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Near-Infrared Reflectance Spectroscopy Calibration for Trypsin Inhibitor in Soybean Seed and Meal

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Abstract: Trypsin inhibitors (TI) are naturally occurring antinutritional factors found in soybean seeds [*Glycine max.* (L.)] that decrease the growth rate of livestock, causing malnutrition and digestion troubles. The current accurate method to quantify TI levels in soybean seeds or meals is by high-performance liquid chromatography (HPLC); however, it is time-consuming, creating bottlenecks in industrial processing. Establishing a near-infrared reflectance spectroscopy (NIR) model for estimating TI in seeds and meals would provide a more efficient and cost-effective method for breeding programs and feed producers. In this study, 300 soybean lines, both seeds and meals, were analyzed for TI content using HPLC, and calibration models were created based on spectral data collected from a Perten DA 7250 NIR instrument. The resulting models demonstrated robust validation, achieving accuracy rates of 97% for seed total TI, 97% for seed Kunitz TI, and 89% for meal total TI. The findings of this study are significant as no NIR calibration models had previously been developed for TI estimation in soybean seed and meal. These models can be used by breeding programs to efficiently assess their lines and by industry to quickly evaluate their soybean meal quality.

Keywords: soybean; trypsin inhibitor; NIR calibration

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1. Introduction

Soybean [*Glycine max* (L.)] is a highly valued row crop, primarily due to its high protein and oil content. The high protein content, balanced amino acid profile, and various vitamins and minerals contained in a soybean have made it a staple in animal feed production [1,2]. In the United States, 70% of soybeans produced are destined for animal feed—totaling 33.12 million tons in 2021 [3]. Despite its nutritional benefits, soybean contains antinutritional factors, such as trypsin inhibitors (TI), that inhibit the absorption and digestibility of nutrients in livestock [2–6]. When livestock consume significant amounts of TI, it can result in severe health implications, such as limited growth, because of the limited protein and nutrient absorption. In more severe cases, overconsumption of TI may lead to the enlargement of the pancreas, liver, intestines, and even result in pancreatitis [7–10]

TI is a naturally occurring protein found in legume species that binds strongly to trypsin, a digestive enzyme in animals, effectively blocking its active site and hindering the digestion process [9,11]. Soybean has two types of TI—Kunitz trypsin inhibitor (KTI) [12] and Bowman–Birk trypsin inhibitor (BBTI) [11,13,14]. The KTI is a monomeric protein

in soybean seeds, consisting of 181 amino acids and 21.5 kDa [12]. It is more abundant than BBTI and forms a strong, irreversible complex with trypsin [15]. In contrast, BBTI is a smaller protein, 7–8 kDa, with various isoforms. Present in lower quantities, BBTI inhibits both trypsin and chymotrypsin [16–18].

To negate the antinutritional effects of TI in soybeans, the raw seeds are heated and processed into a meal. The benefits of heating soybeans were first observed in 1917, and the practice was widely adopted even though the reasons behind its improvement of the feed's nutritional value were not yet understood [19]. In recent years, heating raw soybean to 121 °C for 15 min is the industry's standard practice to deactivate TI within soy meal for livestock consumption [20]. However, this process requires careful control; overheating the soy meal can destroy beneficial nutrients, while insufficient heating may leave TI concentrations too high for animal consumption. As such, TI concentration and nutritional value of the meal is regularly checked to maintain product quality.

To date, numerous methods for detecting TI in soybeans have been published [17,21–24]. However, these existing techniques for measuring TI concentrations are often time-consuming. High-performance liquid chromatography (HPLC), an analytical chemistry technique that separates compounds within a mixture for quantification, is a widely used and efficient method at the time of this study [22,24]. Although HPLC is known for its accuracy and sensitivity, it has several disadvantages. The HPLC method is time-consuming, requires extensive sample preparation, and demands costly maintenance, including expensive reagents, specialized equipment, and skilled personnel, particularly when analyzing complex protein mixtures. The initial equipment cost of a high throughput system, such as the one used in this study, can range from USD 40,000 to USD 120,000 [25]. The estimated cost in 2025 is USD 1.89 per sample [22]. Approximately 13 min per sample is required for HPLC analysis, not including the time needed to grind and prepare samples. These factors make HPLC less practical for high-throughput or routine analysis. Given these limitations, there is a growing need in the animal feed industry for more efficient and practical methods to measure TIs. The creation and adoption of a near-infrared reflectance spectroscopy (NIR) calibration for quantifying TIs on an industrial scale would allow for a more time-efficient evaluation of a soybean seed and meal.

