

ISOLATION AND CHARACTERIZATION OF GAMMA ORYZANOL FROM RICE BRAN OIL SOAPSTOCK

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Abstract

This research investigates the effect of ethanol concentrations in an ethyl acetate/ethanol mixture and the crystallization conditions on the recovery and purity of gamma oryzanol isolated from dried rice bran soapstock, using two-step crystallization. The ethanol concentrations were between 0, 10, 20, 30, and 40% v/v, and the crystallization parameters included the solvent to crude gamma oryzanol rich fraction (ORF) ratio, crystallization temperature, and time. In the first crystallization, the solvent to ORF ratio was between 2.5:1, 5:1, 7.5:1, and 10:1 (v/v), the crystallization temperature was between -60, -45, -30, -15, and 0°C, and the time between was 5, 7.5, 10, 12.5, and 15 h. The liquid phase was subsequently subjected to a second crystallization for the gamma oryzanol crystal, where the crystallization temperatures and time were between 0, 5, 10, 15, and 20°C and 1, 6, 11, and 15 h, respectively. The optimal ethanol concentration in the solvent mixture was 30% v/v. The optimal first-crystallization solvent to ORF ratio, crystallization temperature, and time were 7.5:1 (v/v), -15°C, and 7.5 h, respectively. The optimal second-crystallization temperature and time were 5°C and 6 h, with the gamma oryzanol recovery and purity of 58.03-61.17% and 87.10-90.29%, respectively. The physicochemical properties of the gamma oryzanol crystal also indicated good agreement between the isolated gamma oryzanol and the standard.

Keywords: Rice bran oil soapstock, gamma oryzanol, isolation, purity

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Introduction

Rice bran, a by-product of rice milling, contains 12-23% oil (Wongwaiwech *et al.*, 2018). Rice bran is also rich in nutraceuticals, including gamma oryzanol, tocopherols, and tocotrienols, making crude rice bran oil highly nutritious. However, high contents of free fatty acids (FFA), waxes, gums, and pigments in crude rice bran oil complicate the refining process to improve the odor, appearance, and shelf life to meet the standards of edible oil (Gunstone, 2011).

In rice bran oil refining, the FFA and mucilaginous impurities are removed by alkaline neutralization (Engelmann *et al.*, 2017). The alkaline neutralization process nevertheless causes nearly 90% gamma oryzanol loss through wet soapstock (Krishna *et al.*, 2001). At present, rice bran soapstock is mostly converted into detergent soaps (Narayan *et al.*, 2006), feedstock (Ju and Vali, 2005), and lecithin products (Thurman, 1961). In fact, the residual gamma oryzanol contained in rice bran soapstock presents a promising opportunity for gamma oryzanol production on a commercial scale (Narayan *et al.*, 2006).

Research has shown that gamma oryzanol helps reduce plasma cholesterol (Wilson *et al.*, 2007; Kumar *et al.*, 2009), platelet aggregation, and cholesterol absorption (Seetharamaiah *et al.*, 1986; Sharma *et al.*, 1986). It is also used in cosmetics for anti-itching and anti-dandruff properties (Krishna *et al.*, 2001; Patel and Naik, 2004) and in food products (Patel and Naik, 2004). Therefore, many extraction methods have been proposed for isolating gamma oryzanol from alkaline oil cake (soapstock).

Nishihara and Shibuya (1968) isolated gamma oryzanol from an alcoholic solution (ethanolic and methanolic) of rice bran oil soapstock using CO₂ as an anti-solvent. They successfully derived white-crystal gamma oryzanol with 85% purity; however, gums and waxes were mostly insoluble in a gaseous solvent mixture. Das *et al.* (1998) isolated gamma oryzanol from rice bran oil soapstock using calcium ion-induced precipitation. Although the recovery and purity were high

(76% and 96%), the technique was highly complex and required a toxic solvent (chloroform).

Narayan *et al.* (2004) and Zullaikah *et al.* (2009) experimentally isolated gamma oryzanol from rice bran oil and rice bran soapstock by using preparative high performance liquid chromatography (HPLC); Narayan *et al.* (2004) reported 90% gamma oryzanol recovery with 90-98% purity. Nonetheless, the method was ideal for laboratory-scale production due to low productivity. Zullaikah *et al.* (2009) isolated gamma oryzanol from rice bran oil at a very low temperature (-60°C) by a mixture of ethyl acetate and methanol as the crystallization solvent using two-step crystallization; they reported 57-59% gamma oryzanol crystal recovery with 93-95% purity. However, the sub-zero temperature (-60°C) during the first crystallization necessitated a special cooling device. Kaewboonnum *et al.* (2010) isolated gamma oryzanol from rice bran oil soapstock with a 20:80 (v/v) ethyl acetate/methanol mixture using two-step crystallization (3°C and 1 h for the first crystallization, followed by 5°C and 24 h for the subsequent step). However, the recovery and purity of gamma oryzanol were both relatively low (55.17% and 74.60%, respectively).

