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RESEARCH ARTICLE

# Determination of Protein, Fat, Starch, and Amino Acids in Foxtail Millet [*Setaria italica* (L.) Beauv.] by Fourier Transform Near-Infrared Reflectance Spectroscopy

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**Abstract** Quantitative detection of protein, fat, starch, and amino acids in foxtail millet using Fourier transform near-infrared spectroscopy (NIRS) was investigated. Foxtail millet samples ($n$=259) were analyzed using NIRS. Spectral data were linearized with data from chemical analyses. Calibration models were established using a partial least-squares (PLS) algorithm with cross-validation. Optimized models were tested using external validation set samples with coefficients of determination in the external validation ($R^2_{val}$) of >0.90. Residual predictive deviation (RPD) values were nearly equal to or >2.5 for crude protein, alanine, aspartic acid, glutamic acid, isoleucine, leucine, and serine. However, for glycine, histidine, phenylalanine, proline, threonine, tyrosine, and valine, the $R^2_{val}$ values were >0.83 and RPD values were nearly equal to or >2.0. For crude fat, total starch, arginine, and lysine, the $R^2_{val}$ values were >0.70 and RPD values were >1.5. NIRS is a rapid determination tool for foxtail millet breeding, and for quality control.

**Keywords:** foxtail millet, protein, starch, amino acid, NIRS

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## Introduction

Foxtail millet [*Setaria italica* (L.) Beauv.] is an important nutritious food in traditional diets, especially for people in China, Japan, and India (1). In northern China, it is widely used as a nourishing gruel or soup for pregnant and nursing women (2). The main components of foxtail millet include starch, protein, amino acids, fat, vitamins, and minerals (3-6). Foxtail millet also has been used as a Chinese traditional medicine to invigorate the stomach, strengthen the spleen, and quench thirst (7). In recent years, more foxtail millet products, including millet porridge, millet wine, and millet nutrition powder have entered into the daily lives of people and the planting area of foxtail millet has increased. Determination of the principal constituents of foxtail millet has become a key issue for breeding programs. However, reference analysis methods are complex, expensive, and time-consuming.

Near-infrared spectroscopy (NIRS) together with chemometrics is a well established technique for determining the components of many agricultural products (8,9). It is a rapid, cost-effective, nondestructive method, allowing for simultaneous determination of principal constituents in a sample using multivariate data analysis (10,11). In early studies, NIRS has been applied to determine the nutritional and functional components, such as protein, amino acids, crude fat, total starch, isoflavone, lutein, and carotene, in many crops including rice, soybean, maize, buckwheat, oats, wheat, common bean, and kale (12-20).

To the best of our knowledge, no study has been conducted to establish NIRS calibration equations for analysis of the protein, fat, starch, and amino acid contents of foxtail millet. The objective of the present study was to develop a rapid and accurate NIRS measurement method

or these quality parameters.

## Materials and Methods

**Materials** A total of 259 foxtail millet samples were obtained from six provinces in China (67 from Shanxi, 53 from Hebei, 45 from Jilin, 26 from Henan, 21 from Heilongjiang, and 47 from Inner Mongolia). All samples were dry milled to powder in our lab with a Retsch cyclone mill (17-40; Glen Creston, London, England), passing through a 0.25 mm mesh. After grinding, samples were scanned to obtain near infrared spectra.
The 15 amino acid standards of alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val) were purchased from Sigma and Aldrich (Sigma-Aldrich Corporation, St. Louis, MO, USA). All other reagents were of analytical or chromatographic grade.

