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Nondestructive Determination of Lignans and Lignan Glycosides in Sesame Seeds by Near Infrared Reflectance Spectroscopy

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Sesame (*Sesamum indicum* L.) contains abundant lignans including lipid-soluble lignans (sesamin and sesamolin) and water-soluble lignan glycosides (sesaminol triglucoside and sesaminol diglucoside) related to antioxidative activity. In this study, near infrared reflectance spectroscopy (NIRS) was used to develop a rapid and nondestructive method for the determination of lignan contents on intact sesame seeds. Ninety-three intact seeds were scanned in the reflectance mode of a scanning monochromator. This scanning procedure did not require the pulverization of samples, allowing each analysis to be completed within minutes. Reference values for lignan contents were obtained by high-performance liquid chromatography analysis. Calibration equations for lignans (sesamin and sesamolin) and lignan glycosides (sesaminol triglucoside and sesaminol diglucoside) contents were developed using modified partial least squares regression with internal cross-validation ($n = 63$). The equations obtained had low standard errors of cross-validation and moderate R^2 (coefficient of determination in calibration). The prediction of an external validation set ($n = 30$) showed significant correlation between reference values and NIRS predicted values based on the SEP (standard error of prediction), bias, and r^2 (coefficient of determination in prediction). The models developed in this study had relatively higher values (more than 2.0) of SD/SEP(C) for all lignans and lignan glycosides except for sesaminol diglucoside, which had a minor amount, indicating good correlation between the reference and the NIRS estimate. The results showed that NIRS, a nondestructive screening method, could be used to rapidly determine lignan and lignan glycoside contents in the breeding programs for high quality sesame.

KEYWORDS: Sesame (*Sesamum indicum* L.); NIRS; lignan; sesamin; sesamolin; sesaminol triglucoside; sesaminol diglucoside; HPLC

INTRODUCTION

Sesame (*Sesamum indicum* L.) is one of the most important oil crops and has been used as a traditional health food in Korea. The sesame oil and seed are mainly used for commercial products. An advantage of both sesame seed and sesame oil is their resistance to oxidative deterioration, resulting in oxidative stability during storage and processing (*1*, *2*). Sesame seed contains a number of antioxidants including lipid-soluble lignans such as sesamin and sesamolin and water-soluble lignan glycosides such as sesaminol triglucoside and sesaminol diglucoside (*3*). These four antioxidants are principle constituents in sesame seeds, and their amounts are important factors in evaluating seed quality. Chemical structures of lignans and lignan glycosides were shown in **Figure 1**. These bioactive compounds are important reasons to evaluate sesame seed quality. Average contents of lignans and lignan glycosides of Korean sesame varieties were 4.08 and 2.47 mg/g of sesamin and sesamolin and 0.68 and 0.11 mg/g of sesaminol triglucoside and sesaminol diglucoside, respectively (*4*, *5*).

Recently, more emphasis has been placed on evaluating qualitative traits in food processing and plant breeding, especially increasing the bioactive lignan content. Qualitative determinations of sesame oils and seeds have been performed by analytical instruments such as high-performance liquid chromatography (HPLC) and gas liquid chromatography. Lignan is generally determined by an HPLC-UV/vis system equipped with a reversed phase column (*5*, *6*). However, this analytic method is disadvantageous for selecting superior sesame breeding lines because it is time-consuming, costly, and labor intensive. Thus, a rapid and nondestructive method is in high demand to evaluate quantitative and qualitative traits of sesame seed for a sesame breeding program, as in sunflower (*7*).

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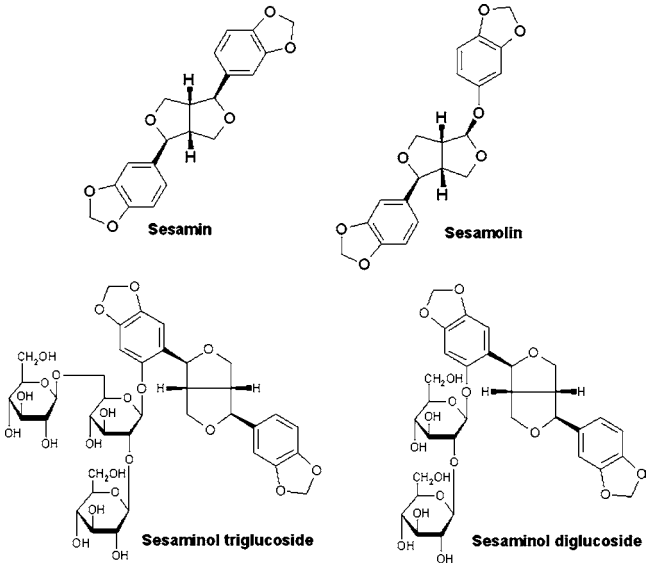

Figure 1. Chemical structures of lignans and lignan glycosides in sesame seeds.