Near-infrared reflectance spectroscopy (NIR) offers a promising alternative for industrial-scale quantification of TIs. Benchtop models, such as the one used in this study, range from USD 15,000 to USD 60,000 [26]. NIR is a secondary analytical technique that quickly identifies and evaluates chemical compositions of a substance [27]. This is achieved by emitting near-infrared light (700–2500 nm) onto a sample, where the wavelengths are absorbed based on the C-O, C-H, and C-N chemical bonds present, and the resulting spectral data are then used to determine composition of various chemical compounds based on pre-existing calibrations [28]. NIR spectroscopy is a non-destructive, fast, and cost-effective method for analyzing agricultural product quality, requiring minimal sample preparation and providing results in approximately 20 s, thereby reducing the need for costly and time-consuming laboratory testing.

It was first developed in 1946 as part of a project funded by the US Department of Agriculture (USDA) to develop a method for grading eggs [29]. Since then, it has become a staple instrument used in breeding programs to evaluate lines for advancement based on desired traits and in the food processing industry to evaluate product quality [30]. NIR's application in agriculture has expanded with successful calibrations for various compounds, including moisture, protein, and oil content, demonstrating its versatility and efficiency [31]. For soybean quality assessment, AACC International (formerly the American Association of Cereal Chemists) currently recommends the NIR method for analyzing protein, crude fat, and moisture content in soybean seeds [32].

The specific wavelengths utilized and available calibrations for NIR analysis vary depending on the manufacturer and the instrument used. In this study, raw spectra data were collected using a DA7250 NIR instrument manufactured by Perten Instruments. The DA7250 NIR can predict the concentration of a sample in under 10 s by utilizing a samples absorption within the 950–1650 nm wavelength range. To our knowledge, no calibration specifically designed for the determination of TI in whole soybean seeds and soybean meal has been developed for the DA7250 or similar NIR models. Developing accurate calibrations for this instrument would significantly reduce the time required to measure TI concentrations in whole seeds and processed meals, thereby leading to a more efficient production process.

As such, the objectives of this study were (a) to develop a DA7250 NIR calibration for the accurate and rapid prediction of TI concentration in whole soybean seeds to be used by breeding programs for evaluating lines potential, and (b) to develop a DA7250 NIR calibration for the accurate and time-saving prediction of TI concentration in soybean meal, enhancing efficiency for industrial feed producers.

2. Materials and Methods

2.1. Plant Material

2.1.1. Whole Seed

A total of 300 soybean plant introductions (PI) were selected from a diverse USDA soybean germplasm collection [33] The 300 samples consisted of soybean in maturity groups 4 and 5, representing diverse origins (China, Japan, USA, Russia, Nepal, Korea, Vietnam, and Morocco) [34]. They were grown in 3 m two-row plots with 76 cm row spacing and harvested in Blacksburg, Virginia in 2020 and 2021. The 300 samples were selected to represent a diverse range of trypsin inhibitor (TI) concentrations, including low, mid, and high concentrations of KTI, BBTI, and total TI (TTI), based on data obtained by HPLC analysis following [22]. TTI was calculated as the sum of KTI and BBTI concentrations per sample. The inclusion of these samples allowed for a model creation that represented naturally occurring TI ranges in soybeans.

