

## Near-Infrared Reflectance Spectroscopy (NIRS) for Tannin, Starch and Amylase Determination in Sorghum Breeding Programs

Mulate Zerihun<sup>1\*</sup>, Glen Fox<sup>2</sup>, Amare Nega<sup>3</sup>, Amare seyoum<sup>3</sup>, Masresha Minuye<sup>3</sup>, David Jordan<sup>4</sup>, Taye Taddese<sup>4</sup>, Alemayehu Assefa<sup>4</sup>

<sup>1,3</sup>EIAR, Melkassa agricultural research center (Food science and nutrition research department) P.O. Box 436, Adama, Ethiopia

<sup>3</sup>EIAR, Melkassa agricultural research center (Sorghum breeding department) P.O. Box 436, Adama, Ethiopia

<sup>4</sup>EIAR, Head Quarter, P.O. Box 2003, Addis Ababa, Ethiopia

<sup>2,4</sup>University of Queensland, Australia

**\*Corresponding author:** Mulate Zerihun, EIAR, Melkassa agricultural research center (Food science and nutrition research department) P.O. Box 436, Adama, Ethiopia, E-mail: mulatezerihun@yahoo.com

### Abstract

The objective was to develop the NIRS calibration model for the analysis of tannin, starch and amylose by partially replacing more expensive and time-consuming wet chemistry analysis. More than 108 sorghum samples were used to develop NIRS models for starch, tannin, and amylose and forty-six additional samples were used for validation. The developed model result showed the determined tannin, starch, and amylose with coefficient for determination being 0.815, 0.983, 0.762 and RMSE value 6713.54, 0.435, 0.270) with tannin, starch, and amylose respectively. The coefficient of determination for validation ( $R^2(v)$ ) were 0.589, 0.910 and 0.697 for tannin, total starch and amylose respectively. The method is rapid (less than 1 min), relatively accurate, involves little or no sample preparation, and provides a direct readout of tannin, starch, and amylose. The test is non-destructive and, therefore, the model can be used for any sorghum grain quality tests. Therefore, the NIRS models can be used to support the sorghum breeding program efforts.

**Keywords:** Sorghum; Tannin; Total starch and amylase; Near-infrared spectroscopy; Grain quality

### Introduction

Sorghum (*Sorghum bicolor* L. Moench) is one of the most important cereals and it is the 5<sup>th</sup> globally in terms of land coverage and production<sup>[1,2]</sup>. In Ethiopia, sorghum is the third (3<sup>rd</sup>) most important staple cereal crop after teff and maize<sup>[3,4]</sup> and its productivity in the regions of Ethiopia could be enhanced through effective breeding programs, modernized agronomic management and integrated drop protection system using locally adapted and well-characterized germ plasm. This crop is grown in almost all regions of Ethiopia as a staple food crop on which the lives of millions of poor Ethiopians depend. It has tremendous uses for the Ethiopian farmer and no part of this plant is ignored. They are using for multiple purposes such as food (injera & bread mostly), animal feed and used as the basic ingredient in some local beverages<sup>[5-6]</sup>.

Traditionally, sorghum breeding programs have focused on increased yield stability and yield potential under abiotic and biotic stresses. In the past few years, sorghum breeding programs designed to improve the nutritional quality of sorghum for human and animal consumption has received more attention. Additionally, food and chemical industries are demanding more quality parameters in sorghum grain requirements. Thus, the physical and chemical characterization of grain is an important element of any modern sorghum breeding program<sup>[8,9]</sup>.

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Characterizing the sorghum grain for the nutritional and minerals profiles is vital for several reasons. Natural variation of sorghum grain composition (protein, fat, tannin, starch and amylose) between accessions can be used for crop improvement<sup>[10]</sup>. The quantification natural variation of sorghum grain composition and to identify the nutritional composition associated with variation in grain composition contents. Starch is the major component of cereal grains and starchy foods, and changes in its biophysical and biochemical properties (e.g., amylose, amylopectin, pasting, gelatinization, viscosity) will have a direct effect on its end use properties (e.g., bread, malt, polymers)<sup>[11]</sup>. The use of rapid and non-destructive methods to study and monitor starch properties, such as gelatinization, retrogradation, water absorption in cereals and starchy foods, is of great interest in order to improve and assess their quality<sup>[12]</sup>.

