Original Article
Journal of Biosystems Engineering

J. of Biosystems Eng. 41(1):51-59. (2016. 3)
http://dx.doi.org/10.5307/JBE.2016.41.1.051

eISSN : 2234-1862
pISSN : 1738-1266

Non-Destructive Sorting Techniques for Viable Pepper (*Capsicum annuum* L.) Seeds Using Fourier Transform Near-Infrared and Raman Spectroscopy

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Received: February 11st, 2016; Revised: February 22nd, 2016; Accepted: February 25th, 2016

Abstract

Purpose: This study examined the performance of two spectroscopy methods and multivariate classification methods to discriminate viable pepper seeds from their non-viable counterparts. **Methods:** A classification model for viable seeds was developed using partial least square discrimination analysis (PLS-DA) with Fourier transform near-infrared (FT-NIR) and Raman spectroscopic data in the range of 9080–4150 cm^{-1} (1400–2400 nm) and 1800–970 cm^{-1}, respectively. The datasets were divided into 70% to calibration and 30% to validation. To reduce noise from the spectra and compare the classification results, preprocessing methods, such as mean, maximum, and range normalization, multivariate scattering correction, standard normal variate, and 1st and 2nd derivatives with the Savitzky-Golay algorithm were used. **Results:** The classification accuracies for calibration using FT-NIR and Raman spectroscopy were both 99% with first derivative, whereas the validation accuracies were 90.5% with both multivariate scattering correction and standard normal variate, and 96.4% with the raw data (non-preprocessed data). **Conclusions:** These results indicate that FT-NIR and Raman spectroscopy are valuable tools for a feasible classification and evaluation of viable pepper seeds by providing useful information based on PLS-DA and the threshold value.

Keywords: FT-NIR spectroscopy, Non-destructive sorting, Pepper seeds, Raman spectroscopy, Viability

Introduction

Pepper (*Capsicum annuum L.*) is an important vegetable, spice, and pharmaceutical material. In the crop business, seed viability is a crucial factor for high productivity. Until now, numerous studies have attempted to improve seed productivity using various procedures such as priming with solutions (Smith and Cobb, 1991; Saracco et al., 1995) and pellet treatment in order to restore germination power and storage ability (Kang and Choi, 2006; Kang et al., 2009). The storage period depends on various parameters such as preservation conditions and life expectancy, which can be divided into microbiotic (a lifespan of less than 2 years), mesobiotic (3 to 5 years), and macrobiotic (more than 5 years). Large quantities of expired seeds are disposed of, resulting in a huge financial loss to farmers and companies. In addition, poor seed quality can lead to low-quality agricultural products and byproducts.

The chemical method using triphenyl tetrazolium chloride is commonly used to test seed viability (Lakon, 1949). Moreover, the physical or physiological properties are

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used to determine the seed germinability. In particular, differences in density can be measured using a float-sink technique (Taylor et al., 1982; Hill et al., 1989), measurement size, weight of seeds (Hoy et al., 1985), or a bioassay germination test using a Fusarium isolate (Vujanovic et al., 2000). However, these methods are time consuming, have a low accuracy, require technical expertise, and are destructive; therefore, quantity loss is unavoidable with these methods. Therefore, there is a need for a non-destructive, robust, and rapid seed quality sorting technique.

Fourier transform near-infrared (FT-NIR) spectroscopy is a rapid and easy-to-use spectroscopic method that uses wavelengths of 780–2500 nm for the quantitative and qualitative measurement of organic materials with C–H, N–H, and O–H bonds. It is commonly used for evaluating the quality of agricultural products. Recently, a few studies have demonstrated the ability of hyperspectral imaging technique (HIT) to sort viable seeds, for instance, radish (Shetty et al., 2011), mottle (Min and Kang, 2003), corn kernel (Min and Kang, 2008), and lettuce seeds (Ahn et al., 2012). Recently, hyperspectral imaging spectroscopy (HSI) has emerged as a viable tool for sorting agricultural products (Kandpal et al., 2016) and microorganisms (Park et al., 2015). HSI provides both spatial and spectral information of target materials that makes 3D hyperspectral cubes. The single slice method allows region of interest (ROI) area to be selected in order to remove redundant information.

