

Feasibility study on use of near infrared spectroscopy for rapid and non-destructive determination of gossypol content in intact cottonseeds

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Research

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Abstract

Background: Gossypol found in cottonseeds is toxic to human beings and monogastric animals and is a primary parameter for the integrated utilization of cottonseed products. It is usually determined by the techniques relied on complex pretreatment procedures and the samples after determination cannot be used in breeding program, so it is of great importance to predict the gossypol content in cottonseeds rapidly and non-destructively to substitute the traditional analytical method.

Results: Gossypol content in cottonseeds was investigated by near-infrared spectroscopy (NIRS) and High-performance liquid chromatography (HPLC). Partial least squares regression, combined with spectral pretreatment methods including Savitzky-Golay smoothing, standard normal variate, multiplicative scatter correction, and first derivate, were tested for optimizing the calibration models. NIRS technique was efficient in predicting gossypol content in intact cottonseeds, as revealed by the root-mean-square error of cross-validation (RMSECV), root-mean-square error of prediction (RMSEP), coefficient for determination of prediction (R_p^2), and residual predictive deviation (RPD) values for all models, being 0.05-0.07, 0.04-0.06, 0.82-0.92, and 2.3-3.4, respectively. The optimized model pretreated by Savitzky-Golay smoothing + standard normal variate + first derivate resulted in good determination of gossypol content in intact cottonseeds.

Conclusions: Near infrared spectroscopy coupled with different spectral pretreatments and PLS regression has exhibited the feasibility in predicting gossypol content in intact cottonseeds, rapidly and non-destructively. It could be used as an alternative method to substitute for traditional one to determine the gossypol content in intact cottonseeds.

1. Introduction

Cotton (*Gossypium*. spp) is one of the important industrial and economic crops (Sunilkumar et al. 2006). Cottonseed, the main by-product of cotton production, can be used to produce food, animal feed, and other products. Cottonseed contains many kinds of nutrients, including proteins, oils, fatty acids, and amino acids, making it a potential food resource for human beings with the rapid growth of global population (Sawan et al. 2006). However, the *Gossypium* species are characterized by the presence of gossypol, which is toxic to human beings and monogastric animals (Lordelo et al. 2005), such that the utilization of cottonseed products is limited.

Gossypol, 1, 1', 6, 6', 7, 7'-hexahydroxy-5, 5'-diisopropyl-3, 3'-dimethyl-(2, 2' binaphthalene)-8, 8'-dicarbaldehyde, is a terpenoid compound that helps cotton defend against biotic stress (Kong et al. 2010; Lin et al. 1993; Blanco et al. 1983). Due to the toxicity of gossypol, breeding for both lower gossypol content in cottonseeds and higher gossypol content in cotton plants has been practiced in many cotton-planting countries. The cottonseed breeding work often requires analyzing a large number of cottonseed samples to measure gossypol content. Conventionally, gossypol content is assayed by UV spectrophotometry which not only involves reagents with great toxicity, but also is not accurate and

reliable. Despite offering a high level of accuracy and sensitivity, HPLC is usually costly and time-consuming. In addition, both classical analytical methods cause undesired destruction of the testing samples which frequently needed to be planted in cotton breeding program. So, a rapid and non-destructive method for gossypol determination is required.

Near infrared (NIR) spectroscopy combined with chemometrics is a rapid, convenient, and environmentally-friendly analytical technique in the quality analysis for crops (Sohn et al. 2008; Huang et al. 2013; Weinstock et al. 2006; Rosales et al. 2011; Bellato et al. 2011; Bala et al. 2013; Hacisalihoglu et al. 2010; Mendoza et al. 2018; Lee et al. 2017; Tierno et al. 2016; Yang et al. 2008; Lin et al. 2013; Kovalenko et al. 2006; Fassio et al. 2004). Although the NIR calibration model for determining gossypol content in cotton powder was developed (Li et al. 2017), it could not be used to non-destructively analyze gossypol content in intact cottonseeds, especially in breeding programs where the genetic materials from genetic modification or cross breeding have a limited availability. It is a challenge to determine gossypol content in intact cottonseeds by NIR, because (i) cottonseed being bigger than other crop seeds, so large voids are left between packed samples in sample cells; (ii) some of immature and wizened cottonseeds can be mixed in the samples, which can introduce irrelevant information into the spectra data; and (iii) the tough and thick shell of cottonseed can impact the penetration of NIR light and result in a lower S/N ratio and poor information. Because of these factors, the spectral data of intact cottonseeds are far more complex than that of other crop seeds, which may contain a large amount of useless and uncorrelated information such as noise and background. To overcome these difficulties, sophisticated chemometric methods are applied to extract useful information from NIR spectra and calibrate robust models for gossypol content in intact cottonseeds. Essentially, these include regression methods such as principal component regression (PCR) (Xie et al. 1997), partial least squares (PLS) (Haaland et al. 1988), support vector machines (SVM) (Nie et al. 2008), least squares support vector machines (LS-SVM) (Shao et al. 2012), and artificial neural networks (ANN) (Makino et al. 2010), coupled with spectral pretreatments such as standard normal variate (SNV) (Barnes et al. 1989), Savitzky-Golay (SG) smoothing (Savitzky et al. 1964), multiplicative scatter correction (MSC) (Hopke et al. 2003), and first derivative (Rinnan et al. 2009).