In recent years, near-infrared reflectance (NIR) and mid-infrared (MIR) spectroscopy have been explored to predict several quality parameters. Near-infrared reflectance spectroscopy (NIRS) is a technique that combines spectroscopy and mathematics to rapidly-produce indirect, quantitative estimates of concentrations of OH-, NH-, CH-, or SH-containing compounds. Near-infrared spectroscopy has been used for accurate measurement of the chemical composition of cereals grain and oil crops, such as moisture, protein, fat, and amylase<sup>[13]</sup>. However, to assess cereals like maize, sorghum, and rice grain quality, in addition to proximate composition, various physicochemical properties like tannin, starch and amylose of the cereals must be analyzed. Several studies were previously carried out to estimate quality-related parameters such as fat acidity and the gelatinization characteristics of cereals<sup>[14,15]</sup>. The physicochemical properties of cereals are known to be correlated with its genetical differences, nutritional and eating quality<sup>[16]</sup>.

All common methods for measuring the physicochemical properties require some specific instruments and chemicals and are labour-intensive and time-consuming. In comparison to wet chemistry procedures, NIRS requires none or simple sample preparation methods and is a rapid and relatively inexpensive technique that facilitates the analysis of several traits simultaneously<sup>[17,18]</sup>. Therefore, there is an intense interest in the sorghum breeding program, food product improvement, culture, and production communities, as well as in the sorghum industry, for a quick and easy method to measure the physicochemical properties of sorghum. Although NIR spectroscopy is used for measuring sorghum physicochemical contents, a method for measuring the physicochemical properties using this technology has not yet been established in Ethiopia. The objective of the present study was to develop and validate NIRS calibration model for tannin, starch, and amylose analysis of sorghum grain.

## Materials and Methods

### Sample collection and preparation

A total of 108 sorghum samples were collected from the germ plasm bank of the national sorghum research program (Melkassa Agricultural Research Center), active sorghum breeding programs and different sorghum production areas for the development and validation of NIRS calibrations. These samples included both white and red types sorghum and different environmental adaptation (highland, mid and lo land) to increase the

range of values for development the calibrations. All samples were ground into flour using cyclone sample mill, UDY-corporation (model 3010-019EN55014 and made in USA-Colorado) with a 1 mm sieve. A sub-sample of the flour was obtained from each sample was used for wet chemical analysis, and the grain sample was used for NIRS analysis. The milled flour was stored in a dark area at room temperature in a glass bottle for further wet chemistry analysis.

### Wet chemistry and reference analysis

The physicochemical properties of sorghum samples were determined with the following methods. The tannin content of sorghum grain was determined through the standard method of vanillin with HCl assay<sup>[19-21]</sup>. 1g of sorghum flour sample was added into a centrifuge tube and added 10 mL 1% HCl/Methanol solution to sample and seal centrifuge tube. Then, the solution was placed in a tube with the sample in solution on a mechanical shaker for 24 hours at room temperature. After 24 hours the samples are centrifuged (table top universal centrifuge, PLC-012 E, U.S.A) at 1000g for 5 minutes. Thereafter 1 mL of supernatant was taken and mixed with 5 mL of Vanillin-HCl reagent in a clean centrifuge tube. The samples were incubated for 20 min at 30°C before reading sample absorbance at 500 nm using a UV-Vis spectrometer. The tannin content of sorghum was calculated in the sample using the following formula:

$$\text{Tannin in mg/g} = ((A_s - A_b) - \text{Intercept}) / (\text{Slope} \times D \times W)$$

Where:  $A_s$  = Sample Absorbance  $A_b$  = Blank Absorbance  
 $D$  = Density of solution (0.791g/ml)  $W$  = Weight of sample in gram here