Raman scattering is an inelastic scattering technique that was discovered and reported by C. V. Raman (Raman, 1928). Raman scattering depends on the polarizability of molecules as well as Rayleigh scattering, which is elastic scattering. In addition, Raman scattering can gain or lose photon energy during interaction with molecules (mainly vibration) so that the scattered photons have a different frequency from the incident photons; this is the major difference between inelastic and elastic scattering, where the latter retains photon energy after interaction with molecules. The Raman spectrum shows unique peaks in the vibrational mode of specific molecules, which are the fingerprints of the molecules. Raman spectroscopy has applications in agriculture (Herrero 2008), food (Li-Chan, 1996; Ilaslan et al., 2015), pharmaceutical (Vankeirsbilck et al., 2002), and biomedical (Fenn et al., 2011) fields where the analysis of small concentrations of molecules is required. In addition, it can distinguish varieties of seeds (Baranski et al., 2005) and analyze the chemical extracts of cocoa seeds (Edwards et al., 2005).

Chemometrics is used to interpret and apply many spectroscopic datasets to practical performance. Partial least square regression is one of the most common multivariate regression methods, and its discriminant analysis application, partial least squares discriminant analysis (PLS-DA), has also been frequently used in agricultural engineering (Giunchi et al., 2008; Almeida et al., 2013).

A number of studies have demonstrated that spectroscopic and multivariate methods can analyze the classification performance of seed viability or quality. Nansen et al. (2015) used hyperspectral imaging based on the reflectance mode in the spectral range of 423.6–878.9 nm (110 spectral bands) to determine the germination of native Australian tree seeds, such as *Acacia cowleana Tate* (Fabaceae), *Banksia prionotes* L.F. (Proteaceae), and *Corymbia calophylla* (Lindl.). They observed and collected hyperspectral reflectance imaging data of the seed germination power for 0, 1, 2, 5, 10, 20, 30, and 50 days of ageing. These data were analyzed with linear discriminant analysis (LDA), and the *Acacia* and *Corymbia* seeds showed over 85% accuracy, while the *Banksia* seeds showed 80% accuracy. Kong et al. (2013) reported that near-infrared hyperspectral imaging in the spectral range from 1039 to 1612 nm could classify four rice cultivars with several multivariate methods, such as PLS-DA or SVM. Raman scattering has also been shown to be a useful spectroscopic method to determine seed quality. Spectroscopic techniques are superior, in terms of rapidity, non-destructivity, and accuracy, to conventional methods in detecting and identifying seed viability.

The main objective of the present study is to classify viable pepper seeds by a spectroscopic method using a multivariate classification method. Both reflectance and scattered spectra of pepper seeds were collected using FT-NIR and Raman spectroscopy, respectively, and a classification model was built by PLS-DA with various preprocessing methods. The classification results were evaluated and compared based on accuracy (%) in accordance with a threshold value.

Materials and Methods

Samples

Non-commercial coated pepper seeds (PR-815) were obtained from a seed company in Korea. The seeds were artificially degraded to evaluate their germination characteristics in association with aging. The degradation treatment was conducted for 168 h at 45°C and 20% moisture content (MC). The seeds that underwent the degradation treatment were maintained at 20°C until they recovered their before-treatment weight. The treated and untreated seeds were stored in a refrigerator at 4°C, according to the rules of the international seed testing association (ISTA). The seeds were exposed to air to each room temperature for testing and analysis.

A germination test was conducted to confirm the germination power of the samples after an experiment in accordance with the rules of ISTA. The seeds were germinated by the Top of Paper method—12 seeds were soaked in water for 15 days at 25°C in a petri dish (90 mm × 15 mm) covered with filter paper.

FT-NIR spectroscopy

FT-NIR spectroscopy (Antaris II FT-NIR Analyzer, Thermo Scientific Co., MA, USA) was performed using a halogen lamp and an InGaAs detector with a spectral range of 12,000–3,800 cm^{-1} (833–2,630 nm), a resolution of 4 cm^{-1}, and a wavenumber accuracy of ±0.03 cm^{-1}. The reflectance spectra of a single pepper seed were acquired, as shown in Figure 1. In this present study, we used a spectral range of 10,000–4,000 cm^{-1} (1000–2500 nm). Each seed was scanned 32 times and the mean spectra were obtained. The dark reference was acquired with a golden slit from each scan.