2.1.2. Meal Preparation

For analysis of soybean meal, meal samples were prepared from the 300 PI lines. First, the starting moisture content of each sample was determined by placing 3 g of whole seed in an oven set to 103 °C for 72 h. After each sample cooled, the final mass was recorded, and moisture content calculated. The whole seeds were then cracked and dehulled by placing 20 g of each sample in a hopper with a roller mill. The moisture content for the remaining soybean meat was readjusted to 15% using the following equation:

$$0.176 \times (X) - 1.176 \times (Y) \times (X) = Z$$

X = Dehulled mass (grams);

Y = Moisture content in decimal form (not percentage);

Z = DI water to be added (milliliters).

Following the addition of the determined amount of water, the samples were then set in an incubator at 65 °C for 15 min [35]. The samples were then added to a roller mill for the creation of flakes. Solvent extraction was performed on the resulting flakes. Each sample was run on a Dionex ASE 350, set to the following method: 65 °C, static time 15 min, 3 cycles, solvent B-Hexane. After the completion of the run, the resulting samples were set under a fume hood overnight to allow for evaporation of the solvent. Each sample was then stored in an airtight bag.

2.2. Spectral Methodology

Approximately 50 g of whole soybean seeds were selected from the 300 lines. Each of the 300 samples were individually placed in the small seed breeding tray and scanned on the Perten DA7250 NIRS instrument (Perten Instruments, Springfield, IL, USA). The spectral data recorded for each sample consisted of 141 datapoints collected across a wavelength range of 950–1650 nm (Operation and Handling—DA 7250TM NIR | PerkinElmer, n.d.). A complete spectral dataset from the 300 samples was then exported from the instrument to be used for model creation. The same method was repeated with approximately 50 g of soybean meal, and the spectra data exported.

2.3. Trypsin Inhibitor Quantification by HPLC

The HPLC method used to quantify TI in both the untreated seed and the meal was conducted following the procedure previously developed by Rosso et. al [22]. Briefly, 10 mg of finely ground soybean seed powder was mixed with 1.5 mL of 0.1 M sodium acetate buffer (pH 4.5). Samples were vortexed and shaken for 1 h at room temperature. The sample was centrifuged at 12,000 rpm for 15 min. One mL of the supernatant was filtered through a syringe with an IC Millex-LG 13 mm mounted 0.2 mm low protein-binding hydrophilic millipore (polytetrafluoroethylene [PTFE]) membrane filter (Millipore, Cork, Ireland). The TI in solution was separated on an Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, CA, USA) equipped with a guard column (4.6 × 5 mm) packed with POROS R2 10 mm Self Pack Media and a Poros R2/H perfusion analytical column (2.1 × 100 mm, 10 um). The mobile Phase A consisted of 0.01% (v/v) trifluoroacetic acid in Milli-Q water, and the mobile Phase B was 0.085% (v/v) trifluoroacetic acid in acetonitrile. The injection volume was 10 µL, and the detection wavelength was 220 nm.

2.4. Model Creation, Cross-Validation, and Statistical Analysis

The method for model creation, cross-validation, and statistical analysis followed the procedure reported by Lord et al., 2021 [35]. The CAMO Unscrambler X software, 10.1 (CAMO Analytics AS) was used for the spectroscopic data pretreatment, model creation, and internal cross-validation of the model. A representative subsample of 124 seeds and 112 meal samples were selected using R 4.3 2 [36] ensuring an equal distribution of low, mid, and high concentrations of each of the six TI values (mg/g): STTI: seed total trypsin inhibitor; SKTI: seed Kunitz trypsin inhibitor; SBBTI: seed Bowman–Birk trypsin inhibitor; MTTI: meal total trypsin inhibitor; MKTI: meal Kunitz trypsin inhibitor; and MBBTI: meal Bowman–Birk trypsin inhibitor. The spectroscopic data for each of the six datasets were then pretreated first with standard normal variation, followed by detrending, which corrected for light scatter and particle size. Models were created using the transformed data and partial least squares regression (PLSR) based on previous studies [34,35]. The number of PLSR components, 10, was determined based on the number of factors that minimized the predicted residual error sum of squares. To perform a 10-fold cross-validation, the samples were randomly divided into 10 equal segments, each consisting of 11 or 12 samples. Unscrambler performed cross-validation by holding out the samples randomly placed in a segment. The model was then recalibrated without the selected samples, and the recalibrated model was used to predict the values of the withheld samples. This was repeated for each segment, until all samples had been withheld. Each model resulted in an R^2 and RMSE value for the calibration and cross-validation of each model. The R^2 demonstrates statistically how well the TI concentrations determined by HPLC match the concentrations predicted from the spectral model. The reported root mean square error (RMSE) values represent the average error present within the model [37–39].

