a spectrum with zero mean and a variance equal to one. Application of

SNVD transformation to raw spectral data reduces the differences in spectra related to physical characteristics such as particle size and path length of the samples (32,33).

Calculated calibration statistics included the standard error of calibration (SEC), the coefficient of determination in calibration (R^2), and the standard error of cross-validation (SECV). The performance of the different equations obtained in the calibration was determined from cross-validation as an internal validation method. Internal cross-validation was used to avoid overfitting of the equations by selecting the minimum number of PLS terms in each model. The best predicted equations for protein content was selected on the basis of minimizing SECV and increasing R^2. Two passes to eliminate outliers were set by 2 outlier detection methods, t and H statistics (Mahalanobis distance) in WinISI software. The t statistics identified outliers having residuals from reference analysis of >2.5 times the SEC. Outliers indicated that their reference values were in doubt and that the samples were in different populations due to atypical spectra. The ratio of the standard deviation (SD) of reference data to SECV, designated RSC, was calculated as a criterion for evaluating the performance of calibrations.

After calibration, the developed regression equations allowed for accurate analysis of many other samples by prediction of data based on the spectra. In addition to the internal cross-validation, the external validations of calibration models were tested for the prediction capacity on the basis of the standard error of prediction (SEP) and the coefficient of determination in prediction (r^2). The ratio of SD for the validation samples to the corrected SEP (designated RSP) was also used as a criterion to evaluate the accuracy of the equations. This RSP value as cutoff point was 3.0 in this study, which is the value recommended for screening purposes. The validation sample set allowed the NIRS equation to be validated for prediction accuracy, using random samples not included in the calibration sample set. The equations selected for protein content in seeds of pea were monitored with the Monitor program in WinISI software, using the validation set (n=37).

Results and Discussion

Protein contents and NIRS spectra The mean protein content of the calibration sample set was 29.54% (range: 22.70 to 33.43%) with a SD of 2.12% as determined by auto-Kjeldahl system (Table 1). There was significant difference among the 78 pea samples for protein content based on their SD. This result suggests that sufficient protein variation exist among the samples to develop useful NIRS equations. And the mean, range, and SD of protein contents in validation sample set were similar to calibration sample set (Table 1).

The log (1/R) spectra of the ground pea samples with the high and low contents of protein are shown in Fig. 1. The patterns of the spectra were seen some differences in each

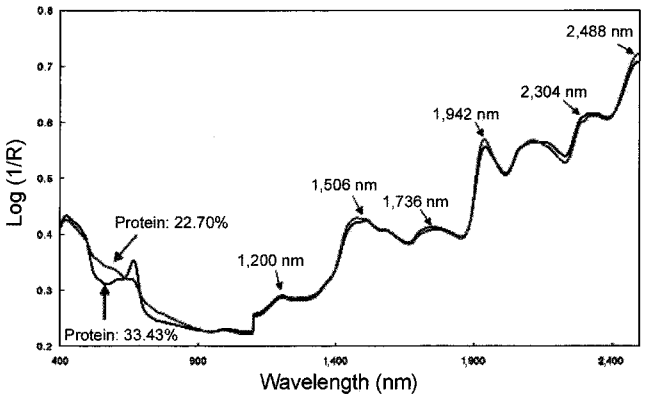

Fig. 1. Raw spectra of NIRS with different protein content in the ground pea seed samples.

peaks between high and low protein content samples, which might be from various chemical differences. The main absorption bands are observed at 1,200 nm related to C-H stretching 2nd overtone, 1,506 nm related to N-H stretching 1st overtone (protein), 1,736 nm related to S-H stretching 1st overtone, 1,936 nm related to O-H bend 2nd overtone (water), 2,304 nm related to C-H bend 2nd overtone (protein), and 2,488 nm attributed to C-H and C-C stretching. The information for the functional groups in the spectrum was determined using WinISI software. Compared with references, absorption bands at 1,936 nm are associated with water, the band at 1,506, 1,736, and 2,304 nm is associated with protein. The overall spectrum shows strong absorption bands related with protein and water, and is similar to that of other legume crop such as soybean, especially in the near infrared region (34).