Raman spectroscopy

The Raman spectrometer (Raman workstation™, Kaiser Optical Systems Inc., Ann Arbor, MI, USA) consisted of a diode laser and a charge-coupled device detector (CCD) with a resolution of 0.3 cm^{-1}. The diode laser wavelength was 785 nm, and the CCD had a holographic transmission grating that enabled the scanning of a plate with 96 square wells (Figure 2). The laser apparatus had a power of 100 mW, a spot size of 3 mm, and an exposure time of 0.5 s. The spectral range was 150–1800 cm^{-1} and each plate was scanned 32 times.

Data analysis

Specific Raman bands are difficult to detect in Raman spectra owing to the fluorescence effects of materials. Therefore, the fluorescence effects in the Raman spectra of organic samples must be removed. Polynomial fitting is frequently used to remove these effects from Raman spectra (Lieber et al., 2003). In this study, 16th order polynomial fitting was implemented to revise the standard line and remove these effects.

To reduce the noise from the environment and instruments, a few preprocessing methods were used, such as minimum, maximum, and range normalization, multiplicative scatter correction (MSC), standard normal variate (SNV), and 1st and 2nd derivatives with the Savitzky-Golay algorithm. The meaningful spectral ranges for data analysis with FT-NIR and Raman spectroscopy are 9080–4150 cm^{-1} (1400–2400 nm) and 970–1800 cm^{-1}, respectively. A classification model for germinated seeds was developed with the PLS-DA method and was evaluated based on accuracy (%). NIR and Raman spectra were acquired from 288 samples—144 sound and 144 degraded (treated) seeds. Then, a germination test was performed and the assigned sample class numbers were confirmed. In particular, the germinated samples of the untreated seeds were assigned a class number of 1 and the ungerminated samples of the degraded seeds were assigned a class number of 2. The samples were divided into two groups;

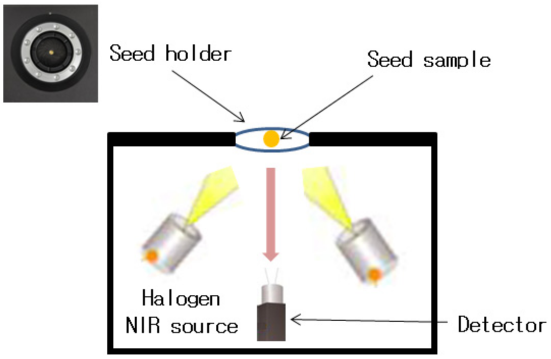


Figure 1. Schematic plot of the NIR spectrometer and sample holder.

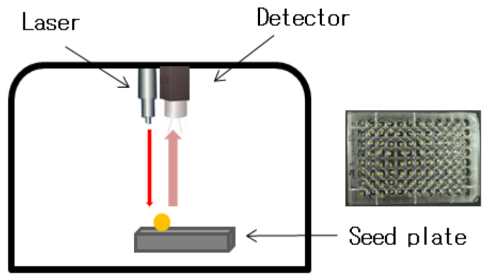


Figure 2. Schematic of the Raman spectrometer and a seed plate with ninety-six wells.

70% for calibration and 30% for prediction to validate the developed model.

PLS-DA

PLS-DA was implemented to develop a classification model for seed germination. The PLS model is a representative regression method that predicts measurement values, for example, the sugar content of fruits. PLS-DA predicts an artificially assigned class number such as {−1, 1} instead of measurement values for the sugar content of fruits.

In this study, non-viable seeds are assigned a class number of 1 and viable seeds are assigned a class number of 2. The threshold value was determined to be 1.5 for classifying the predicted results. The predicted result values are assigned a value of 1 or 2 depending on whether they are greater or smaller than the threshold value, respectively. Samples with predicted values greater than 1.5 are considered non-viable, whereas those with values smaller than 1.5 are considered viable (Johan et al., 2008).

To find an optimal number for latent variables, the root mean square error of validation (RMSEV) was calculated (Lee et al., 2002; Nicola et al., 2005).

$$RMSEV = \sqrt{\frac{\sum_{i=1}^{n}(y_{real} - y_{pred})^2}{n}} \tag{1}$$

where, y_{real} = real class number (1 = germinated, 2 = ungerminated seeds), y_{pred} = predicted value with the PLS-DA model.