**Reference analysis of protein, fat, starch, and amino acids** Briefly, crude protein was measured using the micro-Kjeldahl method with an analyzer unit (Kjeltec 2300; Foss Analytical AB, Höganäs, Sweden) and a conversion factor of 6.25 was used to quantify the crude protein content in accordance with National Standards of the P. R. China, GB/T 5511-2008 (21). Crude fat was determined using a Soxhlet extractor (SCT-02; Hangzhou Huier Instrument Co., Ltd., Hangzhou, China) according to National Standards of the China, GB/T 5512-2008 (22). The total starch content of foxtail millet was determined using an automatic polarimeter (WZZ-1S; Shenguang Instrument Co., Ltd., Shanghai, China) according to National Standards of the P. R. China, GB/T 5006-1985 (23). Amino acids were obtained via hydrolyzation of protein using hydrochloric acid with an extraction procedure based on the method described by Wu *et al.* (24). Amino acids were identified using HPLC (Alltech 626; Alltech Associates, Deerfield, IL, USA), with an Agilent ZORBAX Eclipse AAA Column (4.6×150 mm, 5 μm; Agilent Technologies, Palo Alto, CA) according to a previous method (25). All chemical analyses were performed in duplicate.

**Spectra measurement** A Multi-Purpose Analyzer Fourier transform near-infrared reflectance (FT-NIR) spectrometer (Bruker Corporation, Ettlingen, Germany) was used to obtain spectra. Approximately 15 g of foxtail millet powder was packed into a sample cup (diameter 50 mm, height 75 mm). Samples were irradiated using near-infrared monochromatic light and spectra were collected using a lead sulfide detector in the wave number range of 4,000-12,000 1/cm. Each sample was scanned twice and the two spectra were recorded and averaged automatically using OPUS software 6.5 (Bruker Corporation, Ettlingen, Germany).

**NIRS analysis** In order to correlate spectral data with reference data, multivariate analysis was performed using the spectral analysis program OPUS-QUANT, version 6.5 (OPUS, Bruker). After FT-NIR data acquisition, several data pretreatment methods were tested on the FT-NIR spectral dataset, including first derivative (FD), min-max normalization (MMN), multiplicative scatter correction (MSC), straight line subtraction (SLS), and standard normal variate (SNV). Partial least-squares (PLS) regression was used for model regression. To build up a robust PLS model, data for all 259 average spectra were divided into a calibration group and a validation group using a ratio of 4:1 (207 for calibration, and 52 for validation). The samples selected for calibration and validation were representative of a large range of content and a good uniformity of gradient distribution was achieved. For PLS, a leave-one-out cross-validation was used. The coefficient of determination ($R^2$) was calculated for both cross-validation and external validation. For all of the parameters analyzed, the mathematical pretreatment that yielded the minimum value for the root mean square error of cross validation (RMSECV) was considered to be optimal (26). To obtain the calibration equations, the optimal number of latent variables was defined as the number of factors that did not remarkably decrease the RMSECV when the number of factors was increased. In addition, to evaluate the predictive ability of the calibration model, the value of the residual predictive deviation (RPD) was used. The RPD value is defined as the ratio of the standard deviation (SD) of the chemical method to the standard error of prediction (SEP). Usually, the higher the RPD value, the greater the ability of a calibration model to predict the chemical composition in samples (27).

## Results and Discussion

**Protein, fat, starch, and amino acid contents** All of the amino acid peaks were clearly separated within 11 min. and the retention times in chromatographic profiles for the foxtail samples were consistent with the times for the standard mixtures of amino acids. Descriptive statistics for the reference measurements of crude protein, crude fat, total starch, and amino acid contents are listed in Table 1. All of the constituent contents varied over a wide enough range to enable satisfactory calibrations. The variation was probably due to the genetic diversity of the entries,