3. Results

3.1. Sample Concentration of Trypsin Inhibitor

As expected, the STTI subsample had the highest concentration of TI, as its seeds were raw and without any heat treatment. The total TI in seeds ranged from 2.62 to 13.13% of the total seed content (Table 1). TI concentration in soybean varieties have been reported to range from 0.07 to 18.7%, with anything less than 6% being considered low and greater than 10% high concentration [40]. Therefore, the broad range of TI concentrations in our subsample set effectively captured the diversity found in soybean germplasm, providing a robust basis for calibration model development. However, the preparation of soybean meal resulted in a notable decrease in total TI concentration by at least 2 mg/g across all samples, reflecting the effectiveness of processing in reducing antinutritional factors. The reduction in TI was expected, given the heat treatment applied during meal preparation, which is known to denature these proteins.

Table 1. TI concentration determined by HPLC in the six models: seed total trypsin inhibitor (STTI), seed Kunitz trypsin inhibitor (SKTI), seed Bowman–Birk trypsin inhibitor (SBBTI), meal total trypsin inhibitor (MTTI), meal Kunitz trypsin inhibitor (MKTI), and meal Bowman–Birk trypsin inhibitor (MBBTI).

TI Model	Range %	Mean % [1]	SD	CV
STTI	2.62–13.13	8.39	1.87	0.22
SKTI	0.49–7.78	4.40	1.06	0.24
SBBTI	0.34–7.79	3.99	1.35	0.33
MTTI	0.38–10.25	4.02	1.55	0.37
MKTI	0.24–10.76	2.98	1.26	0.42
MBBTI	0.0–4.27	1.14	0.72	0.63

[1] Mean and range reported in mg/g of sample (%). SD: standard deviation. CV: coefficient of variance.

3.2. Calibration Model Performance

The calibration and validation statistics for each model are presented in Table 2, and the model fit is shown in Figure 1. The STTI and SKTI validation models both resulted in a 97% rate of accuracy, based on the R2 values. Given that the STTI range was 2.62–13.13%, and the model's RMSE was 1.579, a 13.7% error rate was indicated in the validation model. This is considered a moderate range of error and may be acceptable depending on the intended application of the model. The SKTI validation model had a more acceptable error rate of 10.3% calculated from the reported RMSE value of 0.74. The MTTI resulted in a moderately successful validation model as well, with an 86% accuracy rate and a moderate error rate of 15%.

Table 2. Calibration and validation for the TI models, with R^2 representing model accuracy and RMSE the average error rate.

TI Model	Sample Size	Calibration		Validation	
		R^2	RMSE	R^2	RMSE
STTI	124	0.937	1.460	0.968	1.579
SKTI	124	0.979	0.676	0.975	0.741
SBBTI	124	0.027	1.269	0.017	1.287
MTTI	112	0.892	1.398	0.864	1.560
MKTI	112	0.059	1.116	0.052	1.126
MBBTI	112	0.021	0.641	0.016	0.648