Data analysis and model development, including polynomial fitting, preprocessing methods, and PLS-DA modeling are executed using MATLAB (7.0.4, The Mathworks, Natick, MA, USA) software.

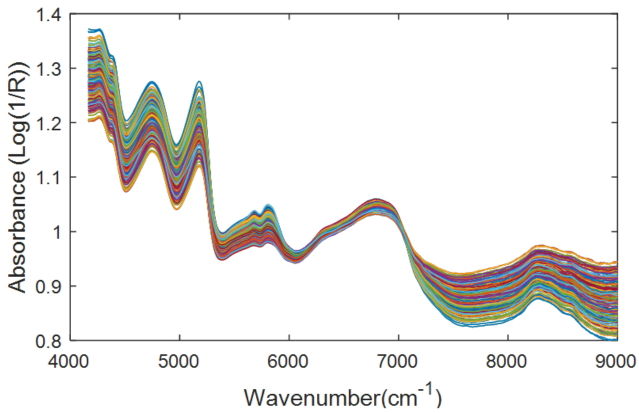
Results and Discussion

Germination tests

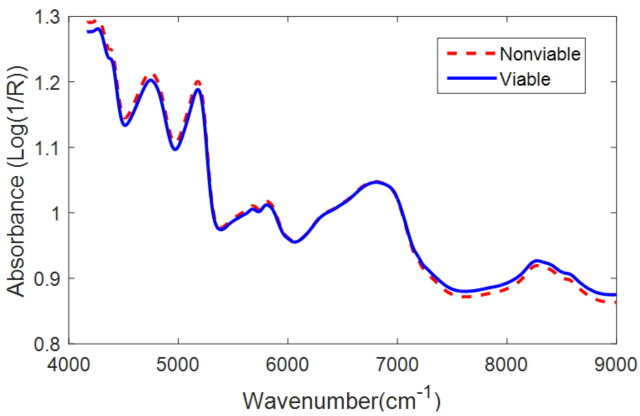
The pepper seeds generally initiated germination in 4 days and accomplished germination in 168 h. Out of the 144 untreated seeds, 141 germinated and showed a seed viability of 97.9%, and we observed that the degraded seeds did not germinate in 168 h. In the germination test, 288 seeds—141 viable seeds and 147 non-viable seeds—were used for analysis.

FT-NIR data analysis

Figures 3(a) and (b) illustrate the FT-NIR absorbance spectra of 288 pepper seeds and the mean spectrum of the viable and non-viable seeds, respectively. It can be observed that the viable and non-viable seeds have similar absorbance characteristics, with an exception in the 9000–5000 cm^{-1} range where different characteristics are seen. Some bands at 6942 cm^{-1}, 6896 cm^{-1}, 6325 cm^{-1}, 6171 cm^{-1}, 5897 cm^{-1}, and 5796 cm^{-1} represent C–H bonds in CH_2 and CH_3 functional groups. At around 4875 cm^{-1}, an N–H bond in protein is observed. Such functional groups are associated with abscisic acid (ABA), which is a germination-inhibiting material (Muhammad et al., 1998). A C–H bond represents lipid and lipid peroxidation in cell damage and can be associated with the aging process (Shetty et al., 2011). Seeds without a cell wall tend to expose some nutritious factors during the germination process, such as protein, amino acid, and sugar, and these exposed materials increase germination-inhibiting activities (Kang and Choi, 2006). In the degradation process,



(a)



(b)

Figure 3. (a) FT-NIR spectra and (b) mean spectrum of viable and nonviable pepper seeds.

structural changes and nutrient exposure due to cell wall breakdown may affect the absorbance spectra.

Raman spectra analysis

Figure 4 shows the Raman scattering spectra of 288 pepper seeds. Figure 4(a) illustrates the raw Raman spectra of pepper seeds before removal of the background due to fluorescence using the 16th order polynomial fitting method. The Raman peaks obtained for the pepper seeds are shown in Figure 4(b). Figure 4(c) shows the mean plot of the Raman spectra for the viable and non-viable seeds. Significant peaks associated with the essential constituents of pepper seeds are observed around 1520 cm^{-1} (C=C), 1440 cm^{-1} (=CH_2), 1263 cm^{-1} (=CH), 1154 cm^{-1} (C–C), and 1090 cm^{-1} (C–O) (Baranski et al., 2006; Sebastian et al., 2007).