Near infrared spectroscopy (NIRS) is known to play a fundamental role in simplifying the analysis of bioactive compounds without sample preparation. NIRS has been applied for the analysis of qualitative characteristics in food and agricultural commodities (*8*, *9*) and the determination of diverse compounds and the classifications in numerous foods and industrial crops, such as sesame (*10*), soybean (*11*), perilla and peanut (*12*), sunflower (*13*), rapeseed (*14*), rice (*15*), maize (*16*), and conifer (*17*). However, the use of the nondestructive NIRS to determine lignan content in sesame seeds has not been reported.

The objectives of this study were to investigate NIRS analysis for predicting lignans (sesamin and sesamolin) and lignan glycosides (sesaminol triglucoside and sesaminol diglucoside) contents and to develop large scale screening techniques in intact samples for sesame breeding programs.

MATERIALS AND METHODS

Sesame Seed Samples. Ninety-three sesame seeds obtained from breeding lines of Jeonnam Agricultural Research and Extension Services (Naju, Korea) were used to develop the NIRS prediction model for the determination of lignans (sesamin and sesamolin) and lignan glycosides (sesaminol triglucoside and sesaminol diglucoside) contents. All samples used in this study were from germplasm with white seed coat including 20 Korean-recommended sesame varieties. The sesame plants were grown in the greenhouse and harvested in 2004. The harvested seeds were cleaned and dried in the laboratory and then stored in desiccators until NIRS and HPLC analysis.

Chemical Analysis. About 2 g of each sample was homogenized using an Ultra-Turax T8 homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany), extracted in 30 mL of *n*-hexane for 1 day by shaking at 100 rpm using a VS-8480 SRN horizontal shaker (Vison Co., Bucheon, Korea), and filtered through filter paper. The residues were extracted two more times, and the combined extract solution was exactly adjusted to 100 mL. The hexane extract was used for lipid-soluble lignan analysis, while the residue (defatted sesame meal) after hexane extracting was stored at −20 °C for analysis of water-soluble lignan glycosides.

A 10 mL aliquot of *n*-hexane extract was taken and completely vacuum-dried, and then, 3 mL of methanol was added and shaken under warm conditions (under 60 °C). The methanol extract was stored for 1 day at −20 °C, and the upper layer was transferred to a 2 mL autosampler vial before HPLC injection for the determination of lipid-soluble lignans (sesamin and sesamolin). The Agilent 1100 Series HPLC instrument (Agilent Technologies Co., Palo Alto, CA) was a diode array UV/vis detector operated at 290 nm, which also provided the UV spectra of the peaks, and a 150 mm × 4.6 mm i.d., 5 μm, reversed phase column, Capcell Pak C18 UG 120 (Shiseido Co., Tokyo, Japan) was used. The mobile phase was a mixture of methanol:water (75:25, v/v) as an isocratic solvent, and the flow rate was set at 1.0 mL/min. The running time was 20 min for each sample.

The defatted sesame meal (about 0.9 g) in a 50 mL conical tube was extracted with 40 mL of 80% methanol/water for 1 day at room temperature by shaking at 150 rpm, and the supernatant was transferred to a 2 mL autosampler vial before HPLC injection for the determination of lignan glycosides (sesaminol triglucoside and sesaminol diglucoside). The HPLC system was equipped with a 150 mm × 4.6 mm i.d. Develosil ODS-UG-5, reversed phase column (Nomura Chemical Co., Seto, Japan) and an UV/vis detector operated at 290 nm. The mobile phase was a linear gradient from solvent A, methanol:water (30:70, v/v), to solvent B, methanol:water (80:20, v/v), in 40 min, and the flow rate was set at 1.0 mL/min. The running time was 60 min for each sample. Each peak of lignan compounds was identified by further analyzation with LC-MS (HCTultra, Bruker Daltonics Inc., Billerica, MA) and comparison of HPLC retention time and UV spectra with each lignan standard (*4*, *6*).