The total starch content of sorghum grain was determined through the standard method using the Megazyme total starch kit<sup>[22,23]</sup>. Measurement of the starch content of commercial starches<sup>[24]</sup> (Moreels and Amylum 1987) and an improved enzymic method for the determination of native and modified starch analysis were used<sup>[25]</sup> (Karkalas 1985). A sample (100 mg  $\pm$  1 mg) was weighed and added into a 15 mL centrifuge tube and record the weights. A centrifuge tube without sample as an analytical blank. The sample is mixed with 200  $\mu$ L of aqueous ethanol (80 % v/v) and stirred on a Vortex mixer to aid dispersion. An addition of 2 mL of DMSO to each sample and stirred the tube on a Vortex mixer as soon as possible. The tube was placed with the sample in a vigorously boiling water bath for 5 min and 3 mL of thermo stable  $\alpha$ -amylase solution (1 mL  $\alpha$ -amylase diluted with 30 mL 50 mM MOPS buffer at pH 7.0) was added in a boiling water bath for 12 minutes with stirring. Then, 4 mL sodium acetate buffer (200 mM, pH 4.5) was added and incubated with 0.1 mL amyloglucosidase at 50°C for 30 minutes with shaking. Finally, adjusted the volume to 100 mL (or adjust to 10 mL then take 0.1 mL to dilute to 1 mL). 1 mL was taken from the diluted digested and centrifuge at 4000 rpm for 10 minutes. Transferred a 0.1 mL supernatant to a 15-mL centrifuge tube and was added 3.0 mL GOPOD reagent to the tube and incubate at 50°C for 20 minutes with shaking. Prepared glucose control (0.1 mL D-Glucose standard, 1 mg/mL) and blank control (0.1 mL distilled water) and incubated them with 3.0 mL GOPOD reagent at 50°C for 20 minutes with shaking. After prepared the solution read the

absorbance of the sample and Glucose control at 510 nm against the blank control. The total starch content was calculated in sorghum flour (mg starch/mg dry flour) as followed:

$$\text{Starch (mg/mg flour)} = A \times F \times FV/0.1 \times 1/1000 \times 1/FW \times 162/180 = A \times F \times 0.9 / FW$$

Where:

A = Absorbance read against the blank control

F = Conversion from absorbance to  $\mu\text{g}$

=  $100(\mu\text{g of D-Glucose})/\text{absorbance for } \mu\text{g of D-Glucose}$

FV = Final volume of the sample = 100 mL

FW = Initial dry flour weight = flour weight  $\times$  (1 - % moisture content)

0.1 = Volume size of the diluted sample that being analyzed using GOPOD reagent

1/1000 = Conversion from  $\mu\text{g}$  to mg

162/180 = Conversion from free D-glucose to anhydrous-D-Glucose (as in starch)

The amylose content of sorghum grain flour was determined through the standard method using modified Megazyme total starch kit<sup>[26-29]</sup>. A sample (20 mg ( $\pm$  0.1 mg)) dry sorghum flour of lipid-free sample was transferred into a 50mL centrifuge tube. Then, wet the sample and standards with 0.8 mL water to each tube and was add 7.2 mL DMSO. The solution was mixed vigorously for 1 min using a vortex mixer and was heated the tubes in a water bath at 85°C for 15 min with intermittent mixing. The solution was allowed in the tubes to cool at room temperature ( $\sim$ 45 min) and 17mL water was added to each tube through shaking well. Then, 100  $\mu\text{L}$  of the diluted solution (Solution I) and 4.4 mL of distilled water were added into a 15-ml centrifuge tube. Diluted iodine solution (0.5 mL) was added and mixed vigorously. Finally, the color is developed for at least 15 min and them measured the absorbance of the sample and each of the standard mixtures at 640 nm against a reagent blank as the reference. The amylose content was calculated as followed:

$$\text{Amylose (\%)} = (A \times 20/SW - \text{intercept}) \div \text{slope} \times 100\%$$

Where: A = Absorbance read against the blank control

SW = Accurate dry starch weight in mg

= Wet flour weight  $\times$  (1 - % moisture content/100)  $\times$  % starch content/100