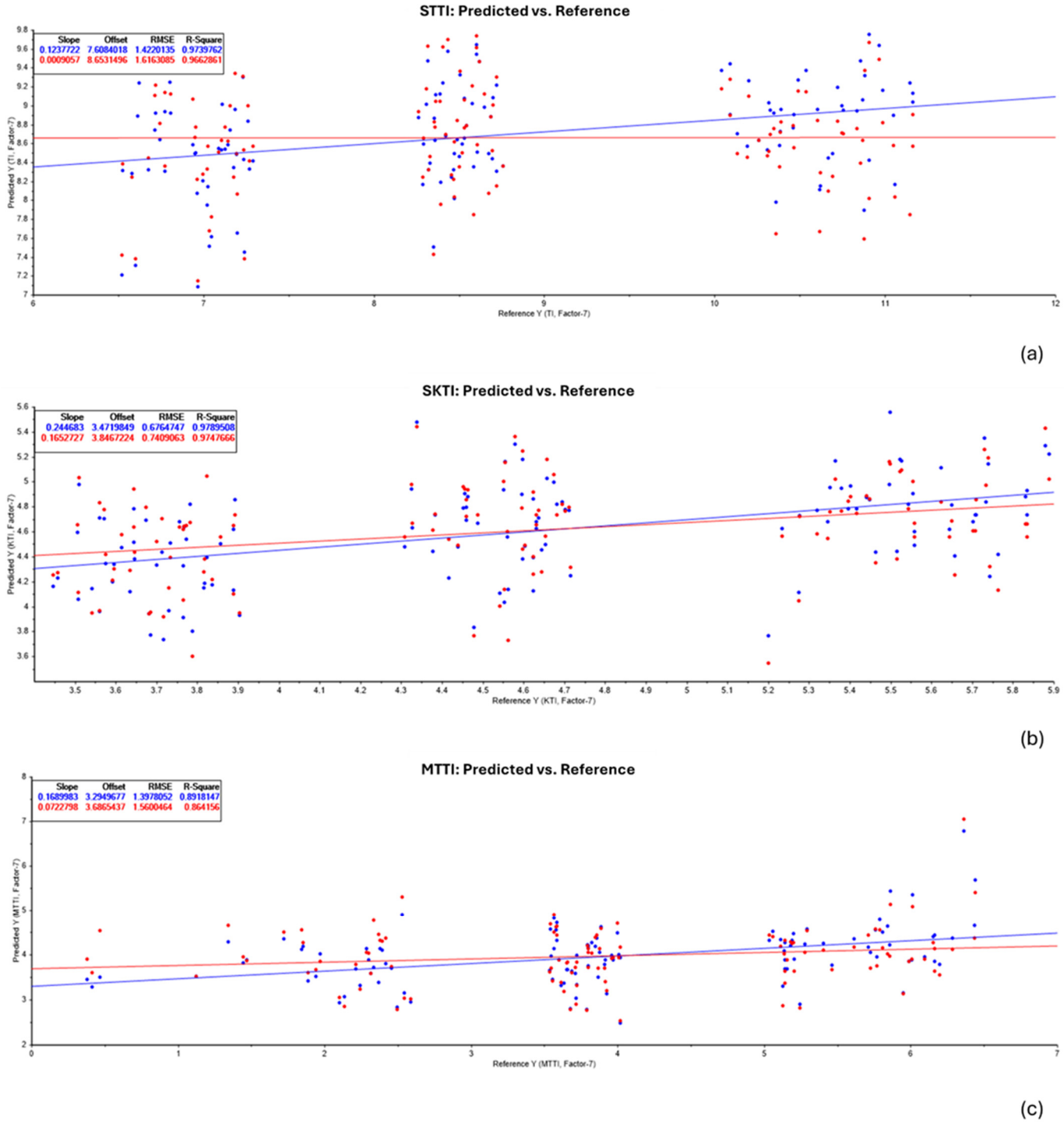

Figure 1. Relationship between the predicted slope (red) and the reference slope (blue) values and fit of (**a**) STTI, (**b**) SKTI, and (**c**) MTTI models.

In contrast, the SBBTI and MBBTI models performed poorly, with validation R^2 values of only 0.017 and 0.016, respectively. As shown in Figure 2, BBTI averaged 47% of STTI and 27% of MTTI composition. Additionally, the low performance for the MKTI model may be due to the reduced concentration of TI by the applied heat treatment, reducing the levels of KTI present to values undeterminable by the NIR instrument used in this study.