In addition, some peaks around 1657 cm^{-1}, 1605 cm^{-1}, 1064 cm^{-1}, and 1006 cm^{-1} represent relevant nutrients such as sugar (glucose) and protein (amino acid) (Li-chan, 1996; Juan et al., 2011). Exposure of soluble sugars, protein, and amino acids due to cell damage in the degradation process may affect the Raman spectra.

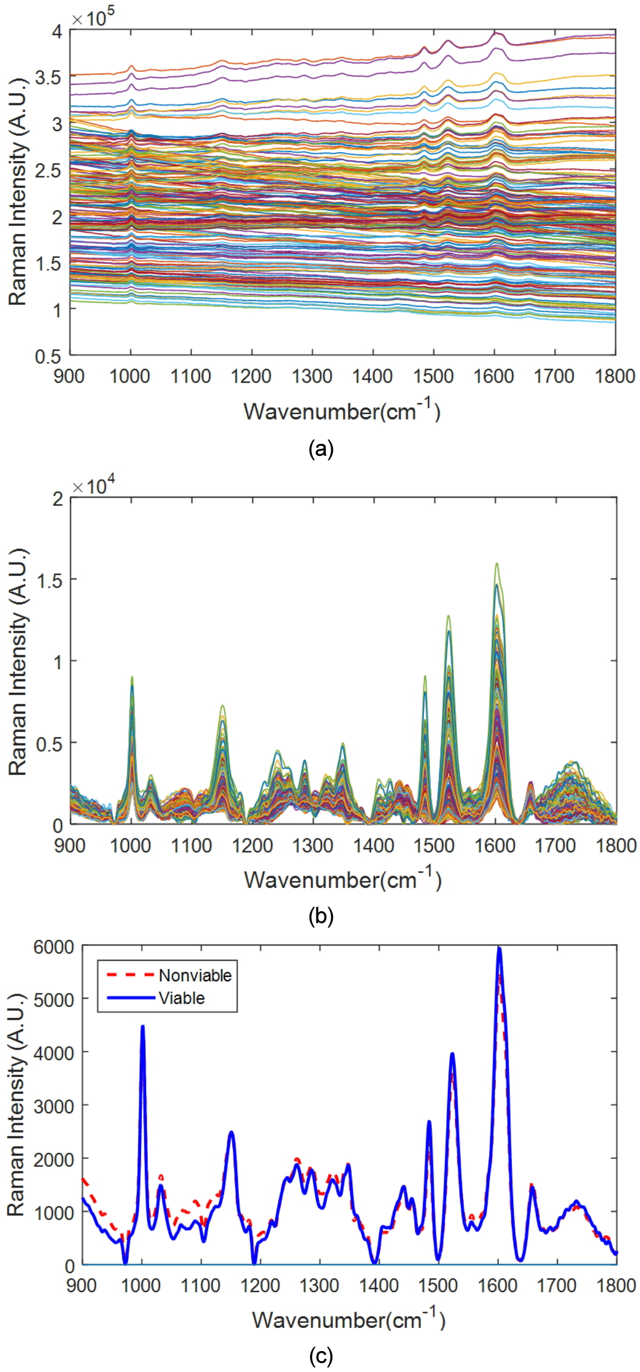


Figure 4. (a) Raman spectra without removed fluorescence background signal, (b) Raman spectra with removed fluorescence background signal, and (c) mean spectrum of viable and nonviable pepper seeds.

Classification analysis of PLS-DA using FT-NIR

A classification model was developed using 141 viable seeds and 144 non-viable seeds, which passed germination tests using the collected FT-NIR spectra. The data were divided into 70% (N = 200) out of 285 for calibration and 30% (N = 85) for validation of a classification model.

To find the optimal number of factors, RMSEV was used in the cross-validation process. Figure 5 illustrates RMSEV in accordance with a number of factors. Twenty latent variables were implemented to select the optimal number for PLS-DA; the 8th factor showed the minimum RMSEV. We selected the number of factors with various preprocessing methods by the same process.

