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Rapid Method for Simultaneous Determination of *γ*-Oryzanol Compounds in Rice (*Oryza sativa*) Grains using UV-Vis Spectroscopy and Chemometrics

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Abstract

Rice (*Oryza sativa*) contains *γ*-oryzanol, which consists of 4 main compounds: Cycloartenol ferulate, 2,4-methylenecycloartanyl ferulate, campesterol ferulate, and *β*-sitosterol ferulate, that contribute to the health benefits of rice. This research aimed to develop a rapid method to determine the 4 major *γ*-oryzanol compounds in 45 varieties covering pigmented (black and red) and non-pigmented (white) rice grain. The method was developed by integrating UV-Vis spectroscopy with chemometrics, specifically principal component analysis (PCA) and partial least square (PLS) regression. Sampling was performed across the Indonesian archipelago collecting 180 samples, which comprises 60 samples for each type of rice in form of rice husk, bran, whole grain, and polished rice. The results of PCA on the spectroscopic data successfully identified distinction for the 3 types of rice attributable by different type and levels of chemical compound in the grain. White rice exhibited a characteristic absorption at 325 nm while in pigmented (red and black) rice showed a maximum absorbance at 280 nm, indicating the presence of different composition of chemical compounds. A reliable model to predict the 4 major *γ*-oryzanol compounds was established with R^2 calibration, R^2 cross-validation, and R^2 prediction higher than 0.9 was obtained using the spectroscopic data in the range from 200 to 400 nm. However, the PLS modeling was unsuccessful for red and black rice samples most likely due to the interfering high absorption of red colored compounds. Compared to the existing techniques for analyzing individual compounds of the *γ*-oryzanol by high-performance liquid chromatography, the newly developed approach using UV-Vis spectroscopy combined with chemometrics is more practical, faster, and cost-efficient and mainly, solvent and residues free.

Keywords: Rice, Bioactive compounds, Ferulic acid esters, Partial least square regression, Principal component analysis

Introduction

Rice is a primary nutrition source for most people around the globe, especially in Indonesia. Within the county, rice serves as a staple food because it provides complex carbohydrates that can be converted to energy for activities [1]. However, rice also contains bioactive compounds that potentially provide health benefits, such as *γ*-oryzanol, tocopherols, tocotrienols, and *γ*-amino butyric acid [2]. Among the bioactive compounds found in rice, *γ*-oryzanol emerges as the predominant compound. This was evidenced in a study investigating the simultaneous analysis of tocopherols, tocotrienols, and *γ*-oryzanol in white and brown rice. The research revealed that the *γ*-oryzanol content in brown (237.64 µg/g) and white (37.75 µg/g) was notably higher compared to the total tocopherol (3.15 - 12.23 µg/g) and total tocotrienol (10.51 - 18.19 µg/g) content [1]. *γ*-oryzanol is a mixture of structurally related components, especially ferulic acid esters with phytosterols and triterpene alcohols referred to steryl ferulate, comprised of 4 major components, namely cycloartenyl ferulate, 2,4-methylenecycloartanyl ferulate, campesteryl ferulate and *β*-sitosteryl ferulate [3].

Numerous research has explored the health benefits of *γ*-oryzanol, focusing on its potential as a treatment for diabetes mellitus. It has been shown to improve insulin sensitivity, lower hyperglycemia, provide antihyperlipidemic effects, and reduce oxidative stress due to its antioxidant properties [4-6]. The bioactive effectiveness of *γ*-oryzanol is believed to be influenced by its specific composition. Studies have indicated that the specific formation of *γ*-oryzanol contributes to its cholesterol-lowering effects, particularly through the release of campesteryl and *β*-sitosteryl into the intestine, which have demonstrated significant cholesterol-lowering properties [7]. In *in-vitro* studies, *β*-sitosteryl and campesteryl played a role in pancreatic cholesterol esterase whilst this effect was not demonstrated by cycloartenyl ferulate and 2,4-methylenecycloartanyl ferulate [8]. Cholesterol oxidation was more effectively inhibited by 2,4-methylenecycloartanyl ferulate than by cycloartenyl ferulate or campesteryl ferulate [9]. Cycloartenyl ferulate and *β*-sitosteryl ferulate suppress intracellular ROS [10]. Therefore, specific knowledge of the composition of *γ*-oryzanol in rice will contribute to the characterization of health benefits of rice in the human diet. Additionally, a detail information about the level of individual compounds would allow for the determination of the synergetic effects among them on the health benefits. Therefore a method for the characterization of *γ*-oryzanol should be very helpful in this kind of studies.

The analysis of *γ*-oryzanol using reversed-phase high performance liquid chromatography (RP-HPLC) for separation, identification and quantification has been conducted in previous studies [11,12]. Research using the UV-Vis spectrophotometric based method was carried out by Bucci *et al*. [13], namely comparing 3 spectrophotometric methods to analyze the total *γ*-oryzanol in rice bran oil. On the other hand, analysis using spectrocopy has advantages over chromatography, including being easier, faster and more environmentally friendly. Although the individualized determination of compounds with overlapping signals in the UV-Vis range is not directly possible, the use of chemometric techniques can indeed make the individual determination of the components in a compound mixture possible. PCA is widely used for multivariate analysis. This multivariate data reduction technique works when there is a correlation between variables. PCA simplifies extensive variables into a more concise dataset that is easier to interpret by transforming the data into matrices: Scores, which convey sample information, and loadings, which

highlight the variables exerting the most significant influence on differences between sample groups [14]. The initial data is transformed into uncorrelated components known as principal components (PCs). Samples with almost the same PCs have high similarities [15]. Hence PCA can be applied to the spectroscopic data resulting from the analysis for a wide range of rice varieties to identify patterns, trends, and outliers, and can improve the efficiency of subsequent analyses.