Spectra Collection and Pretreatment. The spectroscopic analysis was performed using a NIRSystem model 6500 near infrared scanning monochromator (Foss NIRSystems Inc., Silver Spring, MD) in the reflectance mode. Intact seed samples (about 4 g) were placed in a standard ring cup and then scanned. Reflectance energy readings were references to corresponding readings from an internal ceramic disk. Each spectrum was recorded once from each sample and was obtained as an average of 32 successive scans over the sample, plus 16 scans over the standard ceramic, before and after scanning the samples. All spectral data were recorded as the logarithm of reciprocal of reflectance (log 1/*R*) in the wavelength range between 400 and 2500 nm at 2 nm intervals to give a total of 1050 data points per sample. The scanning procedure could be completed within 1.5 min per sample when the NIRS instrument was warmed-up and the stability was confirmed through photometric repeatability (noise test) and wavelength accuracy test.

The NIRS manipulation for scanning, mathematical processing, and statistical analysis was performed with the WinISI II software (Windows version 1.60) (Foss and Infrasoft International LLC, State College, PA). In WinISI software, the Score program was used to choose samples for spectrum outliers and select samples to represent the whole sample set ($n = 93$) before calibration and validation in this study. The distance between a sample and its neighbor was measured as the H distance called the neighborhood H. 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 (*18*).

The samples ($n = 93$) were randomly split into two sets using the WinISI program. The calibration set (63 samples) was used to calibrate and cross-validate the equation derived, and the other 30 samples as an external validation set were used to test the goodness of fit of the developed equations.

Data Processing. The equations for NIRS prediction were developed using the Global program in WinISI software with the modified partial least squares (MPLS) regression using wavelengths of the entire visible (400−1100 nm) and near infrared (1100−2500 nm) region at every 8 nm. Besides MPLS, regression methods such as PLS (partial least squares), principle component regression, and multiple linear regression were tested to develop the calibrations for lignan content in sesame seeds. No scatter correction (log 1/*R*) and scatter correction using standard normal variate and detrending (SNVD) were evaluated. 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 samples (*19*, *20*). Various mathematical treatments were applied for equation development. For example,

2,5,5,1, the first number indicated the order of derivative (two is the second derivative of log 1/*R*), the second number was the gap in data points over which the derivative was calculated, and the third and fourth numbers represented the number of data points used in first smoothing and second smoothing (*21*).

The application of the second derivative algorithm to the raw spectra (log 1/*R*) resulted in an increase of the complexity of spectra and a clear separation between peaks, which overlapped in the raw spectra. 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 performances of the different equations obtained in the calibration were 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 (*22*). The best predicted equations for each chemical constituent were selected on the basis of minimizing the SECV and increasing the R^2 (*23*). Two passes to eliminate outliers were set by two outlier detection methods, *t* and *H* statistics (Mahalanobis distance) in WinISI software. The *t* statistics identify outliers that have residuals from reference analysis of greater than the 2.5 times SEC. Outliers indicated that their reference values were in doubt and the samples were in different populations because of atypical spectra. The ratio of standard deviation (SD) of reference data to SECV was calculated to evaluate the performance of calibrations (*24*).

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 predication (r^2). The validation sample set allowed the NIRS equation to be validated for prediction accuracy, using random samples not included in the calibration sample set (*23*). The equations selected for lignans and lignan glycosides in intact sesame seeds were monitored with the Monitor program in WinISI software, using the validation set.

RESULTS AND DISCUSSION

Spectroscopic Analysis. The NIR reflectance spectrum and SD of absorbance of the intact sesame seed samples are shown in **Figure 2**. The main absorption bands are observed at 1208 nm related to C−H stretching second overtone (−CH_2), 1496 nm related to C−H stretching first overtone, 1724 nm related to C−O (oil) and C−H stretching first overtone (−CH_2), 1942 nm related to O−H bending second overtone (water), and 2308 nm related to C−H bending second overtone (oil). In addition, significant bands with chemical information appear at 1450 nm related to O−H stretching first overtone (water) and C═O stretching third overtone (−C═O), 1760 nm related to C−H (oil) and C−H stretching first overtone (−CH_2), and 1946−1956 nm related to C═O stretching second overtone (−CO_2R). Minor bands appeared at 1024, 2058, 2146, and 2174 nm and were attributed to oil and hydrocarbons. The information of functional group in the spectrum was searched from WinISI II software (Windows version 1.60), which gave chemical information associated with absorption bands in the specific NIR region. The overall spectrum showed strong absorption bands related with oil and water and were similar to other oil crops such as perilla, peanut, and soybean, especially in the near infrared region (*11*, *12*).

