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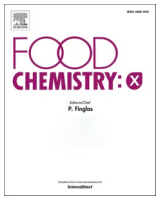

Raman spectroscopy coupled with the PLSR model: A rapid method for analyzing gamma-oryzanol content in rice bran oil

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ABSTRACT

Rice bran oil (RBO) is widely used in food, nutraceutical, and cosmetic industries, due to its γ-oryzanol content, a key quality indicator. This study developed a rapid, non-destructive method for quantifying γ-oryzanol in RBO using Raman spectroscopy combined with partial least squares regression (PLSR). The optimal PLSR model, based on orthogonal signal correction (OSC)-pretreated data of Raman spectra from 800 to 1800 cm^{-1}, demonstrated high accuracy with a strong R^2-Pearson correlation coefficient of 0.9827 and low root mean square error of prediction (RMSEP) of 0.5314. Principal component analysis (PCA) of OSC-pretreated data showed improved sample grouping by concentration of γ-oryzanol compared to untreated data. Additionally, Bland-Altman plots comparing results from Raman and HPLC methods showed random scatter within ±2 SD of the mean difference, confirming the method's reliability. This study indicates that Raman spectroscopy can serve as a reliable method for determining γ-oryzanol content in RBO products within the related industries.

1. Introduction

Rice bran oil (RBO), an edible oil, has become more popular as a cooking oil and nutraceutical product (Pal & Pratap, 2017). It is produced from rice bran, a byproduct of the rice (*Oryza sativa* L.) milling process, using cold press and solvent extraction methods (Ghosh, 2007; Patel & Naik, 2004; Wongwaiwech, Weerawatanakorn, Tharatha, & Ho, 2019). RBO is widely recognized as a rich source of various bioactive compounds that contribute to health promotion. (Nagendra Prasad, Sanjay, Shravya Khatokar, Vismaya, & Nanjunda Swamy, 2011). Crude RBO contains approximately 20 % (*w*/w) total lipids, consisting of 18–29 % saturated fatty acids (SFA: palmitic acid, stearic acid, and myristic acid), 37–43 % monounsaturated fatty acids (MUFA: oleic acid), and 37–44 % polyunsaturated fatty acids (PUFA: linoleic acid, and linolenic acid) (Anwar, Anwer, & Mahmood, 2005). The fatty acid profile of RBO is close to the ideal fatty acid composition of healthy edible oils (27–33 % SFA, 33–40 % MUFA, and 27–33 % PUFA) recommended by the American Heart Association (AHA) and World Health Organization (WHO) (Pal & Pratap, 2017). In addition to fatty acids, RBO also composes of various phytochemicals, including gamma-oryzanol (γ-oryzanol), phytosterols (campesterol, *β*-sitosterol, and stigmasterol), ferulic acid, phytic acid, and vitamin E (tocopherols and tocotrienols) (Nagendra Prasad et al., 2011; Patel & Naik, 2004; Sahini & Mutegoa, 2023). These compounds play important roles in the prevention and alleviation of various health conditions, particularly hypercholesterolemia, cardiovascular diseases, cancer, menopausal symptoms, and aging (Anwar et al., 2005; Nagendra Prasad et al., 2011; Ramazani, Akaberi, Emami, & Tayarani-Najaran, 2021). Various reports have recommended that RBO consumption may reduce cardiovascular disease risk by improving serum lipid profiles. RBO is an effective nutritious oil for reducing total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels, as well as increasing high-density lipoprotein cholesterol (HDL-C) level (Jolfaie, Rouhani, Surkan, Siassi, & Azadbakht, 2016; Pourrajab et al., 2022). The unsaponifiable

Abbreviations: RBO, rice bran oil; FTIR, Fourier transform infrared; UV, ultraviolet; NIR, near-infrared; NMR, nuclear magnetic resonance; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; MSC, multiplicative scatter correction; SNV, standard normal variate; OSC, orthogonal signal correction; PLSR, partial least squares regression; PCA, principal component analysis; R^2, correlation coefficient; RMSEP, root mean square error of prediction.