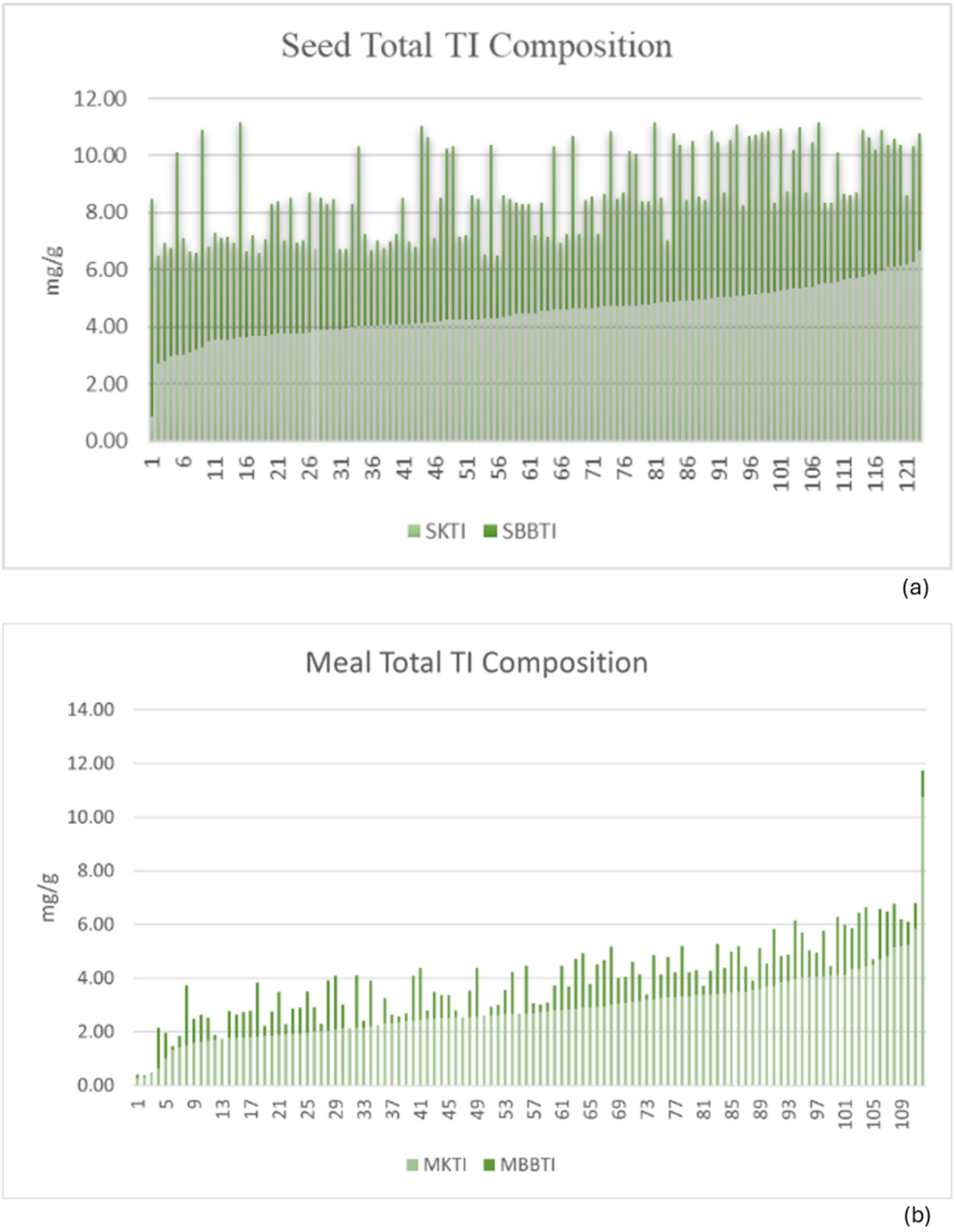

Figure 2. Percentage of KTI and BBTI content for (**a**) whole seed and (**b**) meal samples.