In this research, ethanol was used in combination with ethyl acetate. Ethanol is commonly used in food and pharmaceuticals because of its low toxicity and cost-effectiveness. Besides, ethanol, when combined with ethyl acetate, moderates the solvent polarity, thus enhancing gamma oryzanol crystallization. However, there exists no study on gamma oryzanol isolation using an ethyl acetate/ethanol mixture.

Thus, this research investigates the effect of ethanol concentrations (0, 10, 20, 30, and 40% v/v) in an ethyl acetate/ethanol mixture and the crystallization conditions (i.e., solvent to oryzanol rich fraction (ORF) ratio, crystallization temperature, and time) on gamma oryzanol isolated from dried rice bran soapstock. The isolation was carried out using two-step crystallization. In the first

crystallization, the solvent to ORF ratio was varied between 2.5:1, 5:1, 7.5:1, and 10:1 (v/v), the crystallization temperature between -60, -45, -30, -15, and 0°C, and the crystallization time between 5, 7.5, 10, 12.5, and 15 h. The liquid phase from the first crystallization was subsequently subjected to second crystallization where the crystallization temperatures and time were varied between 0, 5, 10, 15, and 20°C and 1, 6, 11, and 15 h, respectively, for gamma oryzanol crystal. The gamma oryzanol recovery and purity were determined and the physicochemical properties characterized.

Materials and Methods

Materials

Wet rice bran oil soapstock, following Indira *et al.* (2005), was heated to 80°C in a water bath and saponified by adding 1.25% (w/v) NaOH solution with constant stirring for 30 min before centrifugation at 10,000 rpm and 10°C for 15 min to remove the liquid. The solid was then vacuum-evaporated (100°C, 550 mmHg) for 2 h for dried rice bran soapstock (DRBS) with a 4-4.5% moisture content, measured with a moisture meter (AD-4714A, A&D Co., Ltd, Tokyo, Japan). The DRBS was used as the starting material for the gamma oryzanol extraction.

In extraction, 50 g DRBS was extracted in 500 mL ethanol/ethyl acetate mixture (15% ethanol: 85% ethyl acetate) for 4 h in a Soxhlet extractor (Kumar *et al.* 2009). The crude gamma ORF was then centrifuged (10,000 rpm, 10°C, 10 min) to remove the solid before vacuum evaporation (90°C, 550 mmHg) (EW-28615-01 evaporator, Cole Parmer, Vernon Hills, IL, USA). The ORF contained 4.41 g gamma oryzanol/100 g DRBS.

A gamma oryzanol standard (Oryza Oil and Fat Chemical Co., Ltd, Ichinomiya City, Japan) was used for quantification. Meanwhile, ethyl acetate (99.5%), acetonitrile (99.8%), and ethanol (98.5%) were from Sigma-Aldrich (Darmstadt, Germany).

Experimental Methods

In the first crystallization, the mucilaginous impurities (waxes and gums) in the crude ORF solution were removed with a mixture of ethyl acetate and ethanol and stirred for 15 min at room temperature. The ethyl acetate to ethanol ratio was varied between 100:0, 90:10, 80:20, 70:30, and 60:40% (v/v), the solvent to crude ORF solution ratio between 2.5:1, 5:1, 7.5:1, and 10:1 (v/v), the crystallization temperature between 0, -15, -30, -45, and -60°C, and the crystallization time between 5, 7.5, 10, 12.5, and 15 h. After crystallization, the liquid phase (LP) and solid phase (SP) were separated by filtering with Whatman filter paper no.1 under a vacuum condition (350 mmHg). The gamma oryzanol recovery in the LP was determined by spectrophotometry and HPLC.

In the second crystallization, the LP was subjected to different crystallization temperatures (0, 5, 10, 15, and 20°C) and times (1, 6, 11, and 15 h). The gamma oryzanol crystal was precipitated and separated by vacuum filtering. The recovery and purity of the gamma oryzanol were calculated by Equations (1) and (2), respectively:

$$\text{Gamma oryzanol recovery (\%)} = \frac{\text{Amount of gamma oryzanol in precipitate (g)}}{\text{Amount of gamma oryzanol in crude ORF (g)}} \times 100 \quad (1)$$

$$\text{Gamma oryzanol purity (\%)} = \frac{\text{Amount of gamma oryzanol in precipitate (g)}}{\text{Amount of precipitate (g)}} \times 100 \quad (2)$$

Analytical Methods

Quantitative Analysis of Gamma Oryzanol Content

The gamma oryzanol contents were analyzed with a UV-spectrophotometer/NIR (UV-2600, Shimadzu, Corp., Kyoto, Japan) and HPLC, following Joshi *et al.* (2016) and Sakunpak *et al.* (2014). The HPLC consisted of a pump (LPG 3X00), auto-sampler (ACC-3000), diode-array UV/VIS detector (DAD), and Poroshell 120 EC-C18 column (3.0 mm × 150 mm, 2.7 µm). The column temperature and UV detector were set at 25°C and 325 nm. The gradient mobile phase consisted of 100:0, 50:50, and 40:60 (v/v) methanol:acetonitrile

for 5, 10, and 30 min. The flow rate was 1.0 ml/min throughout the HPLC analysis, and the sample injection volume was 20 μ L.