The second derivative spectra had a trough corresponding to each peak in the original spectra, removing the overlapping peaks and baseline effects (*25*). The second derivative variation shown in **Figure 3** fluctuated more at 912, 1148, 1712, 1944, and 2294 nm with higher values of SD spectra. In addition to bands displayed in raw spectra, the mean spectrum of the second derivative (**Figure 3**) showed absorption bands at 1680 nm related to C−H stretching first overtone (aromatic) and 1212, 1726, and 1762 nm related to hydrocarbon (−CH) in the NIR region, showing absorbance peaks similar to a raw spectrum.

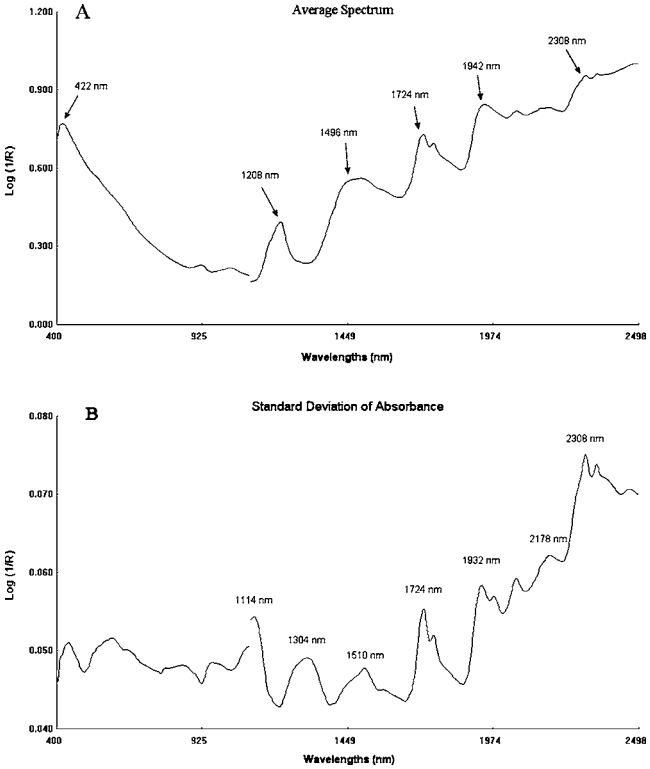

Figure 2. Raw NIR average spectrum of intact sesame seeds (**A**) and SD of absorbance (**B**).

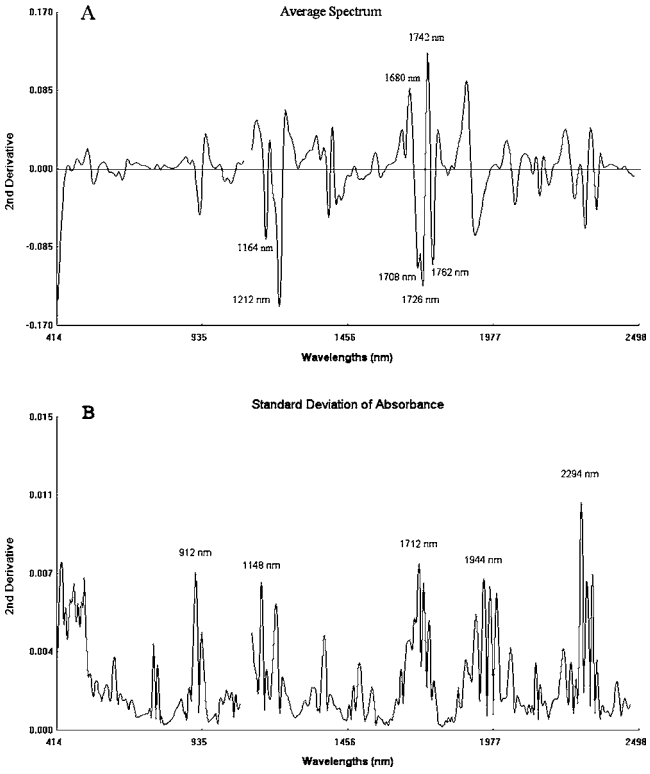

Figure 3. Second derivative of NIR average spectrum of intact sesame seeds (**A**) and SD of absorbance (**B**).