In addition, PLS uses linear combinations of predictor variables compared to the original variables to build a model for quantifying chemical compounds [15]. Regression models employing spectral data present a promising option compared to conventional analytical techniques. Methods like cross-validation and external validation datasets are frequently utilized to evaluate the effectiveness of such models [16]. Previous study on the evaluation of *γ*-oryzanol in germinated brown rice, NIRS was used for the quantitative determination of *γ*-oryzanol in germinated brown rice, and a prediction model was established using PLS with high correlation ($R^2 > 0.9$) [17]. Furthermore, in another study, NIRS spectroscopy with the PLS model accurately quantifies *γ*-oryzanol in rice bran oil [18].

In this research, a method for quantitative analysis of the major components of *γ*-oryzanol was developed using a UV-Vis spectroscopy and chemometrics, specificallys, PCA and PLS for multicomponent analysis, and the HPLC technique as a reference method. To the best of our understanding, this represents the initial documentation of a regression model enabling the rapid determination of the 4 major components of *γ*-oryzanol using UV-Vis spectroscopic data.

Materials and methods

Chemicals

γ-oryzanol standard (42.60 % cycloartenyl ferulate, 41.20 % 2,4-metylenecycloartanyl ferulate, 8.30 % campesteryl ferulate, and 6.10 % *β*-sitosteryl ferulate) was obtained from Chromadex. Inc. (California, USA). Methanol and acetonitrile were HPLC grade from Merck (Darmstadt, Germany), and Whatman Uniflo membrane filter (0.22 µm pore size, PTFE) was obtained from Cytiva Global Life Sciences Solutions (Massachusetts, USA). A 500 ppm standard stock solution was prepared using 10 mg of *γ*-oryzanol standard in 20 mL methanol:acetonitrile (1:1).

Rice samples

Sampling for unhusked rice grains was perfomed across the Indonesian archipelago (**Figure 1**), collecting 45 varieties (**Table 1**) that covered pigmented black (15) and red (15) and non-pigmented white rice (15). The samples were collected in the form of husk, bran, whole grain, and polished rice from the production processes. All samples were collected from June to September 2023 and stored in a freezer before use.

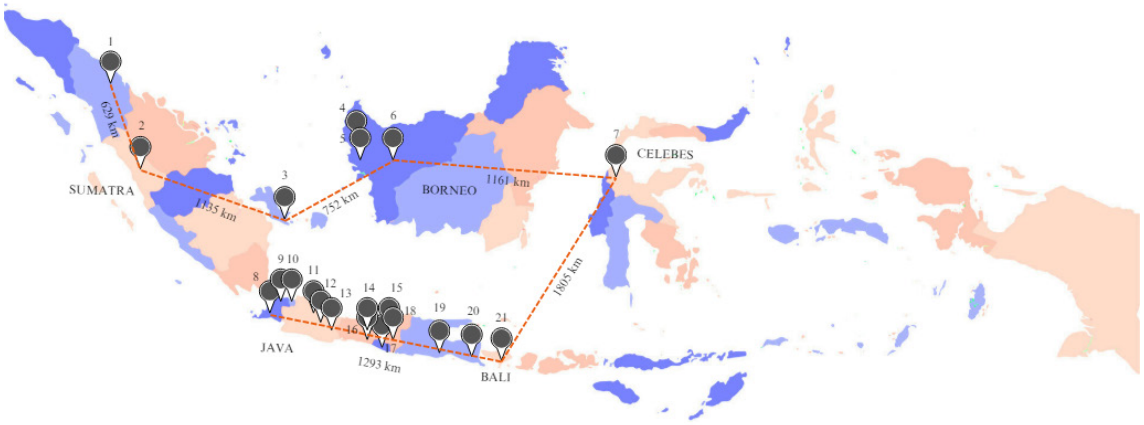

Figure 1 Illustration of sampling at cultivation sites: 1) Simalungun, 2) Limapuluh Kota, 3) South Bangka, 4) Sambas, 5) Landak, 6) Bengkayang, 7) Palu, 8) Lebak, 9) Tangerang, 10) Karawang, 11) Subang, 12) Garut, 13) Tasikmalaya, 14) Wonosobo, 15) Susukan, 16) Purworejo, 17) Yogyakarta, 18) Salatiga, 19) Kediri, 20) Jember, and 21) Tabanan.

Table 1 List of samples.