Due to undesired destruction of the test sample, previous NIR models which can be used in detection of gossypol in cottonseed meal, can be barely applied in breeding trials (Li et al. 2017). In this present study, spectroscopy was investigated the feasibility of analyzing gossypol in intact cottonseeds based on NIR spectrometer. The main aim of this study was to establish an optimal model which could provide a powerful technical support for cotton breeders and other people who work on cottonseeds.

2. Materials And Methods

Samples and preparation

A total of 268 samples of cottonseeds were collected from different growing areas, including Hangzhou (Zhejiang, China), Xiaoshan (Zhejiang, China), Sanmen (Zhejiang, China), Sanya (Hainan, China), Wuhu

(Anhui, China), and Yancheng (Jiangsu, China), in 2012, 2013, and 2014. The cottonseed samples were delinted and dried at 30°C to constant weight. After spectral acquisition by NIR spectroscopy, the intact cottonseed samples were dehulled, and then ground to cottonseed kernel powder for HPLC analysis. The preparations were implemented in the same experimental condition in order to reduce the influence of other physical factors.

Gossypol extraction

0.1 g of cottonseed kernel powder was suspended in 5 mL acetone and sonicated in an ultrasonic bath for 45 minutes. Then, the suspension was filtered through quantitative filter paper followed by a filtration with a 0.45 µm syringe filter (Agela, Newark, USA). The sediment was washed three times by acetone. After this procedure, the extract was adjusted to 25 mL using acetone.

HPLC analysis

HPLC analysis was performed on an Agilent 1100 HPLC system (Agilent, Santa Clara, USA), equipped with an auto-sampler and UV detection. A C₁₈ column (250 mm × 4.6 mm, 5 µm, Dikma, Richmond Hill, USA) was employed as the stationary phase. The mobile phase consisted of methanol/0.2% H₃PO₄ (80/20, v/v). The injection volume was 10 µL and the flow rate was 1.0 mL·min⁻¹. The UV detector was set at 238 nm and the column temperature was 25°C. Each sample was measured three times. The limit of detection (LOD) was obtained at a signal-to-noise (S/N) ratio of three and the limit of quantification (LOQ) at an S/N ratio. To detect the stability of gossypol at room temperature, three samples were randomly employed to determine the changes of peak area within 36 hours. HPLC-grade gossypol was purchased from Sigma (Sigma-Aldrich, St. Louis, USA). Methanol (HPLC grade) was procured from Tianjin Chemical Reagent Company (Tianjin, China). Double deionized water was prepared using Milli-Q-water purification system (Millipore, Molsheim, France).

NIR spectra acquisition

The NIR spectra of intact cottonseed samples were scanned with a Büchi Flex-N500 NIR spectrometer (Büchi, Flawil, Switzerland), equipped with a solid sample module as followings. The NIR spectra were collected across the range 4 000-10 000 cm⁻¹, and were recorded with a spectral resolution of 4 cm⁻¹. Samples were measured three times on a rotating cylinder device at 25 ± 0.5°C and 60% relative air humidity. All the spectra were transformed into absorbance (log (1/R)).

Spectral pretreatment

Before calibration, the spectral data were pretreated for an optimal performance. Eight pretreatment strategies which included one or some combination of Savitzky-Golay smoothing, SNV, MSC, and first derivative (Norris gap) were compared with the raw spectra.

Sampling design

Samples were assigned to calibration and prediction sets using Kennard-Stone (KS) selection (Kennard et al. 1969). The calibration models were established with the calibration set, and the prediction set was used to validate the predictive capabilities and analytical features of the calibration models.