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components especially γ-oryzanol, phytosterols, tocopherols, and tocotrienols are responsible for antioxidant effect as well as reducing cholesterol levels in blood and liver (Nagendra Prasad et al., 2011; Pal & Pratap, 2017; Shibata, Kawakami, Kimura, Miyazawa, & Nakagawa, 2016; Wilson, Nicolosi, Woolfrey, & Kritchevsky, 2007). Among those unsaponifiables, γ-oryzanol distinguishes RBO from other common edible oils, such as olive, canola, sunflower, soybean, coconut, and palm oils, aside from its fatty acid composition. γ-Oryzanol is considered an important phytonutrient, with a content ranging from 1.5 % to 3.0 % in crude RBO (Patel & Naik, 2004; Szcześniak, Ostaszewski, Ciecierska, & Sadkowski, 2016). A number of studies have reported other pharmacological activities of γ-oryzanol, including anti-inflammation, anticancer, antidiabetic, and neuroprotective effects. Moreover, many evidences demonstrated that γ-oryzanol has health benefits for reducing lipid profile, blood glucose, blood pressure, menopausal symptoms, and weight gain (Patel & Naik, 2004; Ramazani et al., 2021). Consequently, RBO and γ-oryzanol have become more popular functional or active ingredients in dietary supplements, pharmaceutical products, and cosmetics (Pal & Pratap, 2017; Patel & Naik, 2004; Punia, Kumar, Siroha, & Purewal, 2021; Wongwaiwech et al., 2019). The primary production of RBO occurs in Asian countries, India, China, Japan, Thailand, and Vietnam (Pal & Pratap, 2017). Quality control of raw materials and finish products is essential in the healthcare industry in order to ensure that the products meet established quality standards. In RBO industry, γ-oryzanol has been used as a chemical marker for the quality control of RBO products. γ-Oryzanol is a mixture of many steryl ferulates, ferulic acid esters of phytosterols and triterpene alcohols. The primary components of γ-oryzanol in RBO include cycloartenyl ferulate, 24-methylenecycloartenyl ferulate, campesteryl ferulate, and sitosteryl ferulate (see Supplementary data: Fig. S1) (Ramazani et al., 2021; Szcześniak et al., 2016).

In recent years, the determination of γ-oryzanol content in RBO has been developed using various analytical techniques. High performance liquid chromatography (HPLC) and ultraviolet spectrophotometry (UV) are common methods for the quantitative analysis of γ-oryzanol (Bucci, Magrì, Magrì, & Marini, 2003). HPLC is widely used for determining the concentration of each component, as well as the total content of γ-oryzanol in RBO (Sakunpak, Suksaeree, Monton, et al., 2014). Whereas, UV spectroscopy is more rapid and convenient for the total content analysis than HPLC. However, the use of a single-wavelength UV spectrophotometric method for analyzing γ-oryzanol content in RBO can lead to low accuracy measurements due to interference from the oil matrix. The application of second derivative spectrophotometry, combined with multi-wavelength measurement and multicomponent analysis, has been developed to enhance the accuracy of γ-oryzanol quantification in RBO (Bucci et al., 2003). Thin layer chromatography (TLC)-densitometric and image analysis techniques have been successfully developed for determining γ-oryzanol content in RBO, showing no statistically significant difference in results (Sakunpak, Suksaeree, Pathompak, et al., 2014). Moreover, proton nuclear magnetic resonance (^{1}H NMR) has been demonstrated to be a rapid method for measuring γ-oryzanol content in RBO by analyzing the intensity of methoxy signal (Endo & Aso, 2019). Vibrational spectroscopic techniques, such as Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy, have been utilized for the identification of γ-oryzanol components or steryl ferulates in RBO (Mandak, Zhu, Godany, & Nyström, 2013). However, quantitative analysis of γ-oryzanol in RBO using Raman spectroscopy has not yet been reported.

Raman spectroscopy is a vibrational spectroscopy technique that relies on the scattering of electromagnetic radiation in the visible light (Vis) to near-infrared (NIR) regions. This occurs when the monochromatic laser light interacts with the electron cloud of chemical bonds within the sample constituents. A change in the frequency of incident light is referred to as inelastic scattering or Raman scattering. The Raman spectra generated from this phenomenon provide valuable information for assessing the chemical compounds in samples, as they reflect differences in structure, atoms, and bonding of the molecules (Shah et al., 2023). Raman spectroscopy is an efficient analytical tool for characterizing both solid and liquid samples that contain complex chemicals without the need for sample preparation. It is a rapid and non-destructive method, exhibiting low sensitivity to water molecules (Tian, Tan, & Li, 2020; Zhang, 2020a, Zhang, 2020b). Consequently, it has been developed for both qualitative and quantitative analysis of raw materials, excipients, and active ingredients in the food, health supplement, pharmaceutical, and cosmetic industries (Arendse et al., 2021; Shah et al., 2023).

The present study developed a rapid method for analyzing γ-oryzanol content in RBO using Raman spectroscopy. Method optimization was carried out to ensure effective acquisition of Raman spectral data. Multivariate analysis was performed using Unscramble X ver.10.5 to develop prediction models for the quantitative analysis of γ-oryzanol content in RBO samples. Partial least squares regression (PLSR) and principal component analysis (PCA) were employed to generate statistical data that represents the relationships between the Raman spectral data variables and γ-oryzanol contents determined by HPLC. An appropriate model with suitable preprocessing methods was evaluated based on several parameters, including correlation coefficients (R^2 model, R^2 Pearson) and the root mean square error of prediction (RMSEP). Consequently, we established an optimal model for analyzing γ-oryzanol content in RBO using Raman spectroscopy combined with PLSR. The reliability of the predicted model's results was compared to those obtained from the HPLC method using Bland-Altman plots and a statistical *t*-test.