Liquid Chromatography-Mass Spectrometry

The components of the gamma oryzanol were characterized with a Dionex Ultimate 3000 HPLC coupled with a Bruker Amazon SL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), following Sakunpak *et al.* (2014). The samples were separated at 25°C on a Poroshell 120 EC-C18 column (3.0 mm $\times$ 150 mm, 2.7 μ m) using a gradient mobile phase consisting of 95:5, 40:60, and 5:95 (v/v) methanol:acetonitrile containing 0.1% formic acid for 2, 20, and 8 min, with a flow rate of 1.0 ml/min. The injection volume was 20 μ L and the UV detector wavelength was 325 nm. The mass spectrometer was equipped with electrospray ionization and a quadrupole-ion trap. The mass spectra were observed in negative ion mode and recorded on a mass to charge ratio (m/z) range of 100-800. The capillary voltage was 4,500 V, the drying gas temperature was 200°C with a flow rate of 4.5 L/min, and the nebulizer pressure was 200 kPa. The gamma oryzanol components were individually identified using the multiple reaction monitoring mode and the data were analyzed with LTQ Orbitrap mass spectrometer software (Thermo Fischer Scientific, Waltham, MA, USA).

Thermal Behavior Analysis

The melting point of the gamma oryzanol was determined with differential scanning calorimetry (DSC) (DSC 7, PerkinElmer Inc.,

Waltham, MA, USA). The baseline was established using 2 empty sealed aluminum pans. Then, 3-5 mg gamma oryzanol crystal was weighed in the aluminum pans and sealed. The DSC chamber was flushed with nitrogen (0.5 mL/min) and the samples were heated from 25°C to 200°C at 10°C/min and maintained at 200°C for 10 min and the melting curve plotted. The samples were then cooled down from 200°C to 25°C at 10°C/min.

Field Emission Scanning Electron Microscope

The particle size and morphology of gamma oryzanol were determined with a field emission scanning electron microscope (FESEM) (JSM 7800F, JEOL, Ltd., Tokyo, Japan). In the analysis, the standard and isolated gamma oryzanol were placed on the metal stub and gold-sputtered and 25,000x images of the shape and surface characteristics of the gamma oryzanol crystal were taken.

Other Analytical Methods

The color values were measured with a Minolta CR-300 Chroma Meter (Minolta Co., Ltd., Osaka, Japan) and expressed in accordance with the Commission Internationale de l'Eclairage L*a*b* system (lightness, redness, and yellowness). The particle size distribution of the gamma oryzanol crystal was determined with a laser scattering particle size distribution analyzer (LA 920, Horiba, Ltd., Kyoto, Japan), following Hong *et al.* (2002). The particle size curve and mean particle size were analyzed with an LA-920 software analyzer (Horiba, Ltd., Kyoto, Japan).

Table 1. Effect of ethanol concentrations in ethyl acetate/ethanol mixture solvent on gamma oryzanol recovery

Ethyl acetate:ethanol (v/v)	First crystallization ¹		Second crystallization ²			
	Gamma oryzanol recovery (%) ³		Gamma oryzanol recovery (%) ³		Purity (%) ³	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
100:0	80.03 ^a ±0.15	75.90 ^a ±1.10	60.10 ^a ±1.51	ND ⁴	90.38 ^a ±1.57	87.40 ^a ±2.00
90:10	79.80 ^a ±0.93	75.98 ^a ±0.93	59.92 ^a ±1.23	57.93 ^a ±1.54	90.31 ^a ±1.23	87.38 ^a ±2.01
80:20	79.79 ^a ±1.01	75.90 ^a ±1.01	60.13 ^a ±1.59	58.03 ^a ±2.01	90.29 ^a ±1.35	87.39 ^a ±1.95
70:30	79.78 ^a ±1.05	75.92 ^a ±1.05	59.97 ^a ±1.45	57.99 ^a ±1.58	90.31 ^a ±1.56	87.40 ^a ±1.69
60:40	74.94 ^b ±1.10	71.94 ^b ±1.10	47.35 ^b ±1.20	44.87 ^b ±2.11	89.93 ^a ±1.61	87.38 ^a ±1.75

¹ Experimental condition was solvent:ORF = 5:1; crystallization temperature = -60°C; crystallization time = 15 h

² Experimental condition was crystallization temperature = 5°C; crystallization time = 15 h

³ The values are the mean of 3 replications $\pm$ standard deviation. Different letters in each column denote statistically significant differences between treatments ($p < 0.05$)

⁴ ND = not determined

Statistical Analysis

The experiments were performed in triplicate, and the means and standard deviations were computed using Statgraphics Centurion XV (Statgraphics Technologies, Inc., The Plains, VA, USA). One-way analysis of variance was carried out to verify the relationship between the isolation conditions and the gamma oryzanol recovery and its physicochemical properties. A Tukey-HSD comparison was used to compare the means, given the 5% significance level ($p < 0.05$).