Figure 4 represented the correlation between each wavelength and reference values for lignans and lignan glycosides in second derivative spectra. Spectra patterns for sesamin and sesamolin were very similar to those for sesaminol triglucoside and sesaminol diglucoside, but the peak and trough of spectra were inversely changed in the whole spectra region. Correlation

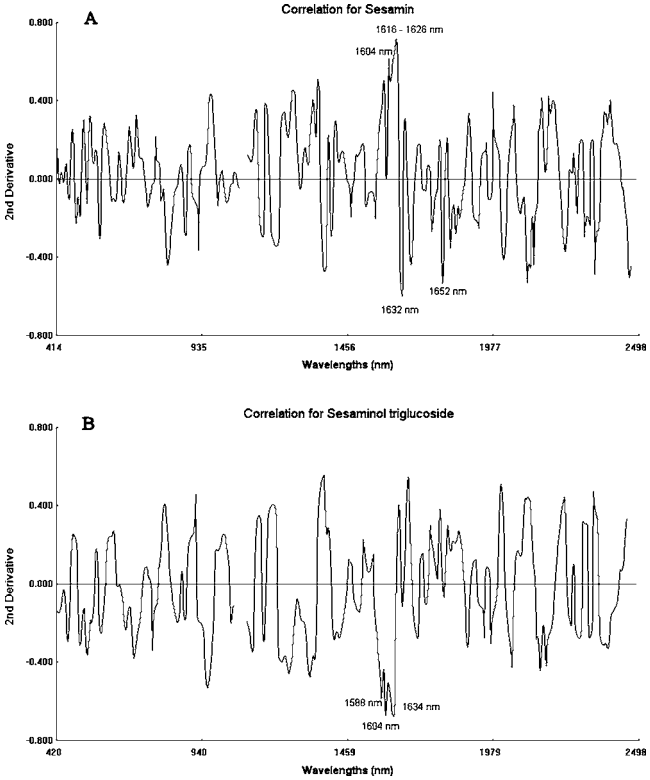

Figure 4. Distribution of correlation coefficients between each wavelength and reference values for sesamin (**A**; mathematical treatment "2,5,5,1") and sesaminol triglucoside (**B**; mathematical treatment "2,8,6,1") in second derivative spectra.

coefficients in the spectra of sesamin type were higher than in those of sesaminol triglucoside type and were high at 1604 ($r = 0.62$), 1616−1626 ($r = 0.67$), 1632 ($r = 0.71$), and 1652 nm ($r = -0.60$), ranging from 1600 to 1700 nm. For spectra of sesaminol triglucoside type, highly correlated peaks were distributed at 1588 ($r = -0.60$), 1604 ($r = -0.63$), and 1634 nm ($r = -0.72$), ranging from 1500 to 1700 nm overlapped with sesamin correlation. Correlation peaks for spectra of sesamin and sesaminol triglucoside were higher overall than those of sesamolin and sesaminol diglucoside, and peak height and distribution for spectra of sesamin and sesaminol triglucoside were much the same as those for sesamolin and sesaminol diglucoside, respectively. This may be due to the similar chemical structure and molecular weight of each of the lignans and lignan glycosides. Highly correlated wavelengths for sesamin and sesaminol triglucoside appeared at 1616−1626 nm associated with C−H stretching first overtone (=CH_2).

Chemical Analysis. The descriptive statistics including mean and SD for content of lignans (sesamin and sesamolin) and lignan glycosides (sesaminol triglucoside and sesaminol diglucoside) of sesame samples used in the calibration and validation sets showed relatively large variation, which were expected to be applicable to wide ranges of samples. Each reference value of lignans and lignan glycosides in a validation sample set was similar to those in the calibration sample set. Mean and SD values of lignans and lignan glycosides were 2.50 and 1.24 mg/g of sesamin, 2.58 and 0.86 mg/g of sesamolin, 0.68 and 0.33 mg/g of sesaminol triglucoside, and 0.05 and 0.03 mg/g of sesaminol diglucoside in the calibration set and 2.32 and 1.20 mg/g of sesamin, 2.54 and 0.67 mg/g of sesamolin, 0.70 and 0.32 mg/g of sesaminol triglucoside, and 0.05 and 0.02 mg/g of sesaminol diglucoside in the validation set, respectively. Total lignans (TLs) represented the sum of sesamin and sesamolin contents, and total lignan glycosides (TLGs) indicated the sum of sesaminol triglucoside and sesaminol diglucoside contents. These mean values for lignans and lignan glycosides were lower than other results reported (*5*, *6*). Normality (*W* test) of the samples was tested for each compound. The *p* values of lignan glycosides with relatively smaller amounts were not above 0.05 at a significant level of 5%, not showing normal distribution of data. The other sample data were normally distributed.