The spectral differences for math treatment effect are shown in Fig. 2. Figure 3 shows the D^2 log (1/R) spectra and mean standard deviation spectrum of calibration samples that are obtained by using the entire wavelength range of 400-2,500 nm. Here, except for visible region, several high standard deviation peaks (about 900, 1,158, 1,350, 1,396, 1,688, 1,746, 1,848, 1,890, 2,246-2,314 nm) are closely connected with the functional groups (C-H, N-H, O-H, and C=O), those peaks act to the NIRS calibration of protein.

Calibration and validation analysis for protein content in the ground pea samples From the development of NIRS models for the protein content in ground pea samples, the statistics of calibrations and cross-validations are shown in Table 2. The MPLS regression model in the whole NIR spectra range (400-2,500 nm) using SNVD of raw reflectance spectra yielded the equations for protein, showing higher R^2 (0.968) and lower SEC in the calibrations and higher 1-VR and lower SECV values in the cross-validations than the different derivative transformation and

Table 1. Laboratory reference value statistics for protein content in the pea samples

Sample set		N	Mean (%)	Range (%)	SD
Calibration	Protein	78	29.54	22.70-33.43	2.12
Validation	Protein	37	28.91	23.71-33.28	2.44

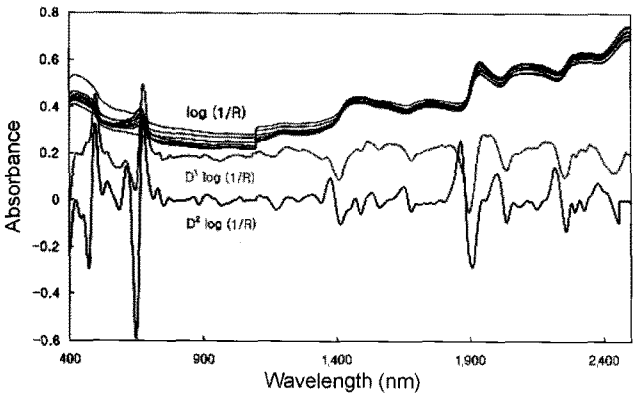

Fig. 2. Raw, 1st (1, 4, 4, 1), and 2nd (2, 8, 6, 1) derivative spectra of the ground pea seed samples.

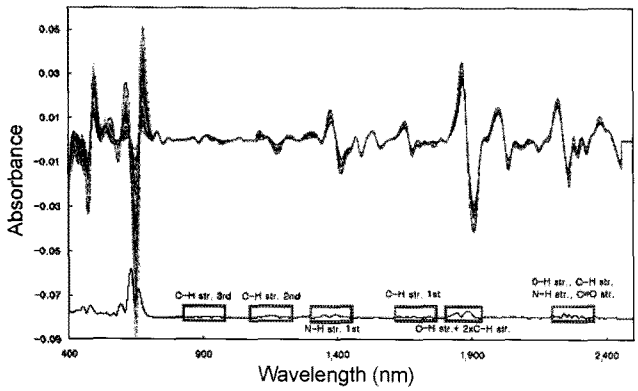

Fig. 3. Second derivative and mean standard deviation spectra of the ground pea seed calibration sample set.

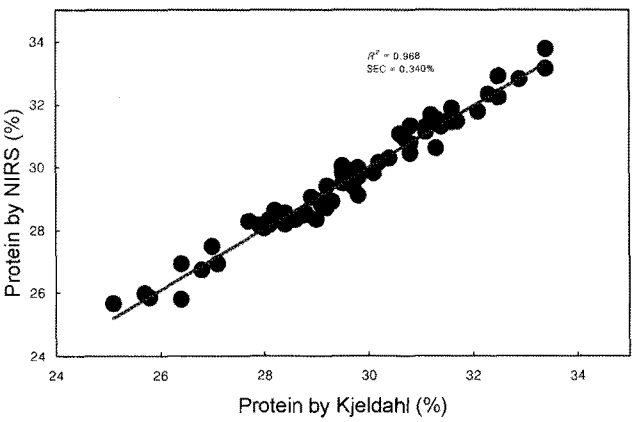

Fig. 4. Scatter plots of protein content in the ground pea samples by Kjeldahl vs. by NIRS for the calibration sample set.

regression methods tested in this study. Using this whole vis-NIR range (400-2,500 nm), higher R^2 and lower SEC values were obtained, better than the visible range (400-1,100 nm) and the near infrared range (1,100-2,500 nm).