No.	Rice type	Sample code	Varieties	Region	Province	Island
1	White Rice	W1	Inpari 30	Subang	West Java	Java
2		W2	Inpari 32	Subang	West Java	Java
3		W3	Inpari 33	Subang	West Java	Java
4		W4	Inpari 42	Subang	West Java	Java
5		W5	Inpari 48	Subang	West Java	Java
6		W6	Mekongga	Subang	West Java	Java
7		W7	Padjajaran	Subang	West Java	Java
8		W8	Cakrabuana	Subang	West Java	Java
9		W9	Ciherang	Subang	West Java	Java
10		W10	Nutrizinc	Subang	West Java	Java
11		W11	Cigeulis	Jember	East Java	Java
12		W12	IR-64	Jember	East Java	Java
13		W13	Siliwangi	Jember	East Java	Java
14		W14	Hipa 21	Kediri	East Java	Java
15		W15	Situ Bagendit	Jember	East Java	Java
16	Red Rice	R1	Nagari Balai Panjang	Lima Puluh Kota	West Sumatra	Sumatra
17		R2	Inpari 24	Gunung Kidul	Yogyakarta	Java
18		R3	Sigara-gara	Simalungun	North Sumatra	Sumatra
19		R4	Inpari 24	Landak	West Borneo	Borneo
20		R5	Inpari Arumba	Subang	West Java	Jawa
21		R6	Pamera	Subang	West Java	Jawa

No.	Rice type	Sample code	Varieties	Region	Province	Island
22		R7	Pamera	Tasikmalaya	West Java	Jawa
23		R8	Ampai Merah	Bangka Selatan	Bangka Belitung	Sumatra
24		R9	Segreng Handayani	Bantul	Yogyakarta	Java
25		R10	Merba	Susukan	Central Java	Java
26		R11	Arum Jenar	Susukan	Central Java	Java
27		R12	Ringkak Merah	Sambas	West Borneo	Borneo
28		R13	Sarinah	Garut	West Java	Java
29		R14	Inpari Arumba	Tabanan	Bali	Bali
30		R15	Pandan Merah	Purworejo	Central Java	Java
31		B1	Jelitheng	Subang	West Java	Java
32		B2	Jelitheng	Gunung Kidul	Yogyakarta	Java
33		B3	Sembada Hitam	Sleman	Yogyakarta	Java
34		B4	Jelitheng	Palu	Central Celebes	Celebes
35		B5	Ciparay Wangi	Tasikmalaya	West Java	Java
36		B6	Ringkak Hitam	Sambas	West Borneo	Borneo
37		B7	Balia	Bengkayang	West Borneo	Borneo
38	Black Rice	B8	Jelitheng	Tabanan	Bali	Bali
39		B9	Pandan Hitam	Purworejo	Central Java	Java
40		B10	Nagari Balai Panjang	Lima Puluh Kota	West Sumatra	Sumatra
41		B11	Wonosobo	Wonosobo	Central Java	Java
42		B12	Jelitheng	Tangerang	Banten	Java
43		B13	Jelitheng	Karawang	West Java	Java
44		B14	Salatiga	Salatiga	Central Java	Java
45		B15	Gantang Hideung	Lebak	Banten	Java

Sample preparation

Dried unhusked rice grains (2 kg) were fed into a rice husking machine to separate whole grains and husks. Subsequently, the whole grain was divided evenly for whole grain samples and grains requiring further process to prepare polished rice samples. Meanwhile, the by-product from the 1st polishing process was collected as rice bran. As a result, 180 samples were obtained, consisting of 4 samples each of husk, bran, whole grain, and polished rice, from each sample. The samples underwent a grinder and sieving through a 40 mesh (400 µm) test sieve. The sieved samples were placed into zip-lock plastics and stored in a freezer (–17 ºC) before being analyzed. Each sample was measured (500 mg), dissolved in 10 mL of methanol, and then exposed to sonication at room temperature for 60 min. The resulting methanolic extract of the samples was filtered using a 0.22 µm membrane filter and then subjected to HPLC and spectrophotometry analysis.

HPLC analysis

The HPLC analysis was conducted following the method outlined by Sabir *et al*. [19], with minor adjustments using an HPLC Shimadzu LC 20A (Tokyo, Japan) with a DAD detector equipped with an Agilent Zorbax Eclipse XDB-C18 (Santa Clara, USA) column (150×4.6 mm id. 5 µm). The mobile phase comprised a combination of methanol and acetonitrile with a ratio of 40:60 (v/v) in isocratic elution mode. The wavelength used for quantification was 325 nm, the flow rate was 1.0 mL/min, the oven temperature was 35 °C, the injection volume was 20 µL, and the total run time was 30 min. Compound identification was conducted by collecting the full DAD spectra (200 - 400 nm) and comparing the spectra as well as the retention times between standard references and samples. Furthermore, linearity tests, as well as determination of the limit of detection (LOD) and limit of quantification (LOQ), were conducted to test the performance of the HPLC method used.

UV-Vis spectrophotometer acquisitions

Before measurement, white rice samples were diluted 10×, while red and black rice samples were diluted 50×. The acquisition method is based on the Bucci *et al*. [13]. Each sample extract was tested using a UV-Vis spectrophotometer Shimadzu UV-Vis 2600 (Tokyo, Japan), with a wavelength between 200 and 600 nm. This was carried out every 1 nm using a 1 cm synthetic quartz cuvette (Hellma Analytics). Samples were analyzed in duplicate.

Principal Component Analysis (PCA)

The PCA analysis was conducted using Unscramble X 10.4 (Camo Software USA, Oslo, Norway). The UV-Vis spectra of 180 samples were imported into Unscrambler X 10.4. PCA was performed to visualize the data structure based on the original groups within the selected wavelength range. Subsequently, PCA was used to analyze the correlation between the 4 major *γ*-oryzanol components and the type of rice.