PLS regression

PLS regression has been widely used as a calibration method to investigate the relationship between the spectral and the corresponding reference data. Before calibration of the PLS models, the data sets (spectral and reference data) were analyzed using 4-fold cross-validation to develop a full-spectra calibration model. The aim of the cross-validation was to find the optimum number of latent variables (LV) for PLS. The root-mean-square error of cross-validation (RMSECV) served as a measure to adjust the parameters, and the number of LV which provide the lowest RMSECV was selected as the best.

Model evaluation

The estimate of the calibration models was based on following quality parameters:

where N is the total number of samples, Y_{nirs} is the predicted value by calibration models, Y_{ref} is the reference value by HPLC, and SD is the standard deviation.

The coefficient for determination of prediction (R_p^2), the root mean square error of prediction (RMSEP), the coefficient for determination of calibration (R_c^2), the root mean square error of cross-validation (RMSECV), and the residual predictive deviation (RPD) were used as criterion to evaluate model performance. An acceptable model should have high R_c^2 and R_p^2 values and low RMSECV and RMSEP values. Meanwhile, the model is considered as robust if the RPD is higher than 2.5.

Software

NIR spectroscopic data (268 samples \times 1501 variables) were exported in text format, organized in Excel spreadsheets, and then transferred into MATLAB R2011a (Math Works, Natick, USA) for chemometric

analysis. All the algorithms in spectral pretreatments, sampling design, and regressions were implemented with MATLAB R2012a.

3. Results

HPLC analysis

The regression equation, correlation coefficient (r^2), limits of detection (LOD), limits of quantification (LOQ), and average recovery of gossypol were illustrated in Table 1. The retention times of gossypol standard and gossypol extraction were 9.91 and 9.60 minutes, respectively (Fig. 1). Table 2 shows the stability for peak area of gossypol determined by HPLC for 24 hours. All the results indicated that the improved HPLC method could be used to detect gossypol content, and the cottonseed extract should be analyzed within 24 hours.

Table 1

HPLC-VU results.

Regression equation	r^2	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	LOQ ($\mu\text{g}\cdot\text{mL}^{-1}$)	Average recovery (%)
$y = 0.42x - 85.055$	0.999	37.5	125.0	95.16-101.72

r^2 , correlation coefficient; LOD, limits of detection; LOQ, limits of quantification; y , peak area; x , concentration ($\mu\text{g}\cdot\text{mL}^{-1}$).

Table 2

The stability of gossypol determined for HPLC during 24 h.

Sample	Time (h)									RSD (%)
	0	3	6	9	12	15	18	21	24	
1	2378.8	2398.0	2397.9	2435.2	2414.2	2421.1	2428.1	2452.7	2465.3	1.14
2	2871.7	2848.1	2860.2	2885.0	2876.6	2886.2	2932.9	2939.8	3010.5	1.76
3	2854.8	2839.8	2862.2	2892.6	2888.7	2918.6	2965.1	2972.7	3007.1	2.01

RSD, relative standard deviation.

NIR spectra analysis

Across the spectral range of 4 000-10 000 cm^{-1} , absorbance values are mainly associated with the combination and overtone bands of the C-H, N-H, O-H, and S-H bonds (Macho et al. 2002), which were quite sensitive to the compositional variations in complex samples. Fig. 2A shows the raw intact

cottonseed spectra in the NIR spectral region. The spectra showed six broad absorption peaks around the 4 200, 4 700, 5 150, 5 580, 6 900, and 8 400 cm^{-1} . The small peak observed at 4 200 cm^{-1} fell within the regions associated with the combination bands of C-H. At 5150 and 6900 cm^{-1} , these could be attributed to the combination and the first overtone bands of O-H, respectively, which were identified as water absorption. The gentle peaks at 5 580 and 8 400 cm^{-1} overlapped with the second and first C-H overtone regions, respectively. It was worth mentioning that the peak at 4 700 cm^{-1} was attributed to the first C-H combination bands of alkenes and aromatic hydrocarbons, which could be identified as the absorption of polyphenolic terpenes, including gossypol and its derivatives.

The raw spectra were homogeneous, so the presence of noise could not be directly identified. Consistent baseline offsets and bias were present in the spectra, which are common features in the NIR spectra. Hence, eight pretreatment strategies were performed to optimize the raw spectra before establishment of the calibration models. The pretreatment spectra of several types of representative strategies were shown in Fig. 2B, Fig. 2C, and Fig. 2D. To different degrees, all these pretreatments could reduce the physical change among samples due to scattering and remove both additive and multiplicative effects in the spectra. It was noted that ten variables were lost after SG smoothing. Hence, the 1491 variables were used for calibration among the models using SG smoothing during the spectral pretreatments.