The summary of the spectral data's contribution to the successful models is shown in Figure 3. In the three graphs, upward peaks represent a portion of wavelength that had a positive correlation to the model creation. The downward peaks have a negative correlation and interference with the model creation. In each of the successful models, the wavelength range of 1450–1470 nm was significant, indicating that the chemical structure of TI may absorb this range of infrared wavelengths. The wavelength range 1410–1430 nm

had a negative correlation with the model creation for both STTI and SKTI. This close range between the negative and positive peak may also be a reason for the error rate in the validation models. The STTI model had a high peak at 1390–1410 nm, not present in the other models.

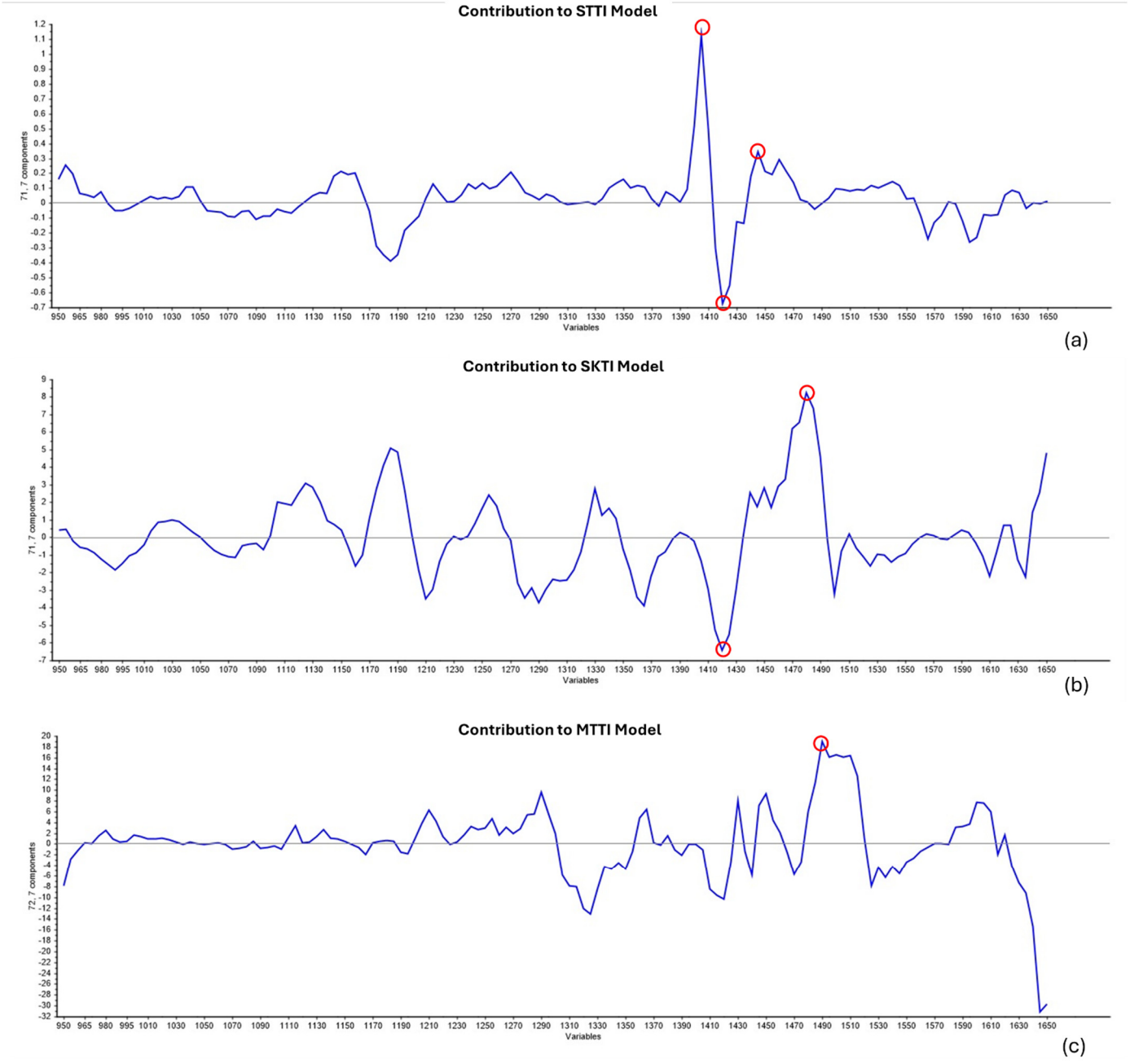

Figure 3. Absorption of each wavelength and its contribution to the model creation: (**a**) STTI; (**b**) SKTI; (**c**) MTTI. Red circles highlight wavelength ranges with significant model impact.