Partial Least Square (PLS) analysis

PLS analysis was conducted using Unscrambler X 10.4 (Camo Software USA, Oslo, Norway). for data processing and model development. The workflow of PLS analysis can be seen in **Figure 2**. The UV-Vis spectra of 180 samples were divided for calibration (70 %) and validation (30 %) sets. The calibration set was established to provide the best regression between the specific levels provided by the chromatographic data and the spectroscopic data set for the *γ*-oryzanol analysis. The efficiency of the model and the most effective wavelength range were assessed using parameters including the coefficient of determination of calibration (R^2C), cross-validation (R^2CV), and prediction (R^2P) in addition to root mean square error of calibration (RMSEC), cross-validation (RMSECV), and prediction (RMSEP). The validation sets used to evaluate the prediction performance of the developed model.

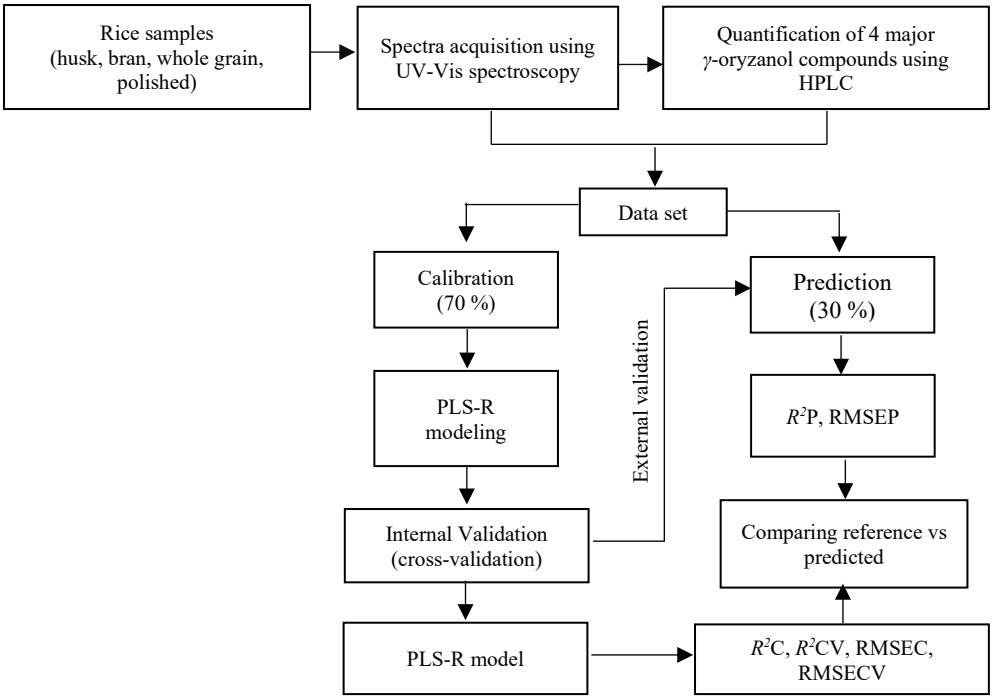

Figure 2 Flow for PLSR development model and external validation.

Results and discussion

HPLC analysis

The HPLC-DAD method used in this study to identify and quantify the 4 major γ-oryzanol compounds was validated with high linearity ($R^2 > 0.9999$) (**Table 2**). The low limits of identification and quantification allowed the method to determine the *γ*-oryzanol compounds at the mg/g level.

Table 2 Parameter of HPLC method performance.

Parameter	CF	24MF	CMF	BSF
Linearity				
- Equation	$y = 3E^7x - 2311.8$	$y = 3E^7x + 350.11$	$y = 3E^7x - 123371$	$y = 3E^7x - 1266.3$
- R^2	0.9999	0.9999	0.9999	0.9999
LOD (mg/L)	0.0001	0.0001	0.0001	0.0001
LOQ (mg/L)	0.0003	0.0002	0.0001	0.0003

Note: CF: Cycloartenyl ferulate, 24MF: 2,4-methylenecycloartanyl ferulate, CMF: Campesteryl ferulate, and BSF: *β*-sitosteryl ferulate.

The identification of 4 major *γ*-oryzanol compounds in the samples was confirmed as the spectra and retention times of the peak in the samples matched with those of the corresponding standard references. The polarity of the compounds determined the arrangement of the peaks in the chromatogram, with the most polar compound being cycloartenyl ferulate, followed by 2,4-methylenecycloartanyl ferulate,

campesteryl ferulate, and *β*-sitosteryl ferulate. The separation result by the HPLC (**Figure 3**) indicated full separation of the compounds both in the samples and standards.