Results and Discussion

Effect of First Crystallization Conditions on Gamma Oryzanol Recovery

Ethyl Acetate/Ethanol Mixture Ratio

Solvent mixture polarity plays a crucial role in the isolation of gamma oryzanol from crude ORF solution. In the first crystallization, the crude ORF solution was mixed, on a trial basis, with ethyl acetate/ethanol mixture (i.e., 0, 10, 20, 30, and 40% v/v ethanol concentrations), and given 5.0:1.0 v/v solvent to the ORF ratio (Kaewboonnum *et al.*, 2010) under constant stirring. The mixture was then retained at -60°C for 15 h (Zullaikah *et al.*, 2009) until the liquid phase (LP) and solid phase (SP) were formed. The LP was filtered through Whatman filter paper no.1 for enriched gamma oryzanol.

Table 1 tabulates the effect of the ethanol concentrations in the ethyl acetate/ethanol mixture on gamma oryzanol recovery. The gamma oryzanol contents in the LP were

insignificantly different ($p > 0.05$) under 0, 10, 20, and 30% (v/v) ethanol conditions. With a 40% (v/v) ethanol concentration, the gamma oryzanol recovery was reduced significantly ($p < 0.05$), which could be attributed to the low solubility of gamma oryzanol in the alcohol (Narayan *et al.*, 2006; Meirelles *et al.*, 2011). In other words, gamma oryzanol was trapped in the residual impurities.

The LP was then subjected to a second crystallization for further isolation of the gamma oryzanol, given the crystallization temperature and time of 5°C and 15 h (Kaewboonnum *et al.*, 2010), respectively. The recovery and purity of the gamma oryzanol were analyzed by spectrophotometry and HPLC. A higher ethanol concentration (40% v/v) significantly lowered the gamma oryzanol recovery (47.35% and 44.87% for spectrophotometry and HPLC, respectively) ($p < 0.05$). However, variations in the ethanol concentrations had no significant effect on the gamma oryzanol purity (89.93-90.38% and 87.38-87.40% for spectrophotometry and HPLC, respectively) ($p > 0.05$). As a result, a 30% ethanol concentration (70:30% v/v) was adopted to isolate the gamma oryzanol from the crude ORF solution.

Solvent to Crude ORF Ratio

The ethyl acetate/ethanol mixture solvent to crude ORF ratio was varied between 2.5:1.0, 5.0:1.0, 7.5:1.0, and 10.0:1.0 (v/v), given 70:30% (v/v) ethyl acetate/ethanol mixture, and retained at -60°C for 15 h (Zullaikah *et al.*, 2009). In Table 2, the gamma oryzanol recovery in the LP significantly increased as

Table 2. Effect of solvent to crude ORF ratio on gamma oryzanol recovery (the first-crystallization stage)

Solvent to crude ORF Ratio (v/v)	First crystallization ¹		Second crystallization ²			
	Gamma oryzanol recovery (%) ³		Gamma oryzanol recovery (%) ³		Purity (%) ³	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
2.5:1	73.92 ^a ±1.17	69.17 ^c ±1.65	49.02 ^a ±2.05	ND ⁴	76.31 ^c ±1.13	71.01 ^c ±1.81
5:1	79.27 ^b ±1.35	76.44 ^b ±1.87	57.61 ^b ±1.98	55.01 ^b ±1.21	89.96 ^{ab} ±1.21	87.35 ^{ab} ±2.15
7.5:1	80.72 ^a ±1.89	77.55 ^a ±2.19	60.79 ^a ±1.82	57.95 ^a ±1.04	89.99 ^a ±1.43	87.41 ^a ±1.69
10:1	80.75 ^a ±2.17	77.56 ^a ±2.62	59.99 ^a ±2.04	57.93 ^a ±0.89	90.01 ^a ±2.01	87.38 ^a ±1.75

¹ Experimental condition was ethyl acetate:ethanol = 70:30 (v/v); crystallization temperature = -60°C ; crystallization time = 15 h

² Experimental condition was crystallization temperature = 5°C ; crystallization time = 15 h

³ The values are the mean of 3 replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p < 0.05$)

⁴ ND = not determined

the ratio increased from 2.5:1.0 to 7.5:1.0 (v/v). This is because the higher solvent to ORF ratio increased concentration gradients between the solute and solvent, thereby enhancing gamma oryzanol solubility in the LP. However, under 10.0:1.0 (v/v), gamma oryzanol recovery was insignificantly different from that of the 7.5:1.0 (v/v) ratio.