2. Material and methods

2.1. Samples and reagents

RBOs with the labeled amount of γ-oryzanol ranging from 2500 to 12,500 ppm from various brands were purchased from supermarkets in Bangkok, Thailand. γ-Oryzanol standard (97 %) was supplied by FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Acetonitrile (HPLC grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Honeywell Burdick & Jackson (Muskegon, MI, Korea). Isopropanol (HPLC grade) was purchased from Honeywell Burdick & Jackson (Ulsan, Korea). Formic acid (90 %) was supplied from Ajex Finechem (New South Wales, Australia).

2.2. RBO sample preparation and arrangement

The RBO with the lowest labeled amount of γ-oryzanol (2500 ppm) was selected as the base RBO for preparing additional samples. A total of 120 RBO samples, with expected γ-oryzanol concentrations ranging from 2500 to 15,000 ppm, were prepared by adding γ-oryzanol standard to the base RBO. Among these, 100 samples were selected using the Kennard-Stone algorithm and used as calibration samples. The remaining 20 samples, not included in the calibration step, were used as validation samples. Additionally, seven commercially available RBO brands served as external validation samples to demonstrate the efficiency of the PLSR-assisted Raman spectroscopic method. The schematic of RBO sample arrangement is shown in Fig. 1.

2.3. Raman spectroscopic analysis

Raman spectra of the RBO samples were recorded using a LabRAM HR Evolution Visible (440–1100 nm) confocal Raman microscope from HORIBA France SAS (Palaiseau, France). A He-Ne laser with a wavelength of 633 nm (17 mW) served as the excitation source. The system utilized a 600 g/mm diffraction grating (500 nm) and a Syncerity OE detector (CCD detector). The instrument was operated with LabSpec software version 6.4.4.16.

The RBO samples (200 μL) were placed in aluminum cups with a

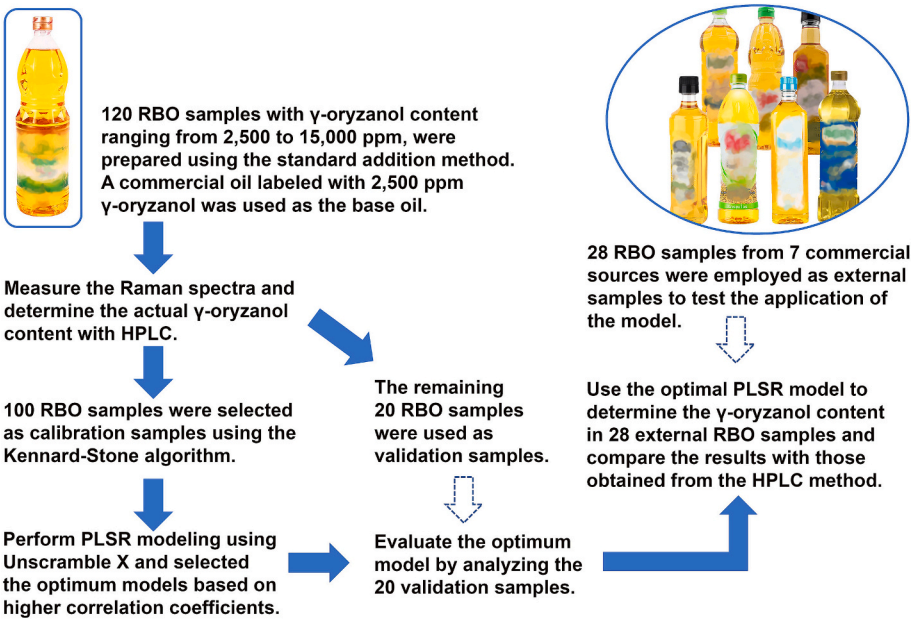

Fig. 1. The schematic of RBO sample arrangement.

diameter of 1 cm and analyzed using the confocal Raman Microscope. To determine the optimal conditions for measuring Raman spectra of RBO, we varied several parameters, including laser type (green laser at 532 nm and red laser at 785 nm), laser power (0.1 %, 1 %, 5 %, 10 %, 25 %, 50 %, and 100 %), acquisition time (5, 15, 20, 30, and 40 s), and accumulation (2, 4, and 8 times), as shown in Table S1 of the Supplementary Data.

The ideal condition was chosen by weighing various factors such as signal-to-noise ratio, signal intensity, resolution, reproducibility, fluorescence interference, and sample damage. After aligning these factors to achieve acceptable quality and accuracy of Raman spectral data within a reasonable analysis time, the optimal Raman analysis for measuring the RBO samples was conducted. This involved using a 10× magnification objective lens, covering a wavenumber range of 200–2000 cm^{-1}, employing a 785 nm laser at maximum power (100 %). The acquisition duration was set to 40 s, with measurements accumulated twice.