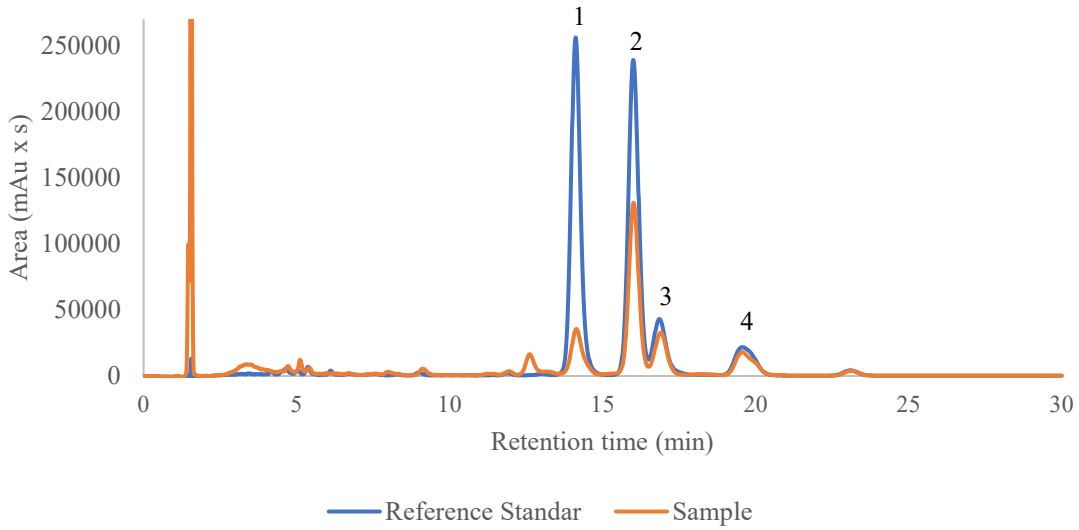

Figure 3 Overlayed chromatograms of 4 major *γ*-oryzanol compounds in standard solution and rice grain sample: 1) Cycloartenyl ferulate, 2) 2,4-methylenecycloartanyl ferulate, 3) campesteryl ferulate, and 4) *β*-sitosteryl ferulate.

HPLC analysis was also used to quantify gamma oryzanol levels in rice. **Figure 4** shows that the *γ*-oryzanol content in pigmented rice (red and black) was higher than that in non-pigmented rice (white). This is consistent with Chinvongamorn and Sansenya [20], indicating that pigmented rice has a higher gamma-oryzanol content than non-pigmented rice. As shown in **Figure 4**, the same trend was observed among the three types of rice: The composition of 2,4-methylenecycloartanyl ferulate was the highest, followed by cycloartanyl ferulate, campesteryl ferulate, and *β*-sitosteryl ferulate.

The levels and composition of individual *γ*-oryzanol compounds differed among the various rice varieties. According to the research by Miller and Engel [21], the composition of *γ*-oryzanol is influenced by environmental conditions. Samples from the Cripto cultivar grown in France had a proportion of campesteryl ferulate 3 times the proportion of campestanyl ferulate. However, samples of the same cultivar grown in Italy showed equal proportions of campesteryl ferulate and campestanyl ferulate. In the Cripto samples grown in Italy the following year, the campesteryl ferulate proportion was half the campestanyl ferulate [21].

Additionally, research by Nakano *et al*. [22], showed that the levels of cycloartenyl ferulate, 2,4-methylenecycloartanyl ferulate, and total *γ*-oryzanol can be influenced by different cultivars and growing seasons, and also in the research by Nurmi *et al.* [23] which found that The content of steryl ferulates is significantly influenced by the genotype and the growing location. Furthermore, when examining the *γ*-oryzanol content in each part of the rice, it can be seen that the highest content is in the bran, followed by whole grain, then the husk and the smallest is in the polished part. This is consistent with previous research

on 16 varieties of Taiwanese rice, where γ-oryzanol was mostly found in the bran fraction, followed by husks, and the lowest was found in polished rice [24].

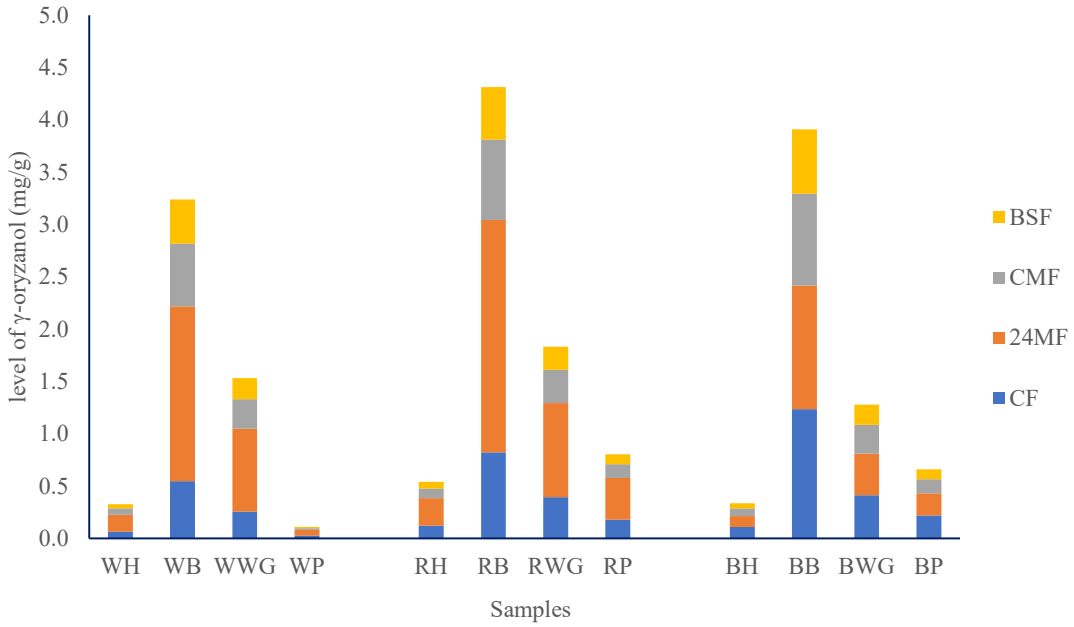

Figure 4 γ-oryzanol level in 3 types of rice extract in various parts of rice.
Note: WH: White (husk), WB: White (bran), WWG: White (whole grain), WP: White (polished), RH: Red (husk), RB: Red (bran), RWG: Red (whole grain), RP: Red (polished), BH: Black (husk), BB: Black (bran), BWG: Black (whole grain), BP: Black (polished), CF: Cycloartenyl ferulate; 24MF: 2,4-methylenecycloartanyl ferulate, CMF: Campesteryl ferulate, and BSF: β-sitosteryl ferulate.