As shown in Table 2, the high gamma oryzanol contents in the LP in turn increased the recovery and purity of the gamma oryzanol in the second crystallization. By comparison, the recovery and purity of the gamma oryzanol under 2.5:1.0 v/v were lowest due to large quantities of residual mucilage in the LP, which subsequently co-precipitated in the second crystallization. On the other hand, higher solvent to ORF ratios (7.5:1.0 and 10.0:1.0 v/v) significantly improved the gamma oryzanol recovery and purity ($p < 0.05$). However, use of large quantities of solvent translates into high production costs. As a result, the solvent to ORF ratio of 7.5:1.0 (v/v) was employed, given the comparably high gamma oryzanol recovery and purity.

First Crystallization Temperature

The first-crystallization temperatures were varied between 0, -15, -30, -45, and -60°C, given 15 h crystallization time. In Table 3, the recovery of gamma oryzanol in the LP was insignificantly different as temperatures fell from -15°C to -60°C ($p > 0.05$). The gamma oryzanol contents in the LP under subzero conditions were higher than at 0°C. This is consistent with Zullaikah *et al.* (2009), who documented that the crystallization temperature

of 0°C achieved low gamma oryzanol recovery and, subsequently, low gamma oryzanol crystal growth.

The first-crystallization LP was further subjected to a second crystallization, given the crystallization temperature and time of 5°C for 15 h (Kaewboonnum *et al.*, 2010). The second-crystallization results showed no significant difference in the recovery and purity, given subzero first-crystallization temperatures (-15, -30, -45, and -60°C). On the other hand, at 0°C, the recovery and purity were comparatively low. The low recovery could be attributed to crystallization of the gamma oryzanol with impurities during the first crystallization. The mucilaginous impurities in the LP after the first crystallization also co-precipitated in the second crystallization under the 0°C condition, resulting in low purity. As a result, the first-crystallization temperature of -15°C was adopted and used in subsequent experiments.

First Crystallization Time

The first-crystallization time was varied between 5, 7.5, 10, 12.5, 15 h, given -15°C crystallization temperature. In Table 4, the gamma oryzanol recovery belonging to the 5 h crystallization time was significantly lower than that of the 7.5 h condition ($p < 0.05$). However, the differences were insignificant between the 7.5, 10.0, 12.5, and 15 h crystallization times ($p > 0.05$).

Additionally, after the second crystallization at 5°C for 15 h (Kaewboonnum *et al.*, 2010), the recovery and purity of the gamma oryzanol improved, given the first-crystallization time between 5 to 7.5 h.

Table 3. Effect of first-crystallization temperatures on gamma oryzanol recovery

Temperature (°C)	First crystallization ¹		Second crystallization ²			
	Gamma oryzanol recovery (%) ³		Gamma oryzanol recovery (%) ³		Purity (%) ³	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
0	44.96 ^b ±0.87	42.34 ^b ±1.98	35.21 ^b ±1.83	ND ⁴	89.90 ^{ab} ±1.89	84.61 ^{ab} ±1.11
-15	80.49 ^a ±1.29	75.02 ^a ±1.78	59.98 ^a ±1.08	57.82 ^a ±0.78	90.19 ^a ±1.59	87.69 ^a ±1.65
-30	80.57 ^a ±0.94	74.98 ^a ±2.12	60.17 ^a ±1.32	57.93 ^a ±1.25	90.15 ^a ±1.65	87.60 ^a ±1.69
-45	80.55 ^a ±1.12	75.11 ^a ±2.45	59.89 ^a ±1.05	57.85 ^a ±1.16	90.21 ^a ±1.54	87.59 ^a ±1.55
-60	80.59 ^a ±1.15	75.09 ^a ±2.38	59.89 ^a ±1.19	57.90 ^a ±1.42	90.18 ^a ±1.72	87.60 ^a ±1.69

¹ Experimental condition was ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1 (v/v); crystallization time = 15 h

² Experimental condition was crystallization temperature = 5°C; crystallization time = 15 h

³ The values are the mean of 3 replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p < 0.05$)

⁴ ND = not determined

However, the recovery and purity of the gamma oryzanol were insignificantly different for the first-crystallization time beyond 7.5 h. As a result, the first-crystallization time of 7.5 h was adopted as it could optimally remove impurities.

Effect of Second Crystallization Conditions on Gamma Oryzanol Recovery and Purity

Second Crystallization Temperature

The second crystallization isolates gamma oryzanol with a higher purity from the LP derived from the first crystallization. The second-crystallization temperatures were varied between 0, 5, 10, 15, and 20°C, given a 15 h crystallization time. In Table 5, the gamma oryzanol recovery at 0 and 5°C were insignificantly different ($p>0.05$). Higher temperatures (10-20°C) lowered the gamma oryzanol recovery as dissolution of the gamma oryzanol was accelerated, adversely affecting the recovery. In this research, the purity of the gamma oryzanol was insignificantly different between different second-crystallization temperatures ($p>0.05$), consistent with Kaewboonnum *et al.* (2010), who reported that 5°C was optimal for gamma oryzanol

crystallization. As a result, the second-crystallization temperature of 5°C was adopted.