The PLS-DA discriminant model was evaluated with

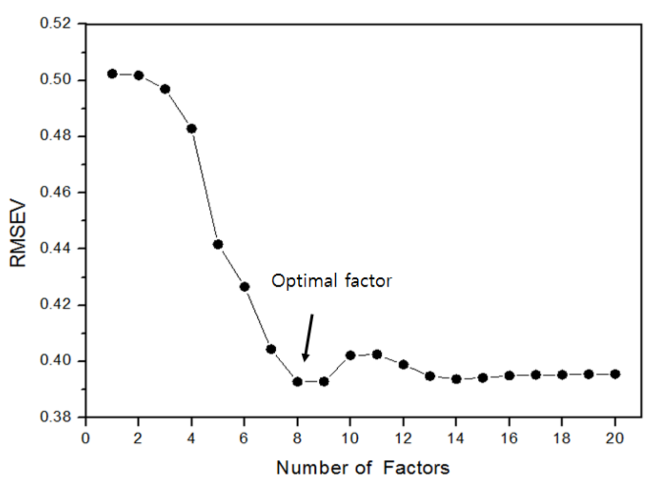


Figure 5. Optimal number of factors from cross validation analysis of PLS-DA model using FT-NIR.

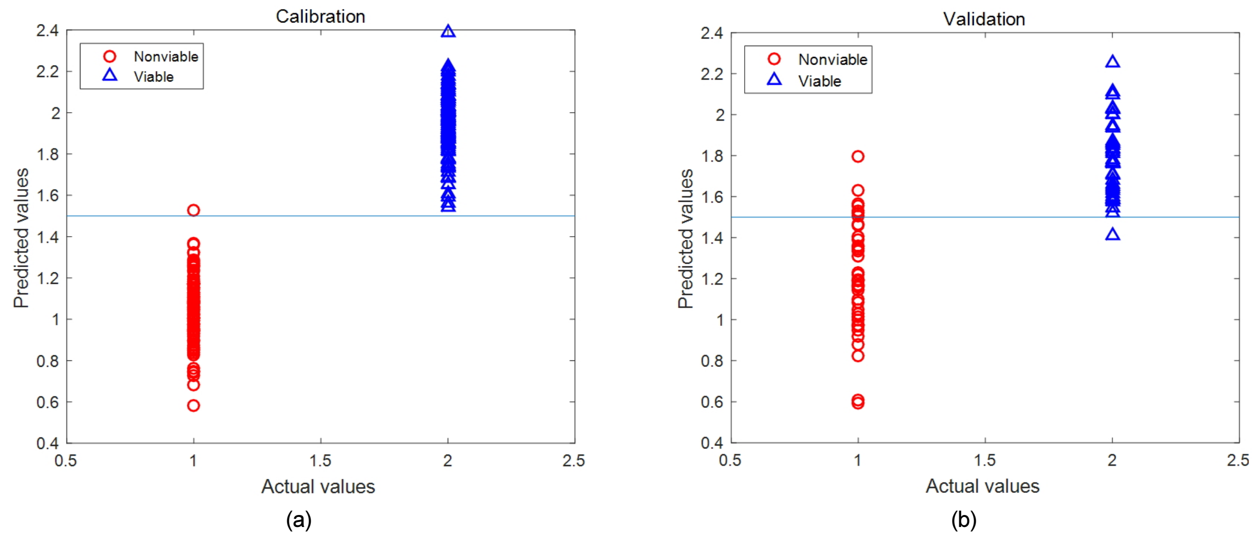


Figure 6. Classification results for (a) calibration and (b) validation of viable and non-viable pepper seeds based on PLS-DA with MSC using FT-NIR spectroscopy.

Table 1. Classification results for calibration of viable pepper seeds based on the PLS-DA model with various preprocessing methods using FT-NIR spectroscopy

Preprocessing	Viable (N=98)		Nonviable (N=102)		Total (N=200)		
	Correct (%)	Incorrect (%)	Correct (%)	Incorrect (%)	Correct(N)	Incorrect (N)	Accuracy (%)
Mean normalization	93.9	6.1	92.2	7.8	186	14	93
Maximum normalization	94.9	5.1	91.2	8.8	186	14	93
Range normalization	94.9	5.1	89.2	10.8	184	16	92
MSC	88.8	11.2	92.2	7.8	181	19	90.5
SNV	89.8	10.2	88.2	11.8	178	22	89
Savitzky_Golay_1st	100.0	0.0	98.0	2.0	198	2	99
Savitzky_Golay_2st	80.6	19.4	85.3	14.7	166	34	83
Raw	93.9	6.1	90.2	9.8	184	16	92