20 = 20 mg of standard AM and AP weight

### NIR Spectral analysis

Spectra of the whole grain sorghum samples of approximately 50g were recorded with a near-infrared spectrometer (model Infratec 9500 (Infratec NIR Systems)). A spinning cup was used for all sample types, i.e., whole-grain sorghum. Reflectance (R) readings at 5 nm increments were collected over a near-infrared wavelength range of 700 to 1100 nm, averaging the values of 108 readings and transforming the values to  $\log_{10}(1/R)$ , giving a total of 80 data points per spectrum. Three replicates for each sorghum samples, with sample repacking, were carried out and averaged for the chemometrics analysis. A commercial spectral data analysis program (The Unscrambler 9.2, CAMO Software), and the chemometric method of partial least squares (PLS)

regression was used to correlate the spectral data and reference data and thereby to develop calibration models. Accordingly, the calibration models to estimate the physicochemical properties of sorghum were developed from the blind spectra of sorghum at first.

### Spectral characteristics of sorghum samples

The Spectral data was stored as a logarithm of the reciprocal of reflectance [ $\log(1/R)$ ]. The software for scanning, mathematical processing, and statistical analysis was supplied with the spectrophotometer by Infratec International (ITI). Calibration models were developed using modified partial least-squares (MPLS) regression and cross-validation techniques. Prior to the PLS regression, spectra had pre-treated by applying a first derivative transformation defined by a combination of four factors where the first is the degree of the derivative, the second is the gap between data points for subtraction, and the third and fourth are the data points used for smoothing. The results of the calibration calculation were monitored by checking the t outliers with  $t > 2.5$ , GH, and X outliers  $> 10$ ; samples with  $t > 2.5$  was deleted from the sample file. The SD between NIRS and reference determinations for the calibration [standard error of calibration (SEC)] and validation sets [standard error of prediction (SEP)] has been calculated. The coefficient of determination of calibration ( $R^2_c$ ) and the coefficient of determination of validation ( $R^2_v$ ) (the fraction of the variance of the reference values explained by the variance of NIRS determinations) was calculated. The calibration models were developed for each wavelength range (NIR) and their combinations, using PLS regression with full cross-validation since the number of samples was insufficient to divide into two separate groups of calibration development and validation. The number of methods were used in the PLS calibration models were those suggested by the Unscrambler software. The ratio of performance deviation (RPD) was calculated as  $SD/SEC$ . Values of RPD were calculated to verify the applicability of the calibrations. RPD is the ratio of the standard error of prediction (SEP) to the standard deviation (SD) of the reference data; in this study, we replaced SEP with SEC (standard error of cross-validation)<sup>[30]</sup>.

## Results and Discussion

### Quality characteristics of sorghum grain

The wet chemistry of sorghum grain in the calibration set for tannin, starch, and amylose were distributed over a wide range (Table 1). The wide range of tannin, starch, and amylose content values are also explained by the use of samples from different locations, including the experimental fields where different agronomic crop management practices were applied. The wide differences between the wet chemistry of the samples were contributed to the robustness of the calibrations. The population sorghum grain samples were analyzed and the maximum and minimum values of 44300.9 and 0.1 mg/g, 72.59 and 65.54%, and 21.78 and 19.66% for tannin, total starch and amylose respectively presented in Table 1. The standard deviation value of tannin was showed high due to the variation of tannin content between populations, this is so important to increase the range of the calibration. These variations in the content are due to differences in the sorghum variety including non-waxy and waxy type,

soil in the paddy field, and the climate of the sorghum-growing district. These factors would produce much larger variations in tannin, total starch, and amylose contents if the sample set included different sorghum varieties from various countries. Thus, the tannin, total starch and amylose properties of the sorghum samples in this study show typical values of commercial sorghum grain.

**Table 1:** Descriptive statistics for wet chemistry analysis of sorghum grain samples.

Parameters	Mean value	Maximum value	Minimum value	Range	SD	Variance
Tannin (mg/g)	11752.60	44300.9	0.1	44300.8	10981.63	1.21E+08
Total starch(%)	69.00	72.59	65.54	7.05	1.548	2.39501
Amylose (%)	20.70	21.78	19.66	2.12	0.464	0.215551