Calibration Model for Lignans. Table 1 showed the statistics of calibration and cross-validation for lignans (sesamin and sesamolin), including the SEC and R^2 values for the equations of best fit obtained for sesamin and sesamolin. The 1-VR (the explained variance), SECV, and number of terms (*T*) are also shown in **Table 1**. The optimum equations for sesamin and sesamolin were the best models with MPLS regression method in the near infrared region (1100−2500 nm) using mathematical treatment 2,5,5,1 (second derivative transformation) with scatter correction (SNVD). Using this near infrared spectra (1100−2500 nm), higher R^2 (0.906−0.947) and lower SEC (0.026−0.046) values were obtained, better than the whole vis-NIR range (400−2500 nm) and the visible range (400−1100 nm). The visible region had a high absorption band at 422 nm of the violet part related to electronic vibration (**Figure 2**) that made a difference to spectra data by seed coat color, not by lignan compounds. NIRS could be sensitive to variations in seed coat color as it is known that color affects the degree of light reflected from the sample. The use of mathematical treatment yielded the equations for lignans with the highest prediction abilities when it was applied over the near infrared segment (1100−2500 nm), not being affected by the color of the seed coat despite a possible change of the visible region (400−1100 nm).

Optimum wavelengths for NIR analysis have generally relied on empirical calibrations to predict qualitative constituents for agricultural products because the broad array of chemical compounds present in the samples led to extensively overlapping and perturbing NIR absorption bands. Our spectra analysis for the correlation between each wavelength and reference value (**Figure 4**) gave good calibration models [lowering SEC, SECV, SEP(C) values], especially for sesamin and sesamolin (data not shown), using more specific near infrared segments (1600−1700 nm) assigned to mainly C−H and C=O stretching vibrations first overtone in hydrocarbons (−CH) and C−O and C−H in oil. A similar result was reported using NIRS in the selected wavelengths (1700−1800 and 2100−2400 nm) for the classification of edible oils from oil crops (*26*).

In **Table 1**, the R^2, SEC, and SECV were 0.947, 0.28, and 0.48 in sesamin, 0.906, 0.26, and 0.41 in sesamolin, and 0.945, 0.46, and 0.78 in TLs, respectively. The equation for sesamolin had moderate SEC values but a little lower R^2 values than the one for sesamin. These may be due to the lower range and SD for sesamolin content of samples used in the calibration. The calibration models developed for sesamin, sesamolin, and TLs had the highest R^2, and the lowest SEC in calibrations and the lowest SECV, the highest explained variance (1-VR) and SD/SECV values in the cross-validations, showed a close relationship between reference values and NIRS estimate values.

Calibration Model for Lignan Glycosides. In developing NIRS models for contents of lignan glycosides (sesaminol triglucoside and sesaminol diglucoside), the statistics of calibrations and cross-validations are shown in **Table 1**. The MPLS regression model using the second derivative transformation with scatter correction (SNVD) of raw reflectance spectra yielded

Table 1. Equation Development Statistics Using MPLS and Scatter Correction for the NIRS Prediction of Lignan and Lignan Glycoside Contents in Intact Sesame Seeds

constituents	spectral range (nm)	math[a]	terms[b]	N^c	calibration SEC	calibration R^2	cross-validation 1-VR	cross-validation SECV	SD/SECV
sesamin	1100–2500	2,5,5,1	8	59	0.28	0.947	0.84	0.48	2.6
sesamolin	1100–2500	2,5,5,1	8	62	0.26	0.906	0.78	0.41	2.1
TLs	1100–2500	2,5,5,1	8	60	0.46	0.945	0.84	0.78	2.6
sesaminol triglucoside	400–2500	2,8,6,1	5	58	0.094	0.910	0.84	0.127	2.6
sesaminol diglucoside	1100–2500	2,5,5,1	8	60	0.084	0.895	0.70	0.014	2.0
TLGs	400–2500	2,8,6,1	5	58	1.011	0.910	0.84	0.136	2.6

[a] Mathematical transformation of spectral data: The first number is the order of the derivative function, the second is the length in data points over which the derivative was taken, and the third and fourth are the segment length over which the function was smoothed. [b] Number of PLS loading factors in the regression model MPLS (modified partial least squares). [c] Samples used to develop the model; 1-VR, one minus the ratio of unexplained variance divided by variance.

the equations of each lignan glycoside. The best equations for sesaminol triglucoside and TLGs were developed using mathematical treatment 2,8,6,1 in the whole visible and NIR spectra range (400−2500 nm). The better equation for sesaminol diglucoside was obtained using mathematical treatment 2,5,5,1 in the near infrared region (1100−2500 nm) showing higher R^2 and 1-VR and lower SEC and SECV values than the different mathematical treatments tested. The equations for each lignan glycoside were selected considering R^2 and SD/SECV values with the highest values, 0.910 and 2.57 of sesaminol triglucoside, 0.895 and 2.01 of sesaminol diglucoside, and 0.910 and 2.60 of TLGs, respectively, as the selection criteria of models. The best prediction models for lignan glycosides were developed with the mathematical approach over the visible and near infrared segment (400−2500 nm), and the results showed that the equations could be used for screening lignan glycoside contents in intact sesame seeds.