Figures 1(a-e) illustrate the FESEM images and particle size histograms of the gamma oryzanol crystals under different second-crystallization temperatures (0, 5, 10, 15, and 20°C). Large particle sizes were present at low temperatures (0 and 5°C), with 75% of the particles larger than 150 μm . At higher crystallization temperatures (10, 15, and 20°C), 74% of the gamma oryzanol crystals were smaller than 150 μm . The results could be attributed to the fact that low crystallization temperatures induced crystal growth and aggregation of crystals, resulting in larger particle sizes.

Second Crystallization Time

The second-crystallization time was varied between 1, 6, 11, and 15 h, given 5°C crystallization temperature. In Table 6, the gamma oryzanol recovery was significantly positively correlated with the crystallization time ($p<0.05$). At 1 h crystallization time, the resulting gamma oryzanol crystal was of small particles (Figure 2(a)). By comparison, extension of the crystallization time from 1 to 6 h

Table 4. Effect of first-crystallization time on gamma oryzanol recovery

Time (h)	First crystallization ¹		Second crystallization ²			
	Gamma oryzanol recovery (%) ³		Gamma oryzanol recovery (%) ³		Purity (%) ³	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
5	44.84 ^a ±0.87	39.29 ^b ±1.05	45.21 ^b ±1.83	42.01 ^b ±1.05	89.91 ^{ab} ±2.09	87.51 ^{ab} ±1.11
7.5	80.06 ^a ±0.99	75.00 ^a ±1.31	59.98 ^a ±1.08	57.98 ^a ±1.68	90.09 ^a ±2.10	87.59 ^a ±1.65
10	80.05 ^a ±1.50	74.98 ^a ±1.10	60.10 ^a ±1.32	57.88 ^a ±1.55	90.12 ^a ±1.84	87.62 ^a ±1.69
12.5	80.05 ^a ±1.20	74.99 ^a ±0.99	59.95 ^a ±1.05	57.91 ^a ±1.41	90.11 ^a ±1.79	87.59 ^a ±1.55
15	80.03 ^a ±1.02	75.01 ^a ±1.01	59.93 ^a ±1.19	57.86 ^a ±1.49	90.13 ^a ±1.83	87.60 ^a ±1.69

¹ Experimental condition was ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1 (v/v); crystallization temperature = -15°C

² Experimental condition was crystallization temperature = 5°C; crystallization time = 15 h

³ The values are the mean of 3 replications $\pm$ standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$)

Table 5. Effect of second-crystallization temperatures on gamma oryzanol recovery

Temperature (°C)	Gamma oryzanol recovery (%) ¹		Purity (%) ¹	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC
0	59.91 ^a ±0.78	ND ²	90.21 ^a ±0.95	87.10 ^a ±0.95
5	59.98 ^a ±0.95	57.68 ^a ±1.14	90.24 ^a ±1.01	87.09 ^a ±1.01
10	58.10 ^b ±0.98	55.61 ^b ±0.89	90.19 ^a ±1.01	87.15 ^a ±0.95
15	55.94 ^c ±0.90	51.15 ^c ±0.93	90.23 ^a ±1.10	87.10 ^a ±1.01
20	51.00 ^d ±0.90	46.00 ^d ±1.06	90.21 ^a ±1.10	87.08 ^a ±1.05

¹ Second crystallization time was 15 h after the first crystallization (ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1 (v/v); crystallization temperature = -15°C, and crystallization time = 7.5 h). The values are the mean of 3 replications $\pm$ standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$)

² ND = not determined

increased the size of the gamma oryzanol crystals (Figures 2(a-b)). However, the crystal sizes (100-300 nm) under prolonged crystallization time from 6 to 15 h were insignificantly different and the crystal shape was similar (Figures 2(b-d)). These could be because the crystallization time of 6 h was sufficient to aggregate the gamma oryzanol crystals.

In addition, the crystalline structure and shape were similar to the gamma oryzanol standard (Figure 2(e)). Besides, variation in the crystallization time had no effect on the gamma oryzanol purity (Table 6). Thus, the crystallization time of 6 h was adopted as the second-crystallization duration.