Kennard-Stone sampling design

The Kennard-Stone algorithm is an effective method for extracting a sample subset in multidimensional space, which includes all the most diverse samples and enables the selection of a subset of representative samples. Therefore, it has been confirmed that a calibration set extracted using KS selection has a better predictive capability than a set randomly built or constructed by other data selection methods such as Kohonen self-organized mapping (Kohonen et al. 1982) and D-optimal designs (de Aguiar et al. 1995). In this study, the total 268 intact cottonseed samples were divided into calibration and prediction sets based on KS algorithm, with the former set consisting of 218 samples and the later one 50 samples. The statistical values of gossypol contents in all cottonseed samples for calibration and prediction set were demonstrated in Table 3, which indicated that the range of variation for gossypol content was broad enough to develop NIR calibration models.

Table 3

Statistical values of gossypol content for calibration and prediction set samples.

a sets	N	Minimum ($\text{g}\cdot\text{kg}^{-1}$)	Maximum ($\text{g}\cdot\text{kg}^{-1}$)	Mean ($\text{g}\cdot\text{kg}^{-1}$)	SD
ation set	218	0.32	1.04	0.63	0.16
tion set	50	0.35	0.95	0.65	0.15
amples	268	0.32	1.04	0.64	0.16

N, number of samples; SD, standard deviation.

PLS regression

The calibration models of gossypol content in intact cottonseeds based on PLS regression were established in the NIR spectral range of 4 000-10 000 cm^{-1} , and the results were summarized in Table 4. The number of LV were selected with the aid of cross-validation using the first minimum RMSECV for all models. The RMSECV and RMSEP values for all the calibration models were between 0.05-0.07 and 0.04-0.06 for calibration and prediction sets, respectively. The values of R_p^2 and R_c^2 ranged from 0.82 to 0.93 and from 0.87 to 0.97, respectively. The RPD values ranged from 2.3 to 3.4.

Table 4

Performance comparison results for calibration models using different spectral pretreatment strategies.

Spectral pretreatments	LVs	Calibration set		Prediction set		
		RMSECV	R_c^2	RMSEP	R_p^2	RPD
Raw	9	0.06	0.94	0.06	0.82	2.3
SG	9	0.07	0.87	0.06	0.86	2.5
SNV	10	0.06	0.92	0.05	0.87	2.8
MSC	8	0.06	0.92	0.05	0.88	2.8
1st D	9	0.05	0.95	0.05	0.87	2.7
SNV+1st D	10	0.06	0.96	0.05	0.89	3.0
MSC+1st D	9	0.06	0.96	0.05	0.88	2.9
SG+SNV+1st D	9	0.05	0.97	0.04	0.92	3.4
SG+MSC+1st D	10	0.05	0.96	0.05	0.89	3.0

LVs, latent variables; RMSECV, root mean square error of cross-validation; R_c^2 , coefficient of determination of calibration; RMSEP, root mean square error of prediction; R_p^2 , coefficient of determination of prediction; RPD, residual predictive deviation; Raw, raw spectra; SG, Savitzky-Golaysmoothing; SNV, standard normal variate; MSC, multiplicative scatter correction; 1st D, first derivate.

4. Discussion

Since NIR spectra of intact cottonseeds were complex and overlapped, suitable spectral pretreatments should be used to optimize the NIR spectra and extract the effective information. In this work, the raw spectra were transformed using by eight pretreatment strategies, including the single pretreatment

strategies (SG smoothing, SNV, MSC, and first derivate), two pretreatments strategies (SNV + first derivate and MSC + first derivate), and three pretreatments strategies (SG smoothing + SNV + first derivate and SG smoothing + MSC + first derivate). In analyzing the results obtained from single pretreatment strategies, the PLS model using eight latent variables based on application of MSC produced better results with low values of RMSECV and RMSEP (0.06 and 0.05, respectively), and the RPD value was increased by 20.36% compared with that of the direct regression model based on raw spectra (Fig. 4). Fig. 3B shows the correlation of model using MSC, presented by plotting predicted and reference values for gossypol content in intact cottonseeds. The samples near the diagonal line indicated that their predicted values were more closed to reference ones and vice versa. In the aspect of two pretreatments strategies, the calibration model based on SNV + first derivate, presented a better predictive ability than that on MSC + first derivate, with the R_c^2 and R_p^2 values of 0.962 and 0.887, respectively. The RPD value of that model was 3.0, increased by 28.14% compared to the model using raw spectra. From all the results of calibration models established, the best model was pretreated using the strategy of SG + SNV + first derivate, and it had the highest R_c^2 (0.97) and R_p^2 (0.93), and the RPD (3.4) increased by 46.28% compared with that of raw spectral model. Furthermore, RMSECV (0.05) and RMSEP (0.04) were the lowest among all the models. The correlation plots between predicted and reference values were focused on the diagonal line (Fig. 3D). It was indicated that the model using SG + SNV + first derivate and PLS was accurate and robust enough to substitute the conventional gossypol analysis methods (HPLC) to measure gossypol in intact cottonseeds.