UV-Vis spectrophotometry analysis

The UV-Vis spectra results showed that white, red, and black rice have distinct pattern (**Figure 4**). For the white rice (**Figure 4**), the spectra within the wavelength range of 200 - 600 nm showing a characteristic absorption (local maximum) at a wavelength of 325 nm. Based on previous studies indicated that γ-oryzanol which composed of ferulic acid esters exhibit maximum absorption at around 327 nm [13,25,26]. However, some other hydroxycinnamic acid derivatives may also contribute to the signal at 325 nm [27]. In contrast, for the red and black rice (**Figure 4**), the spectra within the wavelength range of 200 - 600 nm presentation a local maximumk at a wavelength of 280 nm. The pigmented rice exhibited higher absorption at 280 nm compared to 325 nm. This is attributed to the sub-group of phenolic acid, namely hydroxybenzoic acid aldehydes, that is largely present in pigmented rice which exhibit maximum absorption at a wavelength of 280 nm. Protocatechuicaldehyde and vanillin are the primary phenolic compounds found in pigmented rice. In addition to the aldehydes, anthocyanins are also providing absorption at 280 nm, hence contributing to the peak signals in this wavelength [28].

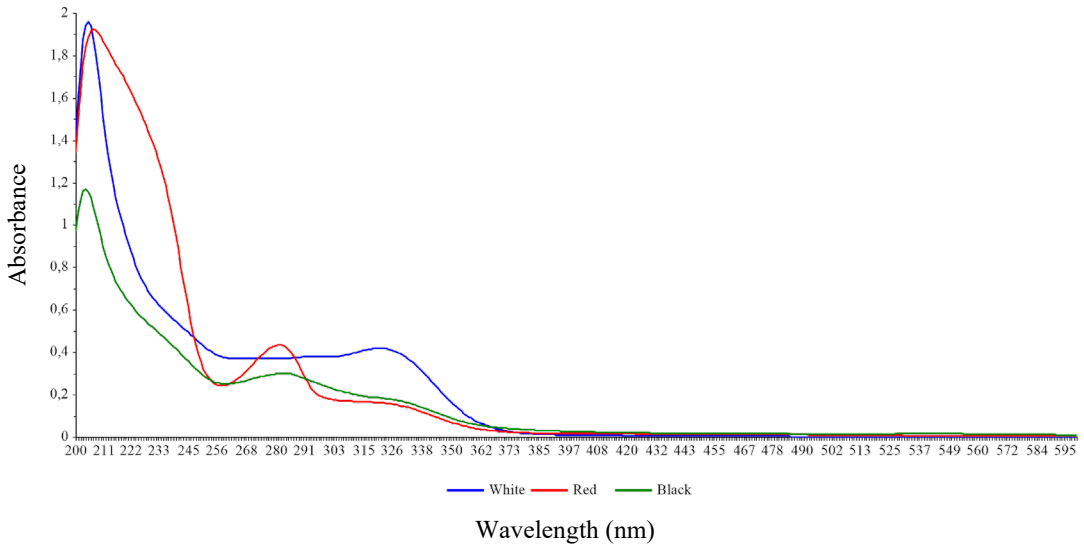

Figure 5 Spectra of white, red, and black rice.

Principal component analaysis

An unsupervised exploratory PCA was carried out on the collected UV-Vis spectra . PCA was used as unsupervised pattern recognition for reducing the original variables into new independent variables referred to as PCs. The 1st 2 PCs are particularly useful for later analysis as they represent the most of data variability [19]. The PCA results revealed 2 components that explained 89 % of the variability in the original data (**Figure 6**).