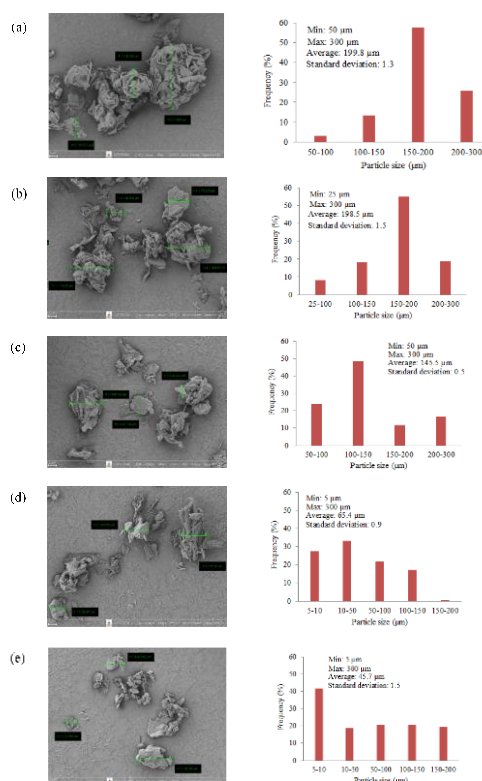


Figure 1. FESEM images (left) and particle size histogram (right) of gamma oryzanol isolated from ORF given the second-crystallization temperature of: (a) 0°C, (b) 5°C, (c) 10°C, (d) 15°C, (e) 20°C

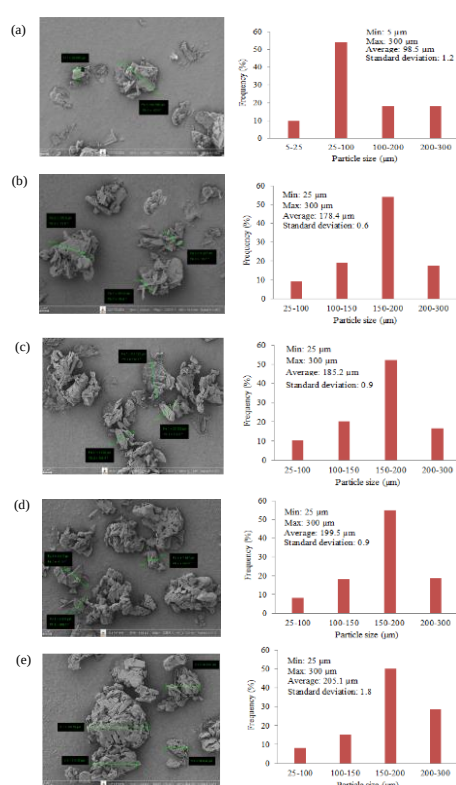


Figure 2. FESEM images (left) and particle size histogram (right) of isolated gamma oryzanol given the second-crystallization time of: (a) 1 h, (b) 6 h, (c) 10 h, (d) 15 h, (e) standard

Table 6. Effect of second-crystallization time on gamma oryzanol recovery

Time (h)	Gamma oryzanol recovery (%) ¹		Purity (%) ¹	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC
1	55.60 ^b ±1.18	51.16 ^b ±1.28	90.01 ^a ±2.55	87.09 ^a ±0.99
6	61.17 ^a ±1.05	58.03 ^a ±1.35	90.29 ^a ±2.12	87.10 ^a ±1.46
11	61.12 ^a ±2.08	57.99 ^a ±1.43	89.99 ^a ±1.31	87.11 ^a ±1.15
15	61.16 ^a ±1.99	58.05 ^a ±1.32	90.21 ^a ±2.10	87.09 ^a ±1.21

¹ Second crystallization temperature was 5°C after the first crystallization (ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1 (v/v); crystallization temperature = -15°C, and crystallization time = 7.5 h). The values are the mean of 3 replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments (p<0.05)

In short, the optimal ethanol concentration in the solvent mixture was 30% v/v. The optimal first-crystallization solvent to ORF ratio, crystallization temperature, and time were 7.5:1 (v/v), -15°C, and 7.5 h, respectively. The optimal second-crystallization temperature and time were 5°C and 6 h, with the gamma oryzanol recovery and purity of 58.03-61.17% and 87.10-90.29%, respectively.

The Physicochemical Properties of Gamma Oryzanol

The physicochemical properties of the gamma oryzanol crystal under the optimal two-stage crystallization condition were characterized using HPLC, liquid chromatography-mass spectrometry (LC-MS), and DSC and the results were compared against the gamma oryzanol standard.

Figure 3 compares the HPLC chromatograms of the gamma oryzanol standard and isolated gamma oryzanol (i.e., gamma oryzanol crystal) with 4 similar peaks: cycloartenyl ferulate (retention time (RT) = 6.235 and 6.243 min), 24-methylene cycloartanyl ferulate (RT = 7.050 and 7.065 min), campesteryl ferulate (RT = 7.763 and 7.765 min), and β -sitosteryl ferulate (RT = 9.006 and 9.089 min). Cycloartanyl ferulate and 24-methylene cycloartanyl ferulate were dominant in the gamma oryzanol crystal, consistent with Patel and Naik (2004), Kumar *et al.* (2015), Chen *et al.* (2012), Krishna *et al.* (2001), and Khuwijitjaru *et al.* (2009). Interestingly, in this study, 24-methylene cycloartanyl ferulate and β -sitosteryl ferulate in the standard and isolated gamma oryzanol were significantly different ($p < 0.05$) (Table 7). This could be