**Table 1. The crude protein, crude fat, total starch, and amino acid contents of foxtail millet**

| Constituent | Calibration set | | | | Validation set | | | |
|---|---|---|---|---|---|---|---|---|
| | Range (g/100 g) | Mean (g/100 g) | SD | SE | Range (g/100 g) | Mean (g/100 g) | SD[1)] | SE |
| Crude protein | 11.85-20.58 | 15.29 | 1.63 | 1.15 | 11.83-20.56 | 15.37 | 1.69 | 1.20 |
| Crude fat | 2.82-4.47 | 3.64 | 0.34 | 0.24 | 2.72-4.45 | 3.57 | 0.37 | 0.26 |
| Total starch | 65.59-74.12 | 70.23 | 2.11 | 1.49 | 65.49-74.11 | 70.18 | 2.15 | 1.52 |
| Alanine | 1.01-2.00 | 1.46 | 0.19 | 0.13 | 1.02-2.02 | 1.51 | 0.22 | 0.16 |
| Arginine | 0.34-0.69 | 0.49 | 0.06 | 0.04 | 0.36-0.71 | 0.50 | 0.07 | 0.05 |
| Aspartic acid | 0.77-1.38 | 1.01 | 0.13 | 0.09 | 0.78-1.42 | 1.06 | 0.16 | 0.11 |
| Glutamic acid | 2.25-4.31 | 3.10 | 0.38 | 0.27 | 2.27-4.38 | 3.12 | 0.41 | 0.29 |
| Glycine | 0.25-0.46 | 0.35 | 0.04 | 0.03 | 0.27-0.45 | 0.37 | 0.05 | 0.04 |
| Histidine | 0.27-0.48 | 0.38 | 0.05 | 0.04 | 0.26-0.43 | 0.35 | 0.06 | 0.04 |
| Isoleucine | 0.46-0.93 | 0.66 | 0.10 | 0.07 | 0.49-0.91 | 0.68 | 0.11 | 0.08 |
| Leucine | 1.45-3.07 | 2.15 | 0.29 | 0.21 | 1.44-3.01 | 2.13 | 0.25 | 0.18 |
| Lysine | 0.29-0.56 | 0.41 | 0.05 | 0.04 | 0.29-0.53 | 0.42 | 0.05 | 0.04 |
| Phenylalanine | 0.64-1.31 | 0.95 | 0.13 | 0.09 | 0.68-1.28 | 0.94 | 0.14 | 0.10 |
| Proline | 0.75-1.60 | 1.10 | 0.19 | 0.13 | 0.76-1.57 | 1.13 | 0.21 | 0.15 |
| Serine | 0.51-0.94 | 0.71 | 0.08 | 0.06 | 0.51-0.95 | 0.72 | 0.09 | 0.06 |
| Threonine | 0.37-0.75 | 0.56 | 0.07 | 0.05 | 0.40-0.74 | 0.55 | 0.07 | 0.05 |
| Tyrosine | 0.35-0.79 | 0.54 | 0.09 | 0.06 | 0.37-0.74 | 0.53 | 0.10 | 0.07 |
| Valine | 0.73-1.09 | 0.89 | 0.07 | 0.05 | 0.75-1.08 | 0.91 | 0.08 | 0.06 |

[1)]SD, standard deviation; SE, standard error; All the chemical analyses were performed in duplicate.

combined with the environmental effects of location and year of harvest. Actually, the protein content is used as a criterion for selection of foxtail millet varieties in the food industry (1). The protein content in the calibration set ranged from 11.85 g to 20.58 g/100 g, with a mean of 15.29 g/100 g. These values are similar to values in the validation set. The crude fat, total starch, and amino acids contents in the calibration set ranged from 2.82-4.47, 65.59-74.12, and 0.25-4.31 g/100 g respectively. In the calibration set, Glu was found to be the most abundant amino acid with a mean content of 3.10 g/100 g. Gly was the least abundant with mean content of 0.35 g/100 g. However, the mean contents of crude protein, crude fat, total starch, and total amino acids in foxtail millet were higher than in other reports (1,3,6), perhaps due to differences in the foxtail millet varieties. Generally speaking, the contents of all the constituents both in the calibration and validation ranged widely, suggesting that the samples used in this study were representative of the natural diversity in foxtail millet.

The correlation coefficients for crude protein, crude fat, total starch, and amino acids are shown in Table 2. Crude protein was significantly ($p$<0.01) correlated with total starch and with most of the amino acids, except lysine. Crude fat was also significantly ($p$<0.05) correlated with total starch and with some of the amino acids. Total starch was significantly ($p$<0.01) negatively correlated with all of the 15 amino acids. The correlations among most of the amino acids were significant ($p$<0.05).