Optimum wavelengths for NIR analysis have generally relied on empirical calibrations to predict qualitative constituents for agricultural products because of the broad array of chemical compounds present in the samples, which lead to extensively overlapping and perturbed NIR absorption bands. The equation for protein using mathematical treatment 2, 8, 6, 1 was selected with higher RSC (SD/SECV) values as the selection criteria of models, rather than the different mathematical treatments. The reliable equation for protein content in the ground pea samples had high value of R^2 (0.968) and RSC (5.088), indicating a close relationship between reference values and NIRS estimated values (Fig. 4). This equation for protein had good accuracy, similar to that of other protein crops (34), with higher R^2 and lower SEC values.

The robustness of the calibration model developed by NIRS analysis was tested through external validation with 37 samples, which were not included in the calibration process. The statistics of external validation for protein in ground pea samples are shown in Table 3 and include r^2, SEP(C) (the corrected standard error of prediction), and RSP [SD/SEP(C)] values, which were factors used to evaluate the reliability of the calibration model. On the basis of lower SEP(C) and higher r^2 and RSP values, an accurate prediction can be monitored with the reliability of the established calibration model. The validation for protein content was confirmed by higher r^2 (0.978) and RSP values (6.513), indicating a good correlation between reference values and NIRS predicted values in the application of the calibration equations (Fig 5).

Table 2. Equation development statistics using MPLS and scatter correction for the NIRS prediction of protein content in the ground pea samples

Transformation	Terms[1)]	Calibration		Cross-validation		
		SEC	R^2	1-VR	SECV	RSC
0, 0, 1, 1	7	0.458	0.955	0.939	0.543	3.907
1, 4, 4, 1	6	0.392	0.964	0.959	0.453	4.683
2, 8, 6, 1	5	0.340	0.968	0.961	0.417	5.088

[1)]Number of PLS loading factors in the regression model MPLS; 1-VR, one minus the ratio of unexplained variance divided by variance.

Table 3. External validation statistics for protein content in the ground pea samples[1)]

N	Mean	SD	Bias	r^2	SEP(C)	Slope	RSP
37	28.91	2.44	-0.125	0.978	0.374	1.038	6.513

[1)]N, number of samples used to monitor the model; SD, standard deviation of mean; bias, average difference between reference and NIRS values; slope, steepness of a straight line curve.

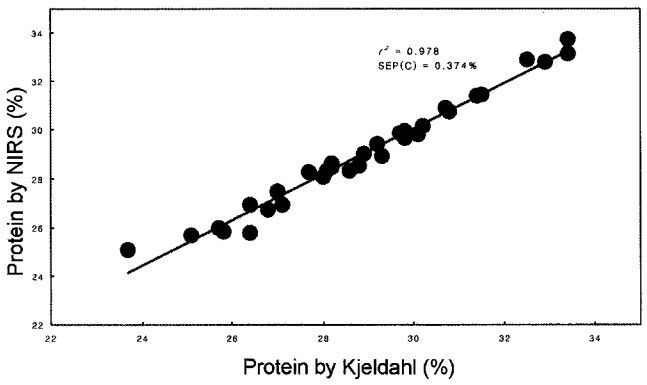

Fig. 5. Scatter plots of protein content in ground pea samples by Kjeldahl vs. by NIRS for the external validation sample set.

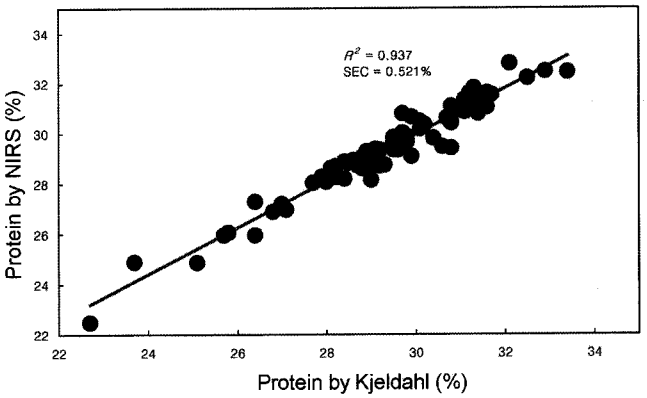

Fig. 6. Scatter plots of protein content in intact pea samples by Kjeldahl vs. by NIRS for the calibration sample set.

Calibration and validation analysis for protein content in intact pea samples Table 4 shows the best NIRS equation statistics of protein content obtained from intact pea seed samples. The best equation condition was obtained at 2, 8, 6, 1 (2nd derivative, 8 nm gap, 6 points smoothing, and 1 points second smoothing) math treatment condition with SNVD scatter correction method and entire spectrum (400-2,500 nm) region (Fig. 6).