Table 2. Classification results for validation of viable pepper seeds based on the PLS-DA model with various preprocessing method using FT-NIR spectroscopy

Preprocessing	Viable (N=43)		Nonviable (N=42)		Total (N=85)		
	Correct (%)	Incorrect (%)	Correct (%)	Incorrect (%)	Correct(N)	Incorrect (N)	Accuracy (%)
Mean normalization	90.7	9.3	85.8	14.2	85	10	88.2
Maximum normalization	86.1	13.9	83.7	16.6	85	13	84.7
Range normalization	88.4	11.6	85.8	14.2	85	11	87.0
MSC	97.7	2.3	83.7	16.6	85	8	90.5
SNV	95.7	4.6	85.8	14.2	85	8	90.5
Savitzky_Golay_1st	58.2	41.8	66.7	33.3	85	32	62.3
Savitzky_Golay_2st	46.6	53.4	54.8	45.2	85	42	50.5
Raw	86.1	13.9	88.1	11.9	85	11	87.0

accuracy of classification (%) that was defined as the rate of corrected classified binary samples. A threshold value of 1.5 was assigned to separate the viable and non-viable predicted values (Figure 6). Tables 1 and 2 show the classification results with PLS-DA for the calibration and validation groups, respectively. As shown in Table 1, most preprocessing methods showed an accuracy of greater than 90% with the first derivative showing 99% accuracy. Table 2 shows that both the MSC and SNV methods gives 90.5% accuracy with a threshold value of 1.5, which also showed the best classification result. MSC and SNV remove scattering due to the irregular shape of materials and noise from the atmosphere. Therefore, PLS-DA with two preprocessing methods can demonstrate classification results without redundant noise from the FT-NIR spectra.

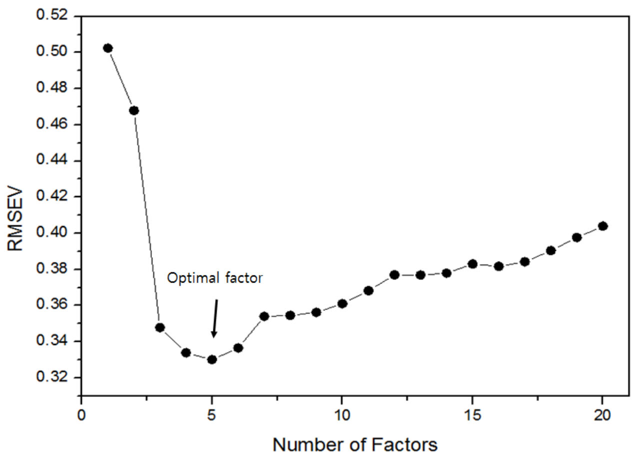


Figure 7. Optimal number of factors from cross validation analysis of the PLS-DA model using Raman spectroscopy.

Analysis of the classification model developed using PLS-DA with Raman scattering

A classification model was developed using PLS-DA with the Raman scattering spectra from which fluorescence noise was removed by polynomial fitting as well as with FT-NIR. The optimal number of factors was 5 according to RMSEV (Figure 7). Assessment of the classification results using PLS-DA is shown in Tables 3 and 4. In the calibration results (Table 3), the 1st derivative has 99% accuracy. Meanwhile, in the validation results (Table 4) with various preprocessing methods, the raw data show 96.4% accuracy in classification (Figure 8).

Table 3. Classification results for calibration of viable and nonviable pepper seeds using the PLS-DA model with various preprocessing method using Raman spectroscopy

Preprocessing	Viable (N=98)		Nonviable (N=102)		Total (N=200)		
	Correct (%)	Incorrect (%)	Correct (%)	Incorrect (%)	Correct(N)	Incorrect (N)	Accuracy (%)
Mean normalization	97.9	2.1	96.1	3.9	194	6	97
Maximum normalization	98.9	1.1	92.2	7.8	191	9	95.5
Range normalization	98.9	1.1	92.2	7.8	191	9	95.5
MSC	98.9	1.1	96.1	3.9	195	5	97.5
SNV	97.9	2.1	96.1	3.9	194	6	97
Savitzky_Golay_1st	100	0	98.1	1.9	198	2	99
Savitzky_Golay_2st	100	0	97.1	2.9	197	3	98.5
Raw	100	0	93.1	6.9	193	7	96.5