**FT-NIR spectra analysis and NIRS model development**

FT-NIR spectra of different foxtail millet samples after second derivative treatment are shown in Fig. 1. According to the spectra, an intense absorbance band can be observed near the infrared region between 9,000 and 4,000 1/cm. The band at 8,262 1/cm is caused by the C-H stretching 2nd overtone of -$CH_3$ and -$CH_2$- groups. Due to the C-H 1st overtone of -$CH_2$- groups, a band at 5793 1/cm is observed and due to the 2nd overtone of the C=O stretching vibration, a band at 5,180 1/cm is present. A shoulder around 4716 1/cm can be attributed to the C–H and C=O stretching vibrations of -HC=CH- (28,29).

Calibrations were developed using PLS regression and leave-one-out validation. The choice of the model was based on RMSECV, latent variables, and RPD values. The number of latent variables is a crucial point for the quality of a calibration model. To avoid overfitting, the optimal number of latent variables was determined using the lowest value of the predicted residual error sum of squares (30). The calibration statistics for the determination of protein, fat, starch, and amino acids using PLS regression are summarized in Table 3. For the calibration models, the coefficients of regression ($r_{cal}$) were >0.95, except for crude fat and total starch. And the $R^2_{cv}$ range was from 0.747 to 0.958.

On the basis of guidelines outlined by Williams and Norris (31), the performance criterion used for FT-NIR calibration models was, models with an $R^2$ value below 0.66 are unusable, models with an $R^2$ value between 0.66

**Table 2. Correlation coefficients among crude protein, crude fat, total starch, and amino acids in foxtail millet**

| | CF[1)] | TS | Ala | Arg | Asp | Glu | Gly | His | Ile | Leu | Lys | Phe | Pro | Ser | Thr | Tyr | Val |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CP | 0.221 | -0.598[b2)] | 0.939[b] | 0.775[b] | 0.938[b] | 0.944[b] | 0.838[b] | 0.839[b] | 0.951[b] | 0.945[b] | 0.228 | 0.880[b] | 0.933[b] | 0.933[b] | 0.924[b] | 0.790[b] | 0.923[b] |
| CF | | -0.529[b] | 0.209 | 0.423[b] | 0.253 | 0.196 | 0.376[b] | 0.300[a] | 0.209 | 0.201 | 0.332[a] | 0.199 | 0.149 | 0.225 | 0.261[a] | -0.128 | 0.286[a] |
| TS | | | -0.601[b] | -0.726[b] | -0.655[b] | -0.612[b] | -0.698[b] | -0.620[b] | -0.632[b] | -0.603[b] | -0.400[b] | -0.590[b] | -0.583[b] | -0.622[b] | -0.631[b] | -0.371[b] | -0.642[b] |
| Ala | | | | 0.759[b] | 0.969[b] | 0.990[b] | 0.838[b] | 0.861[b] | 0.978[b] | 0.995[b] | 0.22 | 0.903[b] | 0.984[b] | 0.982[b] | 0.940[b] | 0.785[b] | 0.937[b] |
| Arg | | | | | 0.836[b] | 0.765[b] | 0.897[b] | 0.884[b] | 0.776[b] | 0.761[b] | 0.510[b] | 0.816[b] | 0.729[b] | 0.780[b] | 0.787[b] | 0.579[b] | 0.793[b] |
| Asp | | | | | | 0.979[b] | 0.907[b] | 0.909[b] | 0.978[b] | 0.974[b] | 0.332[a] | 0.930[b] | 0.959[b] | 0.967[b] | 0.938[b] | 0.769[b] | 0.959[b] |
| Glu | | | | | | | 0.835[b] | 0.876[b] | 0.986[b] | 0.996[b] | 0.242 | 0.927[b] | 0.991[b] | 0.988[b] | 0.956[b] | 0.805[b] | 0.952[b] |
| Gly | | | | | | | | 0.841[b] | 0.849[b] | 0.827[b] | 0.448[b] | 0.778[b] | 0.810[b] | 0.844[b] | 0.852[b] | 0.588[b] | 0.871[b] |
| His | | | | | | | | | 0.876[b] | 0.872[b] | 0.426[b] | 0.938[b] | 0.837[b] | 0.855[b] | 0.817[b] | 0.725[b] | 0.873[b] |
| Ile | | | | | | | | | | 0.985[b] | 0.252 | 0.923[b] | 0.967[b] | 0.969[b] | 0.943[b] | 0.799[b] | 0.968[b] |
| Leu | | | | | | | | | | | 0.227 | 0.924[b] | 0.986[b] | 0.984[b] | 0.946[b] | 0.800[b] | 0.948[b] |
| Lys | | | | | | | | | | | | 0.332[a] | 0.191 | 0.298[a] | 0.276[a] | -0.078 | 0.397[b] |
| Phe | | | | | | | | | | | | | 0.901[b] | 0.899[b] | 0.864[b] | 0.803[b] | 0.904[b] |
| Pro | | | | | | | | | | | | | | 0.984[b] | 0.957[b] | 0.815[b] | 0.918[b] |
| Ser | | | | | | | | | | | | | | | 0.969[b] | 0.766[b] | 0.948[b] |
| Thr | | | | | | | | | | | | | | | | 0.766[b] | 0.922[b] |
| Tyr | | | | | | | | | | | | | | | | | 0.707[b] |