The NIR spectra of these intact seeds generally contained a mass of undesirable features, including noise, overlapping peaks, baseline effects and some systematic behaviors, caused by the seed size, shell, and some other physical factors. Hence, a suitable pretreatment strategy was required for widespread application of NIR technology in crop seed analysis. In this work, it was indicated that an advisable pretreatment strategy before regression was important to refine the effective information from spectral data and eliminate spectral deviation to calibrate an accurate and robust NIR model.

The calibration models reported here confirmed the feasibility of the using of NIR technology for rapid and non-destructive determination of gossypol, an important parameter to cottonseed products, in intact cottonseeds for the first time. The high RPD values (3.4) suggested that this technology could be an effective method for the measurement of gossypol in intact cottonseeds. The optimal model could substitute conventional analysis methods for gossypol, including UV spectrophotometry and HPLC. Because of the potential of high sample throughput and low costs, as well as a significant reduction in toxic chemicals, the application of NIR method could be encouraged and popularized to other similar agricultural products.

5. Conclusions

The calibration and validation statistics obtained in the current work showed the potential of NIRS to predict microelement gossypol content in intact cottonseeds. The optimized model was that pretreated by

Savitzky-Golay smoothing + standard normal variate + first derivative, with RMSECV, RMSEP, R_p^2 , and RPD of 0.05, 0.04, 0.92, and 3.4, respectively, which provided a method to determine gossypol content in intact cottonseeds feasibly.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All co-authors have consent for submission of manuscript.

Availability of data and materials

All relevant data are within this article.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

Li C (Cheng) and Zhu SJ designed the experiments and wrote the manuscript. Li C (Cheng), Zhao TL and Su BS analyzed the data, Li C(Cheng), SU BS, Li C (Cong) participated in experiment. Chen JH assisted in editing the article. Zhu SJ and Chen JH conducted and supervised the experiments.