Table 4. Classification results for validation of viable and nonviable pepper seeds using the PLS-DA model with various preprocessing methods using Raman spectroscopy

Preprocessing	Viable (N=43)		Nonviable (N=42)		Total (N=85)		
	Correct (%)	Incorrect (%)	Correct (%)	Incorrect (%)	Correct(N)	Incorrect (N)	Accuracy (%)
Mean normalization	93.1	6.9	92.9	7.1	79	6	92.9
Maximum normalization	97.7	2.3	90.5	9.5	80	5	94.1
Range normalization	97.7	2.3	90.5	9.5	80	5	94.1
MSC	93.1	6.9	95.3	4.7	80	5	94.1
SNV	93.1	6.9	92.9	7.1	79	6	92.9
Savitzky_Golay_1st	93.1	6.9	85.7	14.3	76	9	89.4
Savitzky_Golay_2st	76.7	23.3	80.9	19.1	67	18	78.8
Raw	100	0	92.8	7.2	82	3	96.4

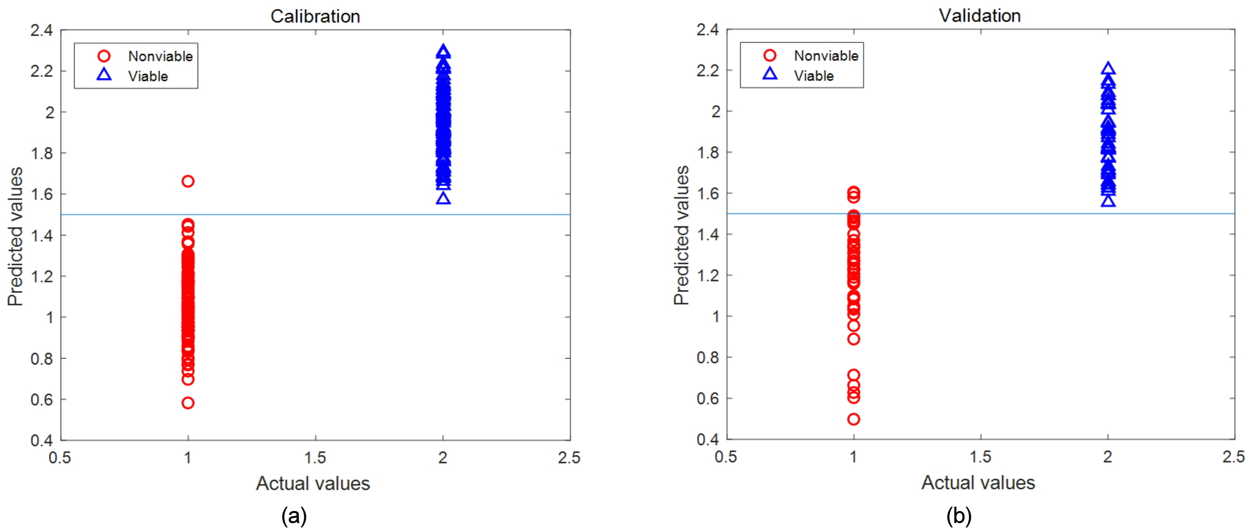


Figure 8. Classification results for (a) calibration and (b) validation of viable and nonviable pepper seeds based on PLS-DA with raw data from Raman spectroscopy.

Conclusion

FT-NIR and Raman spectroscopy have been used successfully to classify viable and non-viable pepper seeds. PLS-DA with various preprocessing methods was employed to compare the classification results and select the optimal preprocessing method.

FT-NIR spectroscopy with both MSC and SNV showed a classification accuracy of 90.5% and Raman scattering with raw data (none-preprocessed data) showed the best classification accuracy of 96.4%. This can be explained by the influence from water on the FT-NIR spectra, making it difficult to find the optimal peaks of specific molecules (Teye et al., 2013). FT-NIR spectroscopy can demonstrate the best classification with two preprocessing methods. However, Raman scattering yields sharper spectroscopic peaks. This study suggests that both spectroscopic methods have potential to determine the viability of pepper seeds.

Conflict of Interest

The authors have no conflicting financial or other interests.

Acknowledgement

This research was supported by research fund of Chungnam National University in 2015.

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