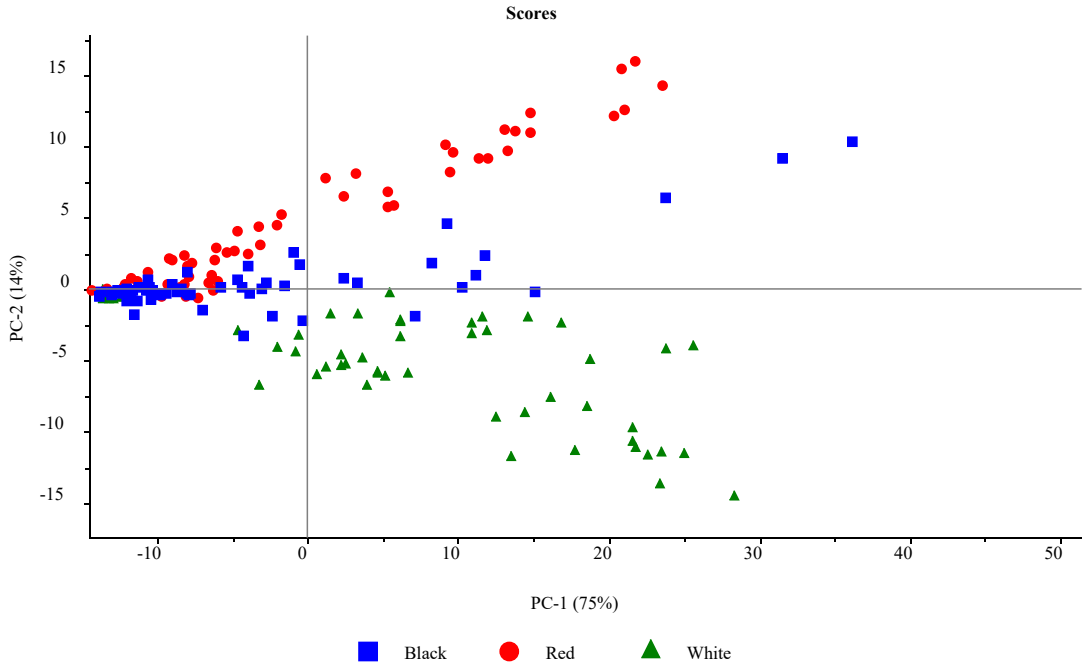

Figure 6 PCA score plot of rice grain spectra data.

The PCA results could be associated with the 3 distinct groups that correspond to the 3 types of rice: White rice, red rice, and black rice, with some overlappings. It can be seen that both PCs would be needed to explain the variability of the sample set. It must be also noted that there are high overlapping characteristics between black and red rice samples, while withe rice samples are arranged in a different area based on PC2 (negative values of PC2 for white samples).

As a conclusion of the PCA results. It has been demonstrated that the UV-Vis spectroscopic data would be useful for the characterization of the 3 groups of rice samples. It can be concluded that the spectroscopic information is directly related to the type of rice sample. Therefore, specific regression models should be developed for each type of rice, as the spectroscopic information is different of each type of rice. Therefore in the next step, individual PLS models will be developed for each kind of rice.

Calibration and validation of the PLS-regression model

The results of the PLS regression analysis between the specific compounds of γ-oryzanol, i.e. cycloartenyl ferulate, 2,4-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate and the spectroscopic data splitted in 3 regions are presented in **Table 3**.

Table 3 Parameter of PLS regression model on 4 major γ-oryzanol compounds.

Parameter	Rice type	Wavelength	Calibration		Cross-validation		Prediction	
			R^2C	RMSEC	R^2CV	RMSECV	R^2P	RMSEP
CF	White Rice	200 - 600	0.9418	0.0514	0.9172	0.0627	0.8062	0.0962
24MF			0.9445	0.1734	0.9234	0.2097	0.6287	0.3168
CMF			0.9890	0.0257	0.9854	0.0303	0.9058	0.0682
BSF			0.9906	0.0170	0.9861	0.0213	0.9170	0.0427
CF	White Rice	200 - 400	0.9285	0.0569	0.9178	0.0625	0.8720	0.0782
24MF			0.9484	0.1672	0.9398	0.1848	0.9265	0.1409
CMF			0.9785	0.0359	0.9739	0.0405	0.9553	0.0469
BSF			0.9865	0.0205	0.9831	0.0234	0.9789	0.0215
CF	White Rice	200 - 250	0.9061	0.0643	0.8531	0.0823	0.8847	0.0739
24MF			0.9212	0.1921	0.8718	0.2506	0.9380	0.1147
CMF			0.9775	0.0349	0.9659	0.0440	0.9547	0.0443
BSF			0.9772	0.0250	0.9618	0.0330	0.9692	0.0225
CF	Red Rice	200 - 600	0.9068	0.1376	0.6159	0.2859	0.4136	0.1850
24MF			0.8723	0.3128	0.5851	0.5770	0.6281	0.4838
CMF			0.8778	0.1043	0.7220	0.1610	0.7123	0.1377
BSF			0.8903	0.0679	0.6852	0.1177	0.7987	0.0754
CF	Red Rice	200 - 400	0.8899	0.1502	0.3508	0.3717	0.3307	0.1976
24MF			0.8398	0.3504	0.6422	0.5359	0.7533	0.3941
CMF			0.8609	0.1112	0.7401	0.1557	0.7951	0.1162
BSF			0.8879	0.0687	0.6475	0.1246	0.7942	0.0763

Parameter	Rice type	Wavelength	Calibration		Cross-validation		Prediction	
			R^2C	RMSEC	R^2CV	RMSECV	R^2P	RMSEP
CF	Red Rice	200 - 250	0.6995	0.2471	0.6620	0.2681	0.4406	0.1807
24MF			0.7346	0.4511	0.7098	0.4826	0.7105	0.4268
CMF			0.8234	0.1254	0.8142	0.1316	0.8036	0.1138
BSF			0.8645	0.0754	0.8562	0.0796	0.7956	0.0760
CF	Black Rice	200 - 600	0.7030	0.2566	0.6156	0.2986	0.4004	0.3576
24MF			0.7030	0.2458	0.6157	0.2862	0.4004	0.3426
CMF			0.7563	0.1767	0.6618	0.2130	0.3092	0.2598
BSF			0.7701	0.1175	0.6992	0.1375	0.2993	0.1840
CF	Black Rice	200 - 400	0.6723	0.2695	0.5722	0.3151	0.3618	0.3689
24MF			0.6723	0.2582	0.5722	0.3019	0.3618	0.3535
CMF			0.7410	0.1822	0.6536	0.2211	0.2319	0.2740
BSF			0.7560	0.1211	0.6585	0.1465	0.2288	0.1931
CF	Black Rice	200 - 250	0.5481	0.3165	0.5023	0.3337	NA	0.4670
24MF			0.5481	0.3032	0.5023	0.3198	NA	0.4475
CMF			0.4800	0.2581	0.3778	0.2889	NA	0.3414
BSF			0.5118	0.1712	0.4529	0.1855	NA	0.2395

Note: CF: Cycloartenyl ferulate, 24MF: 2,4-methylenecycloartanyl ferulate, CMF: Campesteryl ferulate, and BSF: β-sitosteryl ferulate.