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Figures

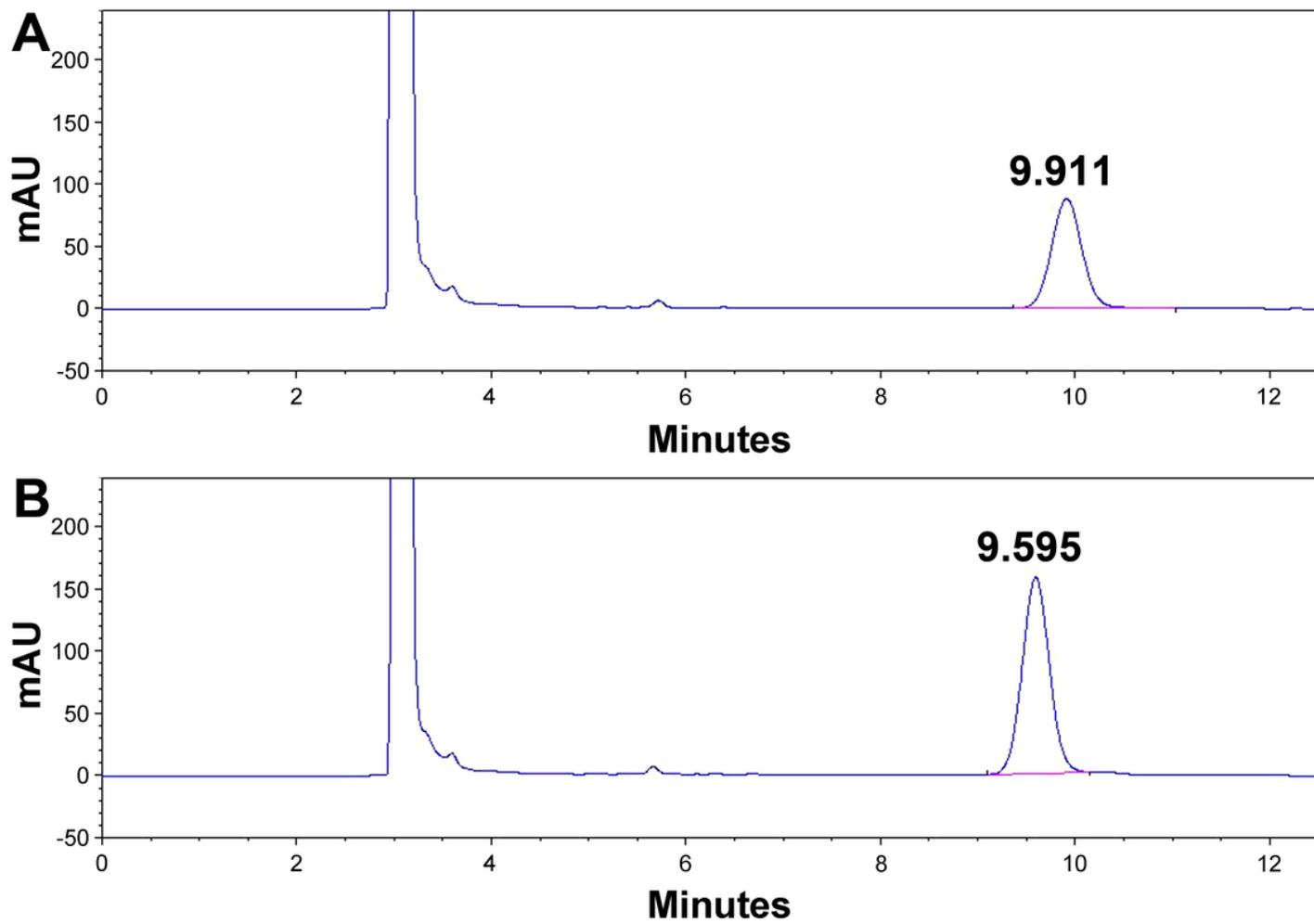


Figure 1

Chromatograms of (A) gossypol standard and (B) gossypol extract in intact cottonseeds.