### NIRS and calibration model development

NIRS calibration equations, developed on the basis of 46 samples and the tannin content had high coefficients of determination for calibrations ( $R^2c=0.815$ ) and slightly lower coefficients of determination for cross-validations ( $R^2cv = 0.589$ ), the total starch content had high coefficients of determination for calibrations ( $R^2c = 0.983$ ) and slightly lower coefficients of determination for cross-validations ( $R^2cv = 0.910$ ), the amylose content had high coefficients of determination for calibrations ( $R^2c = 0.762$ ) and slightly lower coefficients of determination for cross-validations ( $R^2cv = 0.697$ ) (Table 2). However, the difference between  $R^2c$  and  $R^2cv$  was minor, indicating that the calibrations were homogeneous. The SEC and SECV were small (SECV/mean= 3.8, 1.2, and 4.3% for tannin, total starch, and amylase respectively). On the basis of the RPD values, all calibrations can provide meaningful estimates of tannin, total starch, and amylase for breeding programs (Table 2).

**Table 2:** Reference values, NIRS calibration, and cross-validation statistics for tannin, total starch, and amylose the calibration set of sorghum

Parameters	Calibration			Validation		
	slope	RMSE	$R^2$	slope	RMSE	$R^2$
Tannin	0.815	4453.11	0.815	0.619	6713.54	0.589
Total starch	0.983	0.189	0.983	0.911	0.435	0.910
Amylose	0.762	0.227	0.762	0.718	0.270	0.697

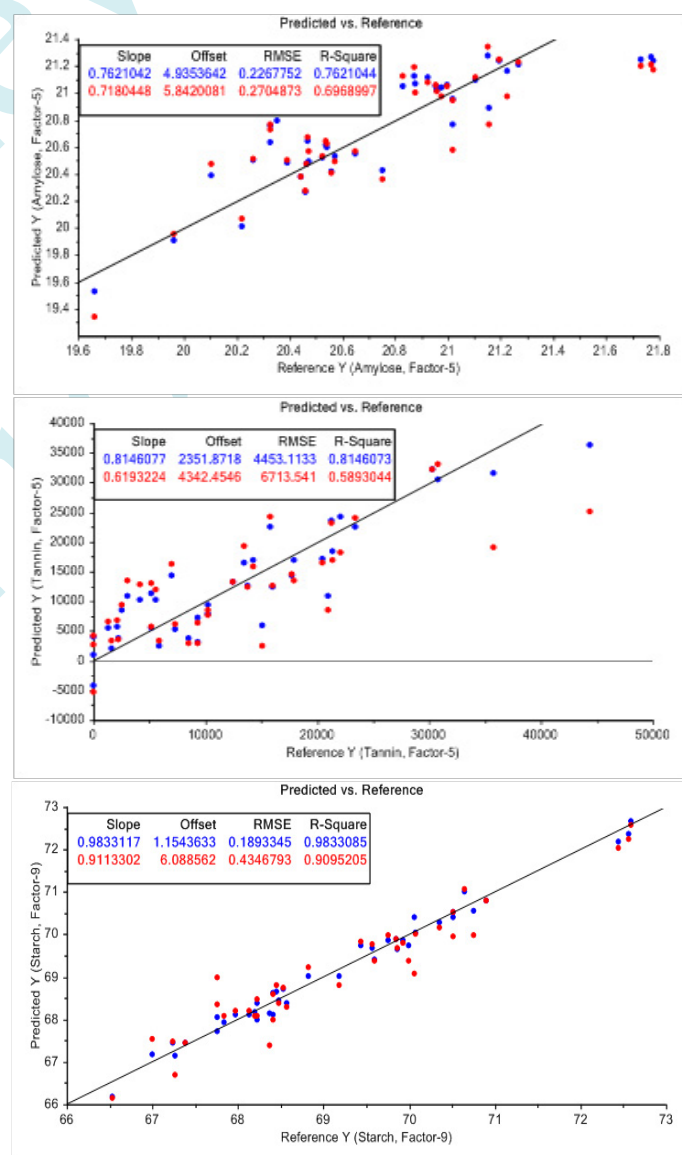
### Independent/External Validation

Independent validation of the calibrations was performed using 46 samples for tannin, total starch, and amylase from the tropical and subtropical breeding programs of sorghum in Melkassa, Ethiopia. The coefficients of determination for independent validation ( $R^2v$ ) were larger than those measured for the cross-validations (Table 2). The SD/SEP ratio for tannin, total starch and amylose were indicating the excellent quality of the calibration. For total starch and amylose, SD/SEP ratios were between 0 and 1, indicated that the calibrations were satisfactory (Table 1). Considered both the SD/SEP ratios and the  $R^2v$  values, our re-

sults were showed that reliable selection for tannin, total starch and amylose are possible by NIRS.