The 3 specific spectroscopic regions were chosen based on the previously described spectroscopic properties of each type of rice. In white rice, the values of R^2 calibration (R^2C), R^2 cross-validation (R^2CV), and R^2 prediction (R^2P) for the 4 compounds exceed 0.9 for any spectroscopic region, indicating a high level of correlation between the reference and predicted values. In red rice, the R^2 values in calibration are relatively high at around 0.8, but the values in cross-validation and prediction are lower. The same trend was observed in black rice, where the R^2 values are around 0.5 - 0.7 in calibration and cross-validation, and below 0.5 in prediction. Overfitting occurs when a model is too complex or fits the training data too closely, making it difficult to generalize to new or unseen data. This can result in the capture of small patterns or noise in the training data that are not representative of the broader data set [29-31]. In this case, interferences from other compounds besides the target compound (γ-oryzanol) may be present, which could prevent successful PLS regression.

Real sample application

Because of the models provided good prediction results for white rice varieties, a total of 16 samples of white rice from the husk, bran, whole grain, and polished rice were used in the validation set for real sample applications. In **Table 4**, a comparison is presented between the predicted values and the reference values.

Table 4 Comparison of predicted (PLS-model) and reference (HPLC).

Rice part	Rice varieties	Sample code	CF		24MF		CMF		BSF	
			Pred. (mg/g)	Ref. (mg/g)	Pred. (mg/g)	Ref. (mg/g)	Pred. (mg/g)	Ref. (mg/g)	Pred. (mg/g)	Ref. (mg/g)
Husk	IR-64	WH12	0.0375	0.0428	0.1927	0.0945	0.0354	0.0323	0.0006	0.0211
	Siliwangi	WH13	0.0399	0.0650	0.0761	0.1493	0.0336	0.0592	0.0245	0.0367
	Hipa-21	WH14	0.0717	0.0794	0.1831	0.1649	0.0712	0.0624	0.0521	0.0421
	Situ Bagendit	WH15	–0.0038	0.0314	–0.1084	0.0597	–0.0187	0.0152	–0.0076	0.0112
Bran	IR-64	WB12	0.4751	0.5369	1.6936	1.3437	0.5500	0.5987	0.3790	0.3884
	Siliwangi	WB13	0.4194	0.5859	1.4172	1.4958	0.4812	0.6459	0.3459	0.4190
	Hipa-21	WB14	0.2922	0.3863	0.9774	0.8107	0.3310	0.3338	0.2371	0.2323
	Situ Bagendit	WB15	0.4891	0.6963	1.6180	1.4959	0.5621	0.5944	0.4123	0.4123
Whole Grain	IR-64	WWG12	0.2622	0.2378	0.9497	0.7926	0.2976	0.3197	0.1988	0.2192
	Siliwangi	WWG13	0.2217	0.2677	0.7338	0.6461	0.2473	0.2821	0.1773	0.1793
	Hipa-21	WWG14	0.1533	0.2107	0.4939	0.4317	0.1664	0.1788	0.1192	0.1249
	Situ Bagendit	WWG15	0.2676	0.3453	0.9021	0.7025	0.3018	0.2844	0.2148	0.2006
Polished	IR-64	WP12	0.0432	0.0301	0.2156	0.0612	0.0399	0.0213	0.0077	0.0132
	Siliwangi	WP13	0.0279	0.0365	0.0667	0.0749	0.0185	0.0275	0.0110	0.0161
	Hipa-21	WP14	0.0051	0.0203	–0.0153	0.0312	–0.0086	0.0078	–0.0079	0.0055
	Situ Bagendit	WP15	0.0246	0.0277	0.0775	0.0486	0.0152	0.0143	0.0045	0.0094

Based on the developed PLS model, the R^2 prediction (R^2P) values for the 4 major γ-oryzanol compounds ranged from 0.8 to 0.9. This indicates a high correlation between predicted and reference values. Additionally, a t-test was used to assess the significance of differences between predicted and reference values at a significance level of 0.05. The t-test results for the 4 major gamma oryzanol compounds showed $p > 0.05$, indicating no significant difference between predicted and reference values. Thus, this developed PLS model can be used for real sample applications in white rice.

Conclusions

The PLS-regression models developed from the UV-Vis spectroscopic data showed good performance in predicting 4 major components of γ-oryzanol in white rice varieties. This result encourages research exploration of functional compounds in rice, especially in Indonesia, to optimize the use of rice as a staple food ingredient with functional effects. Our models provide highly accurate predictions for white rice samples. Additional work should be done for black and red rice samples. UV-Vis spectroscopy and PLS regression can be considered a reliable alternative for routine analysis of 4 major components of γ-oryzanol. This 1st study shows that a simple and fast spectroscopic technique is appropriate for quantifying 4 major components of γ-oryzanol vs conventional chromatographic methods. The research results regarding the distribution of γ-oryzanol in different parts of the rice grain of various varieties will provide useful information for the food industry because of its potential as a nutraceutical and functional ingredient.

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