4. Discussion

The model statistics shown in Table 2 suggest that the NIR calibration models for STTI, SKTI, and MTTI can be reliably used for rapid TI quantification in soybean breeding and industrial applications. In contrast each attempt at a BBTI model was unsuccessful, suggesting that the chemical composition of BBTI may not be suitable for accurate NIR prediction of BBTI concentrations. The molecular size of BBTI in soybean is approximately 8 kDa [32–34], which is much smaller than that of KTI (approximately 20 kDa). In addition

to its smaller molecular size, BBTI consists of seven disulfide bonds, while KTI has only two [41]

Other organic compounds consisting of multiple disulfide bonds, similar to BBTI, have been reported to correspond to peaks around 1700 nm [42] which lies beyond the spectral range of the DA7250 instrument (900 nm–1650 nm). Additionally, the low performance of BBTI may have negatively impacted the overall accuracy of the STTI calibration model, contributing to the 4% decrease between STTI and SKTI. Despite the poor performance of the MKTI and MBBTI models alone, the MTTI models may have been successful in part due to the higher combined concentration of the total TIs compared to the lower concentrations of the individual TI samples.

The wavelength range identified in Figure 3 correlates with a wavelength range previously reported in a study that created an NIR calibration model on another instrument for soy cakes [43]. In this study by Hoffmann, they identified two wavelength ranges related to TI that are beyond the range analyzed by the Perten DA7250 (1640–1830 nm and 2100–2300 nm). A study performed in 2009 also identified peaks in wavelengths greater than 1650 nm for heat-treated trypsin inhibitor activity [44]. This suggests that the error rates and the unsuccessful attempts to create a model for SBBTI, MKTI, and MBBTI may once again be due to the limitations of the instrument used in this study. However, given the popularity of the Perten DA7250 instrument, our models are expected to be broadly adopted by soybean breeders and meal processors.

In conclusion, three models—STTI, SKTI, and MTTI—were successfully developed and validated. The novelty of this study's findings is that they offer a rapid and cost-effective alternative to traditional HPLC methods that was not previously available. Although methods, such as HPLC, will still remain necessary for precise quantification of TI within a seed or meal sample, the Perten DA7250 instrument provides a valuable tool for quick evaluation of a soybean seed for a breeding program and the meal for an industrial feed producer. Future directions include expanding model development to other commonly used benchtop NIRs for further accessibility. Additionally, further research focused on developing accurate models for BBTI quantification using NIR with spectral ranges accommodating BBTI's molecular structure would be beneficial. Soybean breeding programs will benefit from the rapid assessment of TI content, enabling them to efficiently screen and focus resources on varieties that meet their objectives, thereby avoiding investment in non-viable lines. Within the industry sector, the adoption of an NIR model for estimating total TI in meal samples will allow for a more time-efficient and cost-effective evaluation of their production line and final product quality control.

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Abbreviations

The following abbreviations are used in this manuscript:

BBTI	Bowman–Birk trypsin inhibitor
HPLC	high-performance liquid chromatography
KTI	Kunitz trypsin inhibitor
MBBTI	Meal Bowman–Birk trypsin inhibitor
MKTI	Meal Kunitz trypsin inhibitor
MTTI	Meal total trypsin inhibitor
NIR	Near-infrared reflectance
PLSR	Partial least squares regression
RMSE	Root mean square error
SBBTI	Seed Bowman–Birk trypsin inhibitor
SKTI	Seed Kunitz trypsin inhibitor
STTI	Seed total trypsin inhibitor

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