2.4. HPLC analysis

The concentration of γ-oryzanol in RBOs was determined using a Shimadzu HPLC system equipped with an LC-20 AT pump and an SPD-20 A UV detector (Tokyo, Japan), following a modified HPLC method as previously described (Rogers et al., 1993; Yoshie, Kanda, Nakamura, Igusa, & Hara, 2009; Sakunpak, Suksaeree, Monton, et al., 2014). The HPLC analysis utilized a Hypersil GOLD C18 column (Thermo Scientific; 4.6 × 250 mm, 5 μm) with a SecurityGuard cartridge C18 (4 × 3.0 mm). The method employed an isocratic mobile phase consisting of a 70:30 ratio of acetonitrile to methanol, supplemented with 1 % formic acid, at a flow rate of 1.5 mL/min, and a total analytical time of 16 min. Detection of γ-oryzanol absorbance occurred at a wavelength of 320 nm, with an injection volume of 10 μL. The method underwent validation for linearity, accuracy, precision, limit of quantitation (LOQ), and limit of detection (LOD). Linearity was assessed via the standard addition method, employing six concentrations of γ-oryzanol standard (ranging from 10 to 100 μg/mL) in a base RBO solution, with a correlation coefficient (R^2) above 0.999 set as the acceptance criterion. Accuracy was evaluated using the standard addition method with three concentrations of γ-oryzanol standard (10, 50, and 100 μg/mL) in 9 replicates for each concentration, with a percent recovery (% recovery) close to 100 % as the acceptance criteria. Method precision was determined by calculating the percent relative standard deviation (%RSD) of % recovery, with a criterion of less than 2.0 % for acceptance. Isopropanol was utilized to dilute the RBO samples to achieve the appropriate concentration range.

2.5. PLSR modeling for quantitative analysis of γ-oryzanol

The Raman spectra obtained from the RBO samples were pre-processed using techniques such as smoothing, baseline correction, normalization, standard normal variate (SNV) transformation, and orthogonal signal correction (OSC) before performing multivariate analysis. Partial least-squares regression (PLSR) modeling and principal component analysis (PCA) were conducted using Unscramble X, version 10.5 (Aspen Tech, MA, USA). A calibration set consisting of 100 Raman spectra from RBO samples was selected using the Kennard-Stone algorithm. Suitable calibration models were chosen based on higher correlation coefficients (R^2 model). The efficiency of these models was evaluated using 20 test (validation) samples and 28 external samples from 7 different commercial RBOs. The criteria for selecting an optimal model included a higher R^2 of Pearson correlation coefficient (R^2 Pearson) between the actual (HPLC) and predicted values, as well as a lower RMSEP.

The R^2 model values were derived from the calibration plot, which shows the relationship between the predicted values from the Raman PLSR models (Y-axis) and the HPLC values (X-axis) in the calibration dataset. Meanwhile, the R^2 Pearson values and RMSEP were calculated from the prediction (validation) plots of the Raman PLSR model predictions (Y-axis) versus the HPLC values (X-axis) in the test dataset.

2.6. Statistical analysis

The determination results obtained from the PLSR model of Raman spectral data were statistically compared with those from the HPLC method using a *t*-test at a 95 % confidence interval.

3. Results and discussion

3.1. Raman spectroscopic analysis

A suitable condition for analyzing γ-oryzanol content in RBO samples

using Raman spectroscopy was established by optimizing various parameters that influence the Raman spectra. These parameters included the type of laser excitation, laser power, acquisition time, and the number of accumulations. The Raman spectra of the RBO, containing a labeled concentration of 4000 ppm γ-oryzanol, were analyzed using a laser with wavelengths of either 537 nm or 785 nm, with laser power settings of 50 % or 100 %, an acquisition time of 15 s, and 4 accumulations. It was found that the Raman spectra of RBO obtained using laser excitation at a wavelength of 532 nm exhibited inconsistent patterns across repeated measurements. In contrast, excitation with a wavelength of 785 nm resulted in consistent Raman spectra, as shown in Fig. S2 of the Supplementary Data. The laser excitation at 785 nm is commonly used in Raman spectroscopy because it effectively reduces fluorescence interference and enhances measurement sensitivity (Wang, Zhang, Zhang, & Fang, 2013). The effects of various parameters on the resolution of Raman spectra were investigated by varying the laser intensity, acquisition time, and number of accumulations, as shown in Table S1. The highest resolution Raman spectra were observed with a laser intensity of 100 %, an acquisition time of 40 s, and 8 accumulations, without causing any damage to the RBO sample, as demonstrated in Fig. S3 and Fig. S4 of the Supplementary Data. Increasing the laser intensity, acquisition time, and number of accumulations results in higher resolution Raman spectra by reducing unwanted noise and enhancing peak intensity (Wan et al., 2017). However, longer acquisition times and more accumulations extend the overall analysis time. To rapidly obtain high-resolution Raman spectra (within 5 min) while ensuring consistent sample measurements, a laser wavelength of 785 nm, laser intensity of 100 %, acquisition time of 40 s, and 2 accumulations were employed to analyze the RBO samples.

The standard γ-oryzanol, RBO labeled with a γ-oryzanol content of 2500 ppm, along with RBO samples at various γ-oryzanol concentrations (5000, 7500, 10,000, 12,500, and 15,000 ppm) prepared using the standard addition method, were measured under the optimal condition, resulting in the Raman spectra presented in Fig. 2 and Fig. S6. The Raman spectra of the RBO samples exhibited a Raman shift at 1606 cm^{-1}, corresponding to the highest peak in the standard γ-oryzanol spectrum, with intensity increasing in proportion to the γ-oryzanol content. Therefore, Raman spectroscopy, combined with multivariate analysis, can be developed for the quantitative analysis of γ-oryzanol content in RBO.