[1)]CP, crude protein; CF, crude fat; TS, total starch; Ala, alanine; Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, praline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine
[2)][a]Correlation is significant at the 0.05 level (2-tailed); [b]correlation is significant at the 0.01 level (2-tailed).

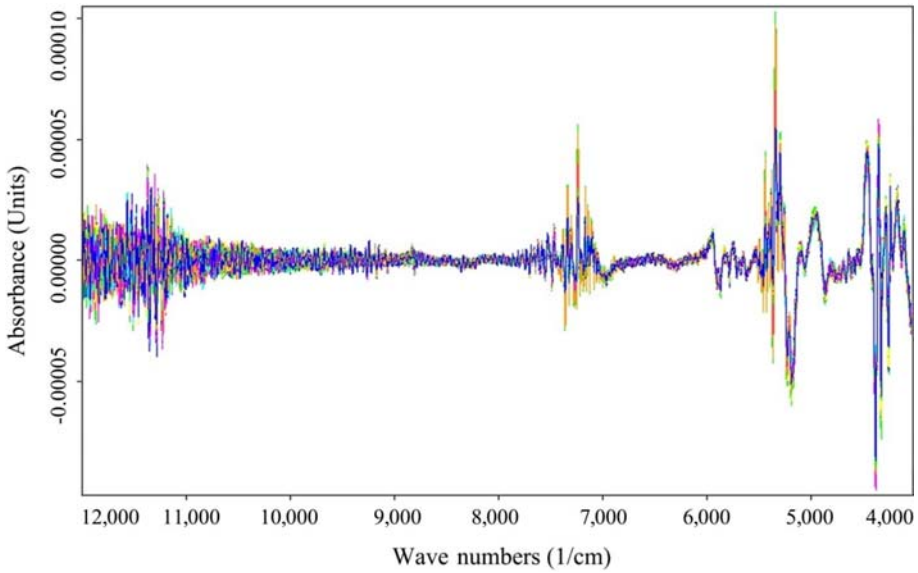


**Fig. 1. FT-NIR spectra of different foxtail millet samples using second derivative treatment.**

and 0.83 can be used for sample screening, (crude fat and total starch), models with an $R^2$ value between 0.83 and 0.92 can be used for many applications (including Arg, Gly, His, Lys, Pro, Thr, Tyr, and Val), and models with an $R^2$ value of 0.92-0.98 are suitable for most applications and quality control (including crude protein, Ala, Asp, Glu, Ile, Leu, Phe, and Ser).