External Validation. The robustness of calibration models developed by NIRS analysis was tested through external validation (prediction) with 30 samples, which were not included in the calibration process. The statistics of external validation for lignans and lignan glycosides in intact sesame seeds included bias, r^2, SEP(C) (the corrected SEP), and SD/SEP(C) values, which were factors used to evaluate the reliability of the calibration model. The r^2 values for sesamin, sesamolin, TLs, sesaminol triglucoside, sesaminol diglucoside, and TLGs were 0.837, 0.784, 0.898, 0.824, 0.739, and 0.807, respectively. The SEP(C) values for each compound were 0.49, 0.31, 0.62, 0.14, 0.01, and 0.17, respectively. On the basis of lower SEP(C) and higher r^2 and SD/SEP(C) values, accurate prediction can be monitored with the reliability of the established calibration models. We obtained relatively higher values of SD/SEP(C), which were 2.5 for sesamin, 2.2 for sesamolin, 3.1 for TLs, 2.3 for sesaminol triglucoside, and 2.0 for TLGs, indicating good correlation between reference values and NIRS predicted values in the application of the calibration equations. However, relatively low values of SD/SEP(C), 1.7 for sesaminol diglucoside showing a minute quantity in sesame seeds, did not indicate good correlation between reference values and NIRS estimate values.

Figure 5 displays NIRS predicted values against laboratory reference values in the validation set for sesamin, sesamolin, and TLs, and **Figure 6** plots those for sesaminol triglucoside, sesaminol diglucoside, and TLGs, showing also the relationship between NIRS and reference. These results demonstrated the accurate prediction capacities of the calibration models for lignans and lignan glycosides using a nondestructive NIRS method in sesame seeds.

It is concluded that the determinations of antioxidant lignan compounds can be predicted with sufficient accuracy using NIRS analysis of unground sesame seeds. This nondestructive NIRS method could highly simplify the analysis of qualitative

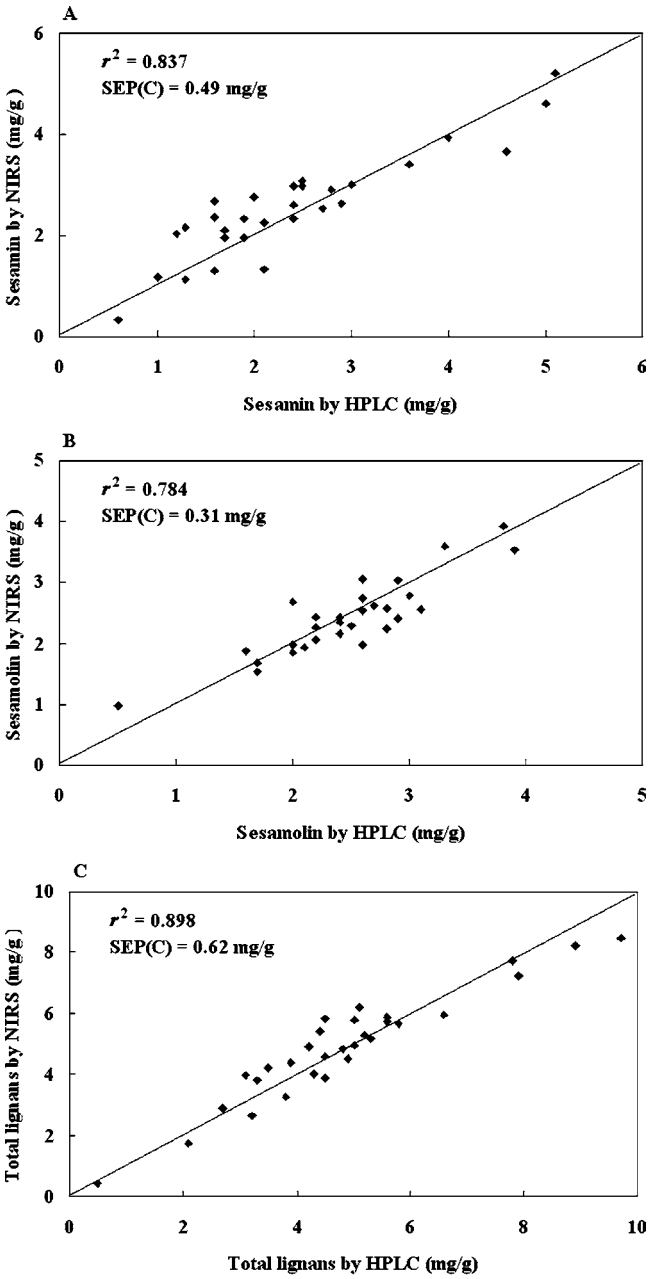

Figure 5. Scatter plots of NIRS vs HPLC values for sesamin (**A**), sesamolin (**B**), and TLs (**C**) contents in the validation set of intact sesame seeds.