3.2. HPLC method validation and quantitative determination of γ-oryzanol

The actual γ-oryzanol content in RBO samples was analyzed using a reverse-phase HPLC method. The optimal ratio of methanol to acetonitrile and the mobile phase flow rate were investigated. The most suitable conditions were obtained using an RP-C18 column (4.6 × 250 mm, 5 μm) with a mobile phase consisting of 1 % formic acid in a methanol and acetonitrile mixture (30:70, *v*/v), a flow rate of 1.5 mL/min, a UV detector set at 320 nm, and a total run time of 14 min. The method demonstrated linearity with an R^2 value greater than 0.999 for the concentration range of 10–100 μg/mL. The accuracy, expressed as % recovery, ranged from 99.87 % to 101.99 %, while the precision, expressed as %RSD, ranged from 0.26 % to 2.26 %. The LOQ, representing the lowest concentration in the linearity range, was established at 10 μg/mL, while the LOD, calculated from a 10:1 signal-to-noise ratio, was 2.5 μg/mL. A summary of the HPLC method performance results is presented in Table S2 of the Supplementary Data.

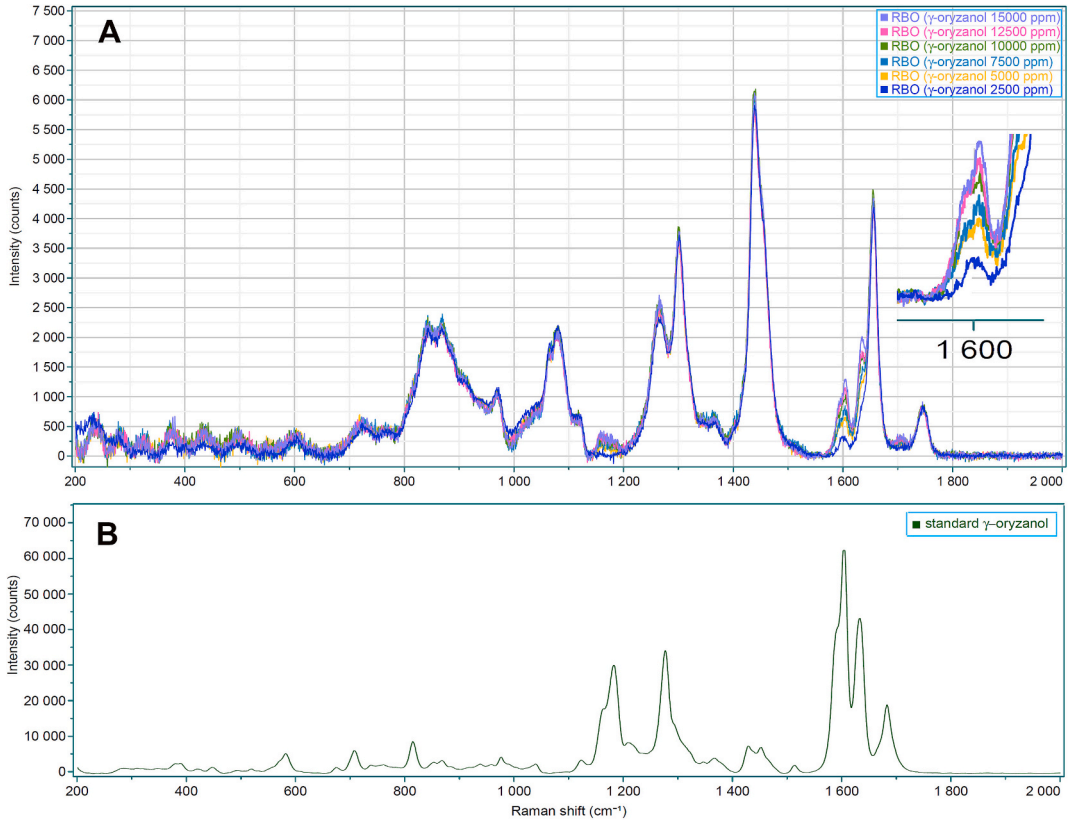

Fig. 2. Raman spectra of RBO samples at various γ-oryzanol concentrations (2500, 5000, 7500, 10,000, 12,500, and 15,000 ppm) prepared using the standard addition method (A) and standard γ-oryzanol (B) were measured using a 10× objective lens, with an acquisition time of 40 s, 2 accumulations, a wavelength of 785 nm, and 100 % laser power.