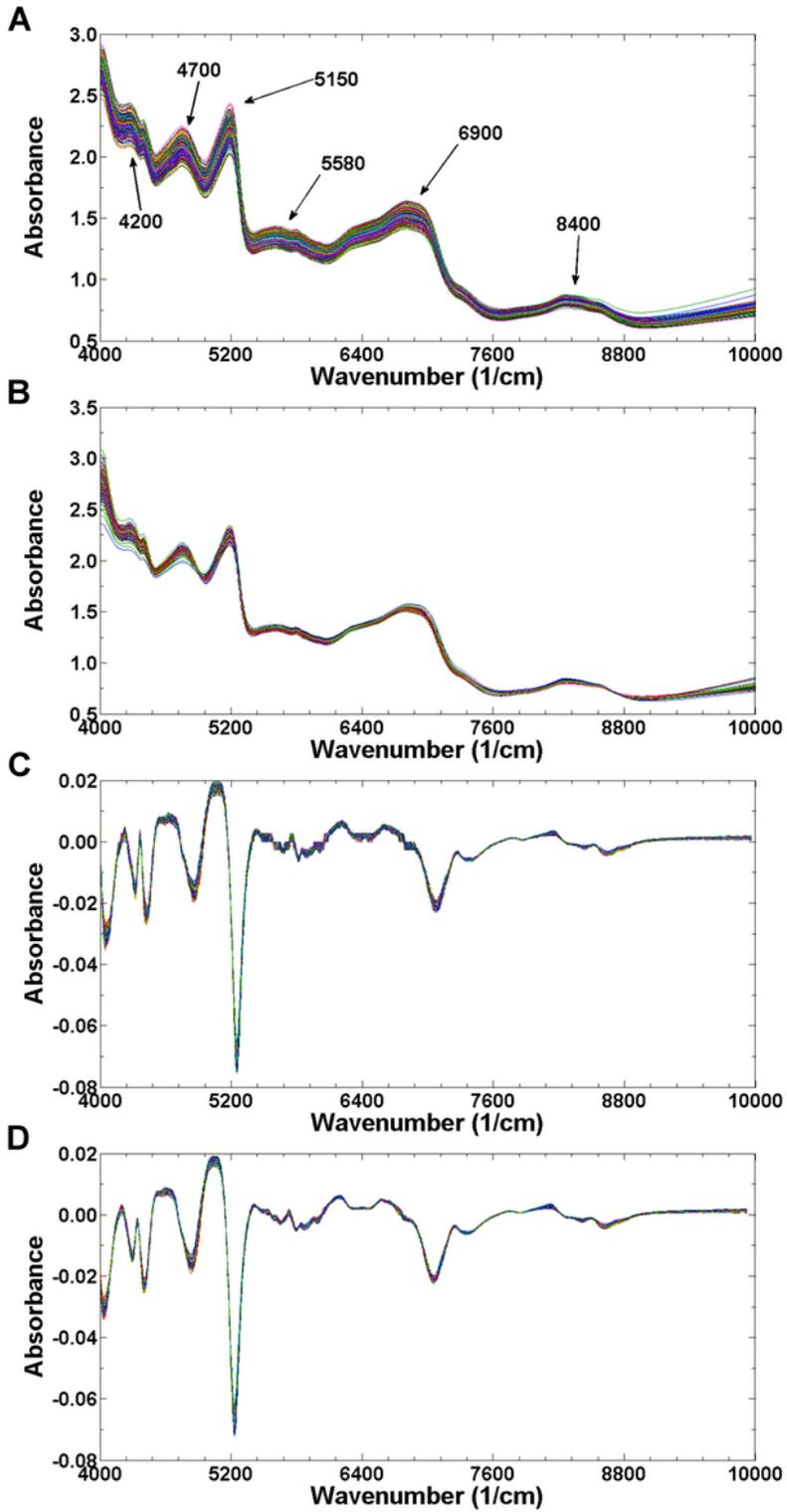


Figure 2

The NIR spectra of intact cottonseeds. (A) The raw spectra, (B) the spectra pretreated by MSC, (C) the spectra pretreated by SNV+ first derivative, and (D) the spectra of pretreated by SG smoothing+ SNV+ first derivative.