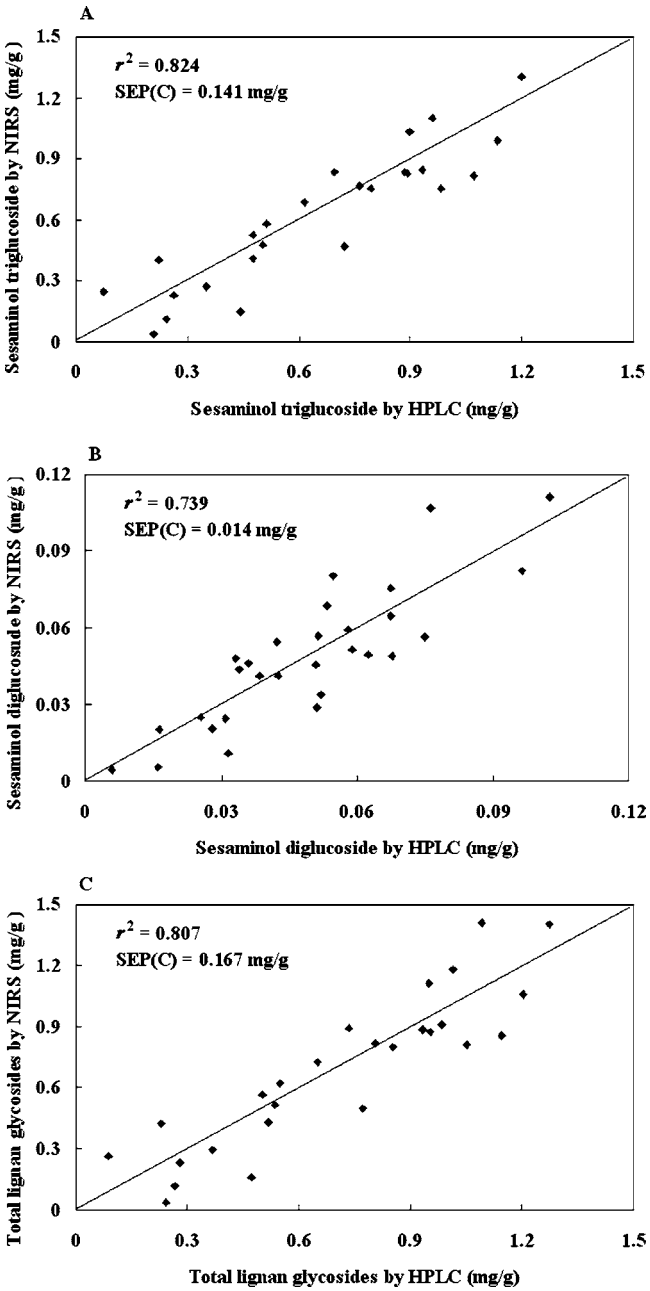

Figure 6. Scatter plots of NIRS vs HPLC values for sesaminol triglucoside (**A**), sesaminol diglucoside (**B**), and TLGs (**C**) contents in the validation set of intact sesame seeds.

factors of interest, because extraction steps with organic solvent were not required and samples without grinding were easily analyzed within a few minutes. These characteristics are 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 can replace chromatographic analytical methods such as HPLC.

The NIRS method is based on the ability of several natural products to readily absorb NIR radiation at a specific region or wavelength. 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 parameter measured for each sample. This relationship can then be used to predict the parameter value in unknown samples. Therefore, it is very reliable in predicting samples that are similar to those used in the calibration. Conversely, samples very different from the calibration samples are often not predicted reliably because of different conditions like species, cultivating area, drying and storage methods, and influences by genetic and environmental variation. The sample set used in this study was not very large and included limited variation. The developed calibration models need to be updated for expanding and increasing the robustness of models by applying to new samples with different cultivation location, growth characteristics, harvest time, cross-population, and qualitative enhancing conditions.

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