3.3. PLSR modeling for quantitative determination of γ-oryzanol

Multivariate data analysis was performed on the Raman spectral data of RBO samples and the γ-oryzanol content of RBO, determined using the HPLC method. PLSR was employed for predictive modeling. The PLSR models were developed using both the original Raman spectral data (unpretreated data) and pretreated data obtained through smoothing, baseline correction, normalization, SNV, and OSC. The suitability of a PLSR model was evaluated based on parameters such as R^2 model, R^2 Pearson, RMSEP, and bias in the predictions of validation and commercial sample sets. Spectral data preprocessing is crucial for obtaining an accurate and precise model. This step can reduce non-chemical biases, such as particle size effects, scattering signals, and noise, thereby enhancing and preserving meaningful information (Afseth, Segtnan, & Wold, 2006; Liu, Delwiche, & Dong, 2009). Various preprocessing techniques, such as smoothing, normalization, multiplicative scatter correction (MSC), and SNV, have been successfully used to improve quantitative models for specific samples (Tian et al., 2020; Zhang, 2020a, Zhang, 2020b; Li et al., 2019).

As shown in Table 1, the PLSR models derived from both untreated and pretreated data of Raman spectra from 200 to 2000 cm^{-1} provided acceptable model parameters, with high R^2 model and R^2 Pearson values, as well as low RMSEP and bias in the predictions for validation and commercial samples. Although the improvements in the models from pretreated data were not significantly greater than those from the original data, the model based on OSC-pretreated data was selected for further analysis. This decision was made because it exhibited the highest R^2 Pearson value, along with the lowest RMSEP and bias in the predictions for commercial samples, compared to the models generated from the original and other pretreated data. The high R^2-Pearson value indicates a strong agreement between the HPLC reference values and the Raman PLSR model predictions, suggesting that the model's outputs are closely aligned with the reference method. Additionally, the low RMSEP value demonstrated that the Raman PLSR model's predictions closely match the HPLC method, further highlighting the model's accuracy.

PCA is a commonly used preliminary step in the PLSR algorithm. Its primary function is to extract relevant and meaningful information from the entire dataset. Through PCA, the original measurement data is transformed into a reduced set of latent variables, or principal components (PCs). These components are visualized using score and loading plots. The score plot typically represents the samples, allowing the structure of the data to be viewed in terms of the PCs. In our study, the score plot of the OSC-pretreated data demonstrated improved sample grouping and clearer separation into three concentration-based groups compared to the score plot of the unpretreated data, as shown by the green, red, and blue data points in Fig. 3A and B. Therefore, PCA plays a crucial role in identifying patterns and relationships within the spectral data of RBO samples. It enables more effective differentiation and grouping of samples by utilizing OSC-pretreated data based on γ-oryzanol concentrations. This indicates that PCA enhances the clarity and separation of data patterns compared to unprocessed data. Consequently, PCA can reveal underlying structures within the data, making it easier to identify meaningful clusters and trends.

These findings indicate that the Raman PLSR model derived from OSC-pretreated data has the potential for high-accuracy quantitative determination of real samples compared to the HPLC method. Furthermore, the PLSR models based on OSC-pretreated data were further developed using narrower Raman spectral ranges of 800–1800 cm^{-1} and 1500–1800 cm^{-1}. As shown in Table 1, the PLSR model obtained from OSC-pretreated data within the spectral range of 800–1800 cm^{-1} was slightly superior to the other models using OSC-pretreated data for the validation samples, with an R^2-model of 0.9791, an R^2 Pearson of 0.9827, an RMSEP of 0.5314, and a bias in predictions of −0.1763. Additionally, the PLSR model from OSC-pretreated data within the 800–1800 cm^{-1} spectral interval was selected as the optimal model due to its high R^2 Pearson value, as well as the lowest RMSEP and bias in the predictions for commercial samples.

The calibration and prediction plots (Fig. 3C and Fig. 3D) showed a linear relationship between the predicted values from the optimal Raman PLSR model (Y-axis) and the HPLC reference values (X-axis), with all data points tightly clustered around the diagonal line. This linearity indicates that the predictions from the Raman PLSR model closely align with the HPLC reference values. Additionally, the slopes of the regression lines were close to 1, and the intercepts were near 0, demonstrating minimal bias in the predictions and low RMSEP. The strong correlation between the predicted and reference values confirms the model's high predictive accuracy and reliability, reinforcing its effectiveness for the quantitative analysis of γ-oryzanol in RBO samples.

3.4. Comparison of γ-oryzanol content determined from Raman and HPLC methods

The γ-oryzanol content in the validation samples was assessed using the optimal PLSR model and compared with the results obtained from the HPLC method. This comparison was evaluated using Bland-Altman plots, as illustrated in Fig. 4 and a statistical *t*-test, as shown in Table 2. The Bland-Altman plot is a graphical method used to assess the agreement between two quantitative measurement methods. It was

Table 1
The resulting PLSR models and their parameters.