Based on several prediction statistics, the best intact seed NIRS equation of protein analysis using MPLS method equation (2, 8, 6, 1; SNVD; 400-2,500 nm) was well predicting the protein contents of external validation sample set, and the SEP(C) value and R^2 of prediction were 0.409 and 0.943%, respectively (Table 5 and Fig. 7). This result indicates that the non-destructive NIRS analysis can be also used as an effective method for measuring pea protein contents.

It is concluded that the determination of protein content can be predicted with reliable accuracy using NIRS analysis of ground and intact pea seed samples. However, the non-destructive NIRS (using intact seed samples) protein analysis equation did not predict the calibration set so accurately as the destructive NIRS (using flour samples) protein equations in this study.

This NIRS method could simplify the analysis of qualitative factors of interest because extraction steps with organic solvents were not required and samples were easily analyzed in a few minutes with or without grinding. Although the information from the NIR spectra tends to be somewhat complicated and difficult to interpret, each chemometric technique establishes a mathematical relationship between variations in the NIR spectra and parameters measured for each sample. This relationship can therefore be used to predict the parameter value in unknown samples. Consequently, it is a very reliable method for predicting

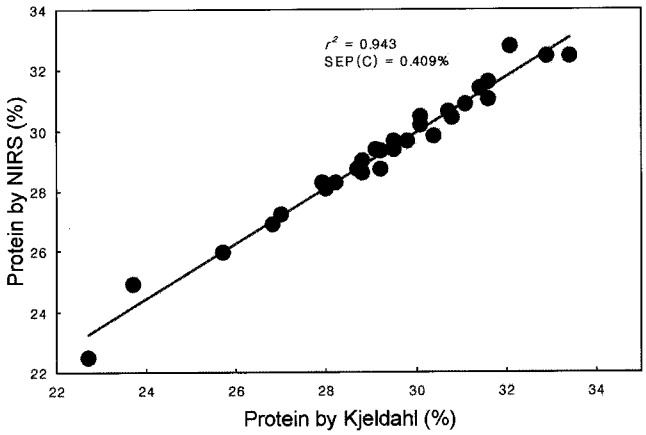

Fig. 7. Scatter plots of protein content in intact pea samples by Kjeldahl vs. by NIRS for the external validation sample set.

Table 4. Equation development statistics using MPLS and scatter correction for the NIRS prediction of protein content in intact pea samples

Transformation	Terms[1)]	Calibration		Cross-validation		
		SEC	R^2	1-VR	SECV	RSC
0, 0, 1, 1	5	0.906	0.784	0.673	1.111	1.928
1, 4, 4, 1	8	0.650	0.893	0.783	0.929	2.306
2, 8, 6, 1	8	0.521	0.937	0.822	0.887	2.415

[1)]Number of PLS loading factors in the regression model MPLS; 1-VR, one minus the ratio of unexplained variance divided by variance.

Table 5. External validation statistics for protein content in intact pea samples[1)]

N	Mean	SD	Bias	r^2	SEP(C)	Slope	RSP
37	28.91	2.44	-0.005	0.943	0.409	1.062	5.966

[1)]N, number of samples used to monitor the model; SD, standard deviation of mean; bias, average difference between reference and NIRS values; slope, steepness of a straight line curve.

samples similar to those used in the calibration. NIRS also has the main advantage that it may be performed simultaneously to the estimation of other quality components such as protein, water, and functional compounds, in addition to oil and individual fatty acids. These analytical characteristics were critical factors for the quality evaluation of nutritional food, the selection of superior breeding lines, and the identification of new germplasm. For the analysis of numerous samples, the NIRS method is capable of replacing the standard analytical methods such as high performance liquid chromatography (HPLC), gas chromatography (GC), Kjeldahl, and soxhlet etc. However, samples very different from the calibration samples are often not predicted reliably. This is likely to be due to different conditions such as species, cultivation area, drying and storage methods, and influences of genetic and environmental variation.

This is the first reported study of a NIRS calibration model developed for the estimation of protein content of pea seed samples. The development of these NIR equations for protein represents only a first step: although NIRS is a practical method, the equations should be updated, expanded, and improved with future samples from different environments and germplasms, and covering a wider range of protein content of the samples used for the calibration models.

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