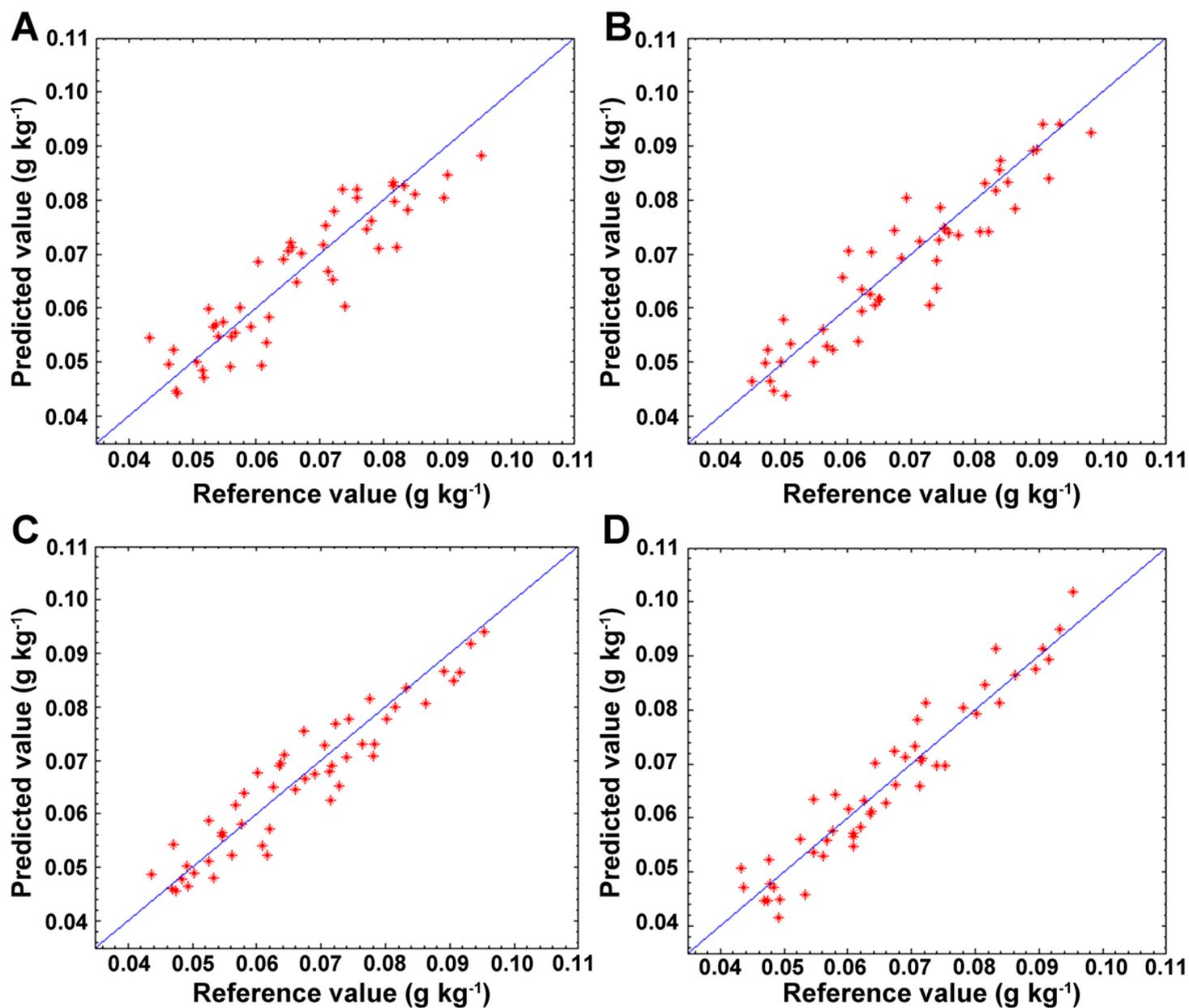


Figure 3

The correlation between predicted and reference values for models of intact cottonseeds. (A) the PLS model based on raw spectra, (B) the PLS model based on the pretreatment of MSC, (C) the PLS model based on the pretreatment of SNV+ first derivate, and (D) the PLS model based on the pretreatment of SG smoothing+ SNV+ first derivate.

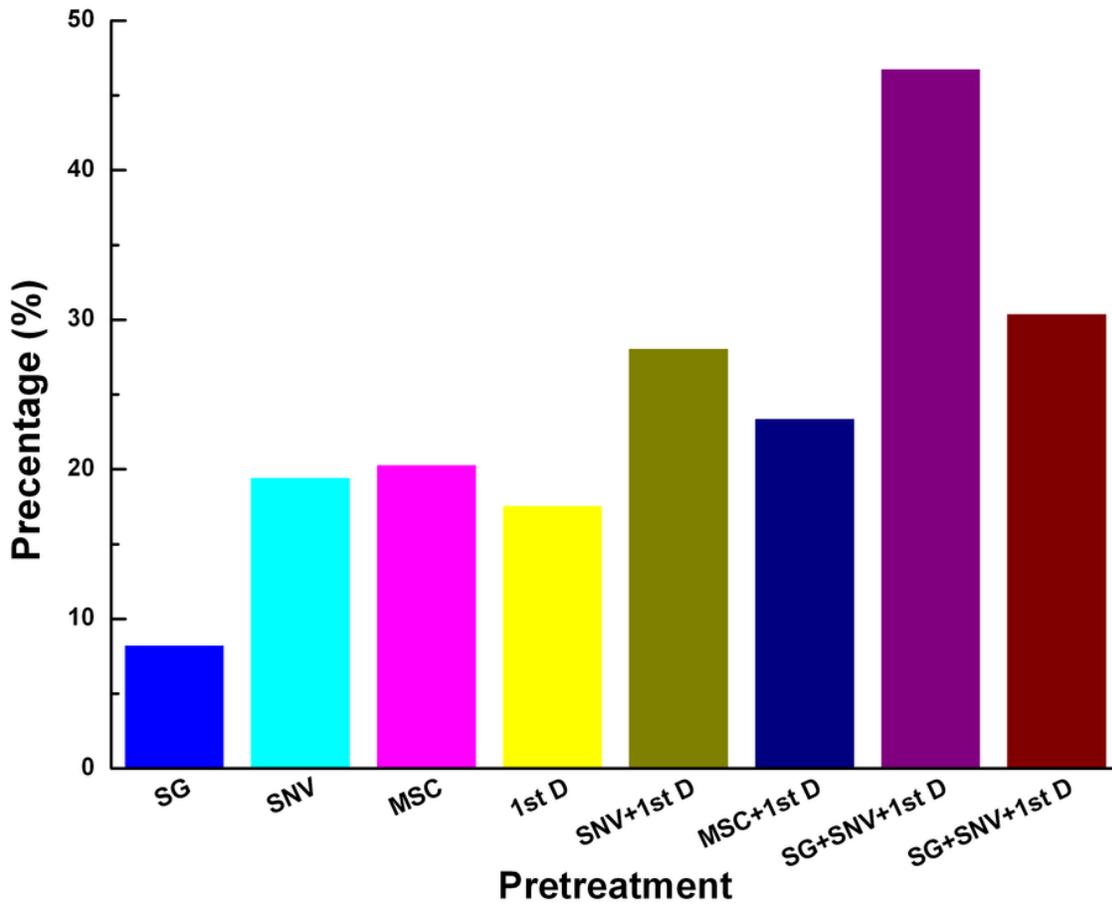


Figure 4

The residual predictive deviation (RPD) for PLS models based on different pretreatment strategies compared with the model using raw spectra.