Data	Calibration		Determination of validation set			Determination of commercial samples		
	R^2 model	Latent factors	R^2 Pearson	RMSEP	Bias	R^2 Pearson	RMSEP	Bias
Original (200–2000 cm^{-1})	0.9905	5	0.9832	0.5129	−0.1388	0.8799	1.0670	−0.5947
Baseline correction (200–2000 cm^{-1})	0.9904	5	0.9862	0.4650	−0.0971	0.8859	0.9462	−0.3849
Smoothing (200–2000 cm^{-1})	0.9779	1	0.9804	0.5906	−0.1100	0.8768	1.1171	−0.6527
SNV (200–2000 cm^{-1})	0.9898	4	0.9883	0.4127	−0.0123	0.8783	1.1047	−0.5854
Area normalization (200–2000 cm^{-1})	0.9907	4	0.9867	0.4471	−0.0590	0.8798	1.1701	−0.6895
OSC (200–2000 cm^{-1})	0.9786	1	0.9822	0.5452	−0.1957	0.9320	0.6808	−0.0190
OSC (800–1800 cm^{-1})	0.9791	1	0.9827	0.5314	−0.1763	0.9304	0.6806	−0.0099
OSC (1500–1800 cm^{-1})	0.9790	1	0.9822	0.5406	−0.1834	0.9240	0.7058	−0.0324

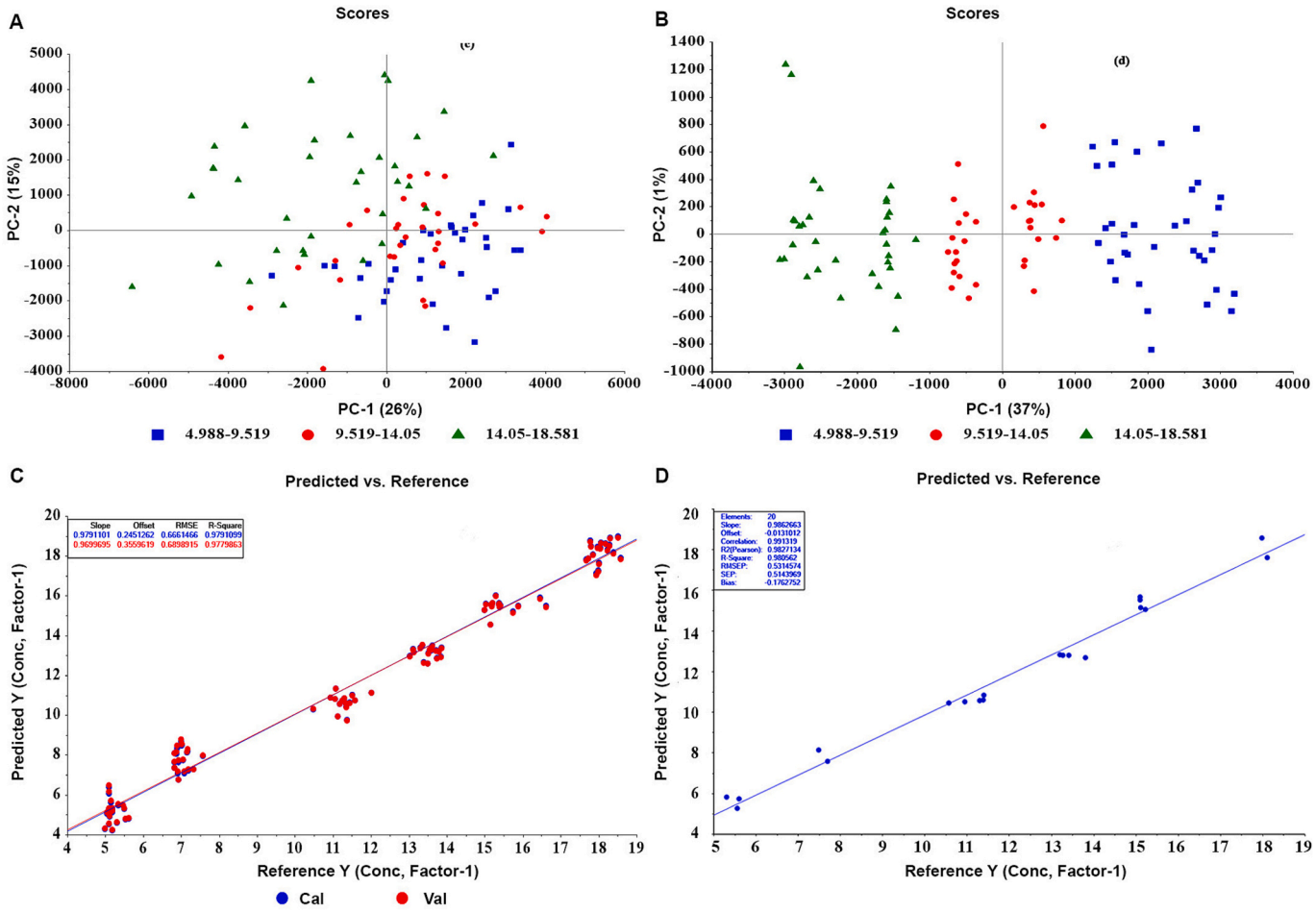

Fig. 3. PCA score plots (PA1 vs PC2) of unpretreated data (A) and OSC-pretreated data (B) ($n = 100$); Calibration plot (C) (n = 100) and prediction plot (D) ($n = 20$) of the optimal Raman PLSR model; The optimal model was developed using OSC-pretreated data (800–1800 cm^{-1}). The relationship between the γ-oryzanol content in RBO samples, as predicted by the optimal model and analyzed by HPLC, was determined using Unscramble X version 10.5.