### Calibration curve regression

To evaluate the prediction performance of the developed models, the predicted of tannin, starch and amylose were plotted against reference values for all dataset (Figure 1). The agreement between these values shows that the models fit well and are not over fitted (built with a low number of factors). One forced fit use more PLS components in the model what need, where  $R^2$  have a high value, but the model fail in predicting new samples. The samples are linearly distributed around a liner line in the reference versus predicted values by PLS models plot (Figure 1). The distribution of calibration and validation points in the plots as shown the Figure 1 indicating absence of systematic trends in the building models, that they presented a normal distribution with satisfactory linearity. The model and calibration graphs have been described below in Figure 1.



**Figure 1:** Tannin, amylose and total starch regressions to NIRS calibration

Breeding programs devoted to developing quality sorghum are being implemented in several countries around the world and these programs require robust, fast, and inexpensive laboratory methods to screen for tannin, total starch, and amylose. We recently developed a colorimetric method for tannin, total starch, and amylase determination, which has proven to be very useful where laboratories with spectrophotometer analysis are in place. The NIRS calibrations described here in for tannin, total starch and amylose a fast and simple screening option because extractions and chemical reactions are not required. However, their application will be limited to laboratories where NIRS equipment and software are available. Use of NIRS for tannin, total starch and amylose analysis's inexpensive and fast; tannin, total starch and amylose analysis by wet chemistry is 20-25 times more expensive and requires more time compared with using NIRS.

Use of NIRS for tannin analysis is cheap and fast; tannin analysis by wet chemistry more expensive and required 10 hr compared to 1 min using NIRS (Table 3). However, the use of NIRS requires a substantial investment in equipment, which depending upon the manufacturer can range from the U.S. \$60000 to the U.S. \$100000. If NIRS is used in large breeding programs, where thousands of samples are analyzed per year or where multiple traits can be analyzed by NIRS, then the equipment will pay for itself in a short period of time.

**Table 3:** Costs and Time comparison between the NIRS and wet chemistry analysis methods for tannin, total starch and amylose determination in sorghum

Activities	Time		Costs (U.S.A. \$)	
	Wet chemistry	NIRS	NIRS	Wet chemistry
Sample preparation	20 min	1 min	1\$	1\$
Tannin extraction and preparation analysis	10 hr	1 min	1\$	20\$
Total starch extraction and preparation analysis	9 hr	1 min	1\$	25\$
Amylose extraction and preparation analysis	8 hr	1 min	1\$	20\$

**Note:** Estimates are for analysis of one sample. Costs estimation from the food science and nutritional quality laboratory in Ethiopia. Time invested includes sample aliquoting and sample cooling time.

## Conclusion and Recommendation

The performances of the NIRS models developed in our study for tannin, total starch and amylose were appreciated using SECV and  $R^2$  statistics. The quality and potential of our NIRS models are due to the use of a core collection of cultivated sorghums that covers a broad diversity of consumers' usages, and the accuracy of the developed models. Tannin, total starch and amylose, which are a very important sorghum chemical quality trait. Sorghum grain models were developed in view of eliminating the tedious and time-consuming grinding step. Current national sorghum breeding program typically evaluates tannin, starch, and amylose at F4, PVT and NVT stages of inbreeding, whereas availability of NIRS makes it efficient to screen germ plasma tra-

ditional breeding stages, thereby reducing the costs of advancing lines with inadequate tannin, starch, and amylose quality. Overall performance of the developed calibration indicates that NIRS can be confidently used and offer a fast and simple screening option for thousands of samples that must be evaluated every cycle because extractions and chemical reactions and sample preparation are not required. It is recommended to verify the accuracy of extreme values by chemical analysis, especially for very advanced breeding material and need validation by using independent samples in different time interval.

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