The optimized models for protein, fat, starch, and amino acids required validation using external validation group samples. As shown in Table 3, the determination coefficient in external validation ($R^2_{val}$) was >0.90, and RPD values were nearly equal to or >2.5 for crude protein, Ala, Asp, Glu, Ile, Leu, and Ser. These were considered to be indicative of good prediction. The $R^2_{val}$ values for Gly, His, Phe, Pro, Thr, Tyr, and Val were >0.83 and the RPD values were nearly equal to or >2.0, which denotes an approximate quantitative prediction. The $R^2_{val}$ values for crude fat, total starch, Arg, and Lys were >0.70 and RPD values were >1.5. These models could be used for sample screening. The NIRS calibration models developed were sufficiently

**Table 3. Statistical descriptions of the NIRS determinations of crude protein, crude fat, total starch, and amino acids in foxtail millet**

| Constituent | Treatment[1)] | Outlier | Latent variable | $r_{cal}$ | $R^2_{cv}$ | RMSECV | $R^2_{val}$ | SEP | RPD |
|---|---|---|---|---|---|---|---|---|---|
| Crude protein | MMN | 3 | 3 | 0.985 | 0.958 | 0.415 | 0.937 | 0.517 | 3.27 |
| Crude fat | FD+MSC | 5 | 5 | 0.926 | 0.785 | 0.168 | 0.755 | 0.186 | 1.99 |
| Total starch | MMN | 7 | 6 | 0.912 | 0.747 | 0.809 | 0.714 | 1.127 | 1.91 |
| Alanine | SLS | 3 | 4 | 0.977 | 0.935 | 0.056 | 0.922 | 0.068 | 3.24 |
| Arginine | FD | 5 | 6 | 0.955 | 0.857 | 0.033 | 0.803 | 0.041 | 1.71 |
| Aspartic acid | FD+SLS | 3 | 4 | 0.984 | 0.944 | 0.042 | 0.904 | 0.053 | 3.02 |
| Glutamic acid | SNV | 3 | 3 | 0.980 | 0.937 | 0.117 | 0.925 | 0.132 | 3.11 |
| Glycine | FD+SNV | 3 | 5 | 0.963 | 0.903 | 0.023 | 0.844 | 0.024 | 2.08 |
| Histidine | MSC | 4 | 6 | 0.965 | 0.885 | 0.017 | 0.866 | 0.025 | 2.40 |
| Isoleucine | FD+MSC | 3 | 3 | 0.982 | 0.941 | 0.032 | 0.912 | 0.038 | 2.89 |
| Leucine | FD+SNV | 3 | 3 | 0.984 | 0.945 | 0.098 | 0.919 | 0.095 | 2.63 |
| Lysine | SNV | 4 | 5 | 0.960 | 0.859 | 0.024 | 0.764 | 0.031 | 1.61 |
| Phenylalanine | FD+SLS | 4 | 4 | 0.971 | 0.929 | 0.039 | 0.897 | 0.049 | 2.86 |
| Proline | MMN | 4 | 4 | 0.960 | 0.901 | 0.083 | 0.849 | 0.087 | 2.41 |
| Serine | FD+SNV | 3 | 3 | 0.979 | 0.948 | 0.029 | 0.908 | 0.036 | 2.50 |
| Threonine | MSC | 4 | 4 | 0.966 | 0.912 | 0.021 | 0.882 | 0.034 | 2.06 |
| Tyrosine | FD+SNV | 5 | 5 | 0.968 | 0.894 | 0.026 | 0.875 | 0.036 | 2.78 |
| Valine | SLS | 6 | 5 | 0.964 | 0.872 | 0.035 | 0.831 | 0.043 | 1.86 |

[1)]MMN, min-max normalization; FD, first derivative; MSC, multiplicative scatter correction; SLS, straight line subtraction; SNV, standard normal variate; $R^2_{cv}$, determination coefficient of cross-validation; RMSECV, root mean square error of cross validation; $R^2_{val}$, determination coefficient of external validation; SEP, standard error of prediction; RPD, residual predictive deviation

accurate for the prediction of protein, fat, starch, and 15 amino acid contents in foxtail millet.

In summary, to optimize the selection process in a breeding program, a large number of phenotypes must be quickly evaluated to decide which progeny will be used to found the next generation. The rst application of FT-NIR to simultaneously analyze the protein, fat, starch, and amino acid contents in foxtail millet is reported. On the basis of the results of chemical analyses, NIRS can used as a screening tool to rapidly analyze the constituents in foxtail millet without the need for costly and laborious chemical analysis. Further development with larger data sets is required to improve the precision and robustness of the NIRS calibration models.

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