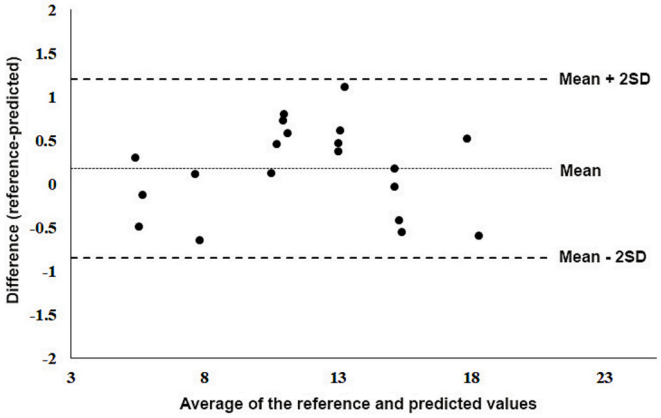

Fig. 4. Bland-Altman plot of the prediction results showed randomly scattered data points for the validation samples within ±2 SD of the mean difference.

introduced by Bland and Altman as a way to evaluate the consistency between two techniques by calculating the limits of agreement. These limits are derived from the mean and standard deviation (SD) of the differences between the two measurements. The plot displays the difference between paired measurements on the Y-axis and their average on the X-axis, illustrating how the difference varies across the range of measurements. Bland and Altman recommended that 95 % of the data points should fall within ±2SD of the mean difference, indicating good agreement. While the standard approach uses the mean of the measurements, variations of the plot can also use differences expressed as percentages or ratios, depending on the context (Bland & Altman, 1986; Giavarina, 2015). In our study, the Bland-Altman plots (Fig. 4), which illustrated the differences between the Raman and HPLC methods for individual samples, showed a random distribution of data points from the test (validation) set within ±2 SD of the mean difference. Furthermore, a t-test analysis comparing the γ-oryzanol content in commercial RBO samples measured using the optimal Raman PLSR model and the HPLC method revealed no significant differences, with a *p*-value greater than 0.05 (p-value = 0.746) (Table 2). These findings suggest strong agreement between the two measurement methods, indicating the absence of any significant systematic bias.

4. Conclusions

A rapid, non-destructive quantitative method for determining total γ-oryzanol content in rice bran oil (RBO) was developed using Raman spectroscopy. Optimal conditions for Raman measurement were established at a Raman shift of 800–1800 cm^{-1} with a 785 nm laser, 100 % intensity, 40-s acquisition, and 2 accumulations. Multivariate analysis and partial least squares regression (PLSR) modeling were applied to the Raman spectra and γ-oryzanol content, as determined by HPLC. The OSC-pretreated PLSR model demonstrated the best predictive accuracy, with lower RMSEP and bias in the predictions. The method effectively discriminated samples by γ-oryzanol concentration and results were

Table 2
Determination results of γ-oryzanol in commercial RBO samples by Raman with the optimal PLSR model and HPLC methods.

Commercial RBO samples*	Replication No.	Raman (μg/mL)	HPLC (μg/mL)
RBO No. 1	1	9.614	9.731
	2	9.118	9.824
	3	9.278	9.869
	4	10.029	9.856
RBO No. 2	1	5.551	5.263
	2	5.042	5.223
	3	5.349	5.067
	4	5.310	5.087
RBO No. 3	1	5.802	5.925
	2	6.048	5.908
	3	5.639	5.964
	4	6.278	5.983
RBO No. 4	1	12.171	12.968
	2	12.260	12.557
	3	12.130	12.371
	4	11.924	12.214
RBO No. 5	1	11.796	12.340
	2	12.106	12.352
	3	12.328	12.205
	4	12.072	12.266
RBO No. 6	1	7.173	7.984
	2	7.179	8.084
	3	7.120	8.041
	4	7.621	8.149
RBO No. 7	1	8.081	8.272
	2	8.351	8.341
	3	8.284	8.254
	4	8.319	8.332
mean		8.642	8.873
SD		2.592	2.705
F-statistic and F-critical		1.088 and 1.905	
t-statistic and t-critical		0.326 and 2.005	
P-value		0.746	

* The seven commercial RBO samples were analyzed with four replications.

consistent with HPLC. These findings support Raman spectroscopy with PLSR as a viable alternative for γ-oryzanol analysis in RBO, with potential for portable spectrometer development for in-house quality control.

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CRediT authorship contribution statement

Pattamapan Lomarat: Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Chutima Phechkrajang:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis. **Pawida Sunghad:** Methodology, Investigation, Formal analysis. **Natthinee Anantachoke:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Natthinee Anantachoke reports financial support was provided by Thailand Research Fund. Natthinee Anantachoke reports article publishing charges was provided by Mahidol University. Natthinee Anantachoke reports equipment, drugs, or supplies was provided by the Center of Excellence for Innovation in Chemistry. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101923.

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