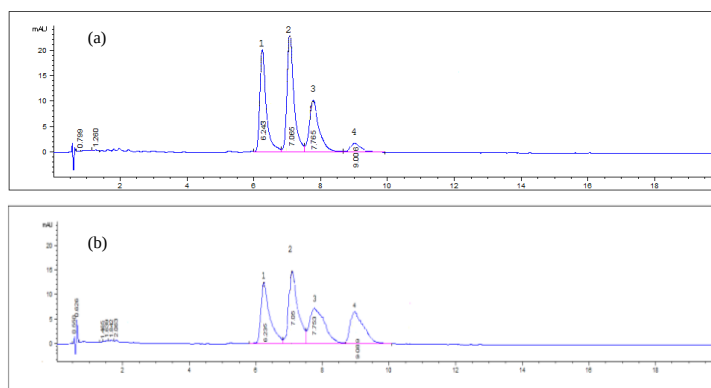


Figure 3. HPLC chromatograms showing retention times (min) of gamma oryzanol analogues of: (a) standard, (b) isolated gamma oryzanol, where (1) denotes cycloartenyl ferulate, (2) 24 methylene cycloartanyl ferulate, (3) campesteryl ferulate, and (4) β -sitosteryl ferulate

Table 7. Components and color characterization of isolated gamma oryzanol from crude ORF

	Gamma oryzanol standard ¹	Isolated gamma oryzanol ¹
Components (%)		
Cycloartenyl ferulate	26.12 $\pm$ 0.94	26.68 $\pm$ 0.43
24-Methylene cycloartanyl ferulate	35.27 $\pm$ 1.05	30.70 $\pm$ 0.76
Campesteryl ferulate	23.20 $\pm$ 0.55	23.21 $\pm$ 0.23
β -Sitosteryl ferulate	15.41 $\pm$ 1.04	19.41 $\pm$ 0.55
Color value		
L*	94.25 $\pm$ 0.16	83.33 $\pm$ 0.95
a*	-0.57 $\pm$ 0.01	-0.01 $\pm$ 0.00
b*	0.76 $\pm$ 0.05	4.69 $\pm$ 0.25

¹ The values are the mean of 3 replications $\pm$ standard deviation. Different letters in each row denote statistically significant differences between treatments ($p < 0.05$).

attributed to different solvent extraction methods and the starting material effect (Kumar *et al.*, 2009).

The 4 characteristic peaks of isolated gamma oryzanol were identified using LC-MS and compared with those of the gamma oryzanol standard and previous studies. In Figures 4(a-d), the isolated gamma oryzanol showed fragmentation patterns, including cycloartenyl ferulate (RT = 12.41 min, deprotonated molecular ion [M-H]⁻ at m/z 601.9 and 587), 24-methylcycloartenyl ferulate (RT = 13.75 min, [M-H]⁻ at m/z 615.9 and 601.0), campesteryl ferulate (RT = 15.11 min, [M-H]⁻ at m/z 575.9 and 561.0), and β -sitosteryl ferulate (RT = 17.51 min, [M-H]⁻ at m/z 589.9 and 575.0). The results were consistent with those of the gamma oryzanol standard and in cold-pressed rice bran oil (Kumar *et al.*, 2009; Das *et al.*, 1998) and soapstock (Sakunpak *et al.*, 2014). The melting points of the isolated gamma oryzanol and gamma oryzanol standard were between 125-130°C, which were slightly different from those of isolated oryzanol from rice bran oil soapstock of Rao *et al.* (2002) (120-125°C) and of Das *et al.* (1998) (132-135°C).

The HPLC, LC-MS, and DSC results of isolated gamma oryzanol confirmed that the

gamma oryzanol derived from crude ORF of this research was comparable to the gamma oryzanol standard and those of previous studies. However, the gamma oryzanol standard had a higher lightness (L*) (94.25±0.50) than the isolated gamma oryzanol (83.33±0.98) ($p < 0.05$). On the other hand, the redness (a*) and yellowness (b*) of the gamma oryzanol standard were lower than those of the isolated gamma oryzanol. The high redness (-0.01±0.00) and yellowness (4.69±0.25) of the isolated gamma oryzanol could be attributed to the impurities, e.g., triglyceride and mucilage.

Conclusions

This research investigated the effect of ethanol concentrations (0, 10, 20, 30, and 40% v/v) in an ethyl acetate/ethanol mixture and the crystallization conditions (solvent to ORF ratio, crystallization temperature, and crystallization time) on the recovery and purity of gamma oryzanol isolated from dried rice bran soapstock. A two-step crystallization was performed to isolate gamma oryzanol. The results showed that the optimal ethanol concentration in the solvent mixture was 30% v/v. Given 70:30 (v/v) ethyl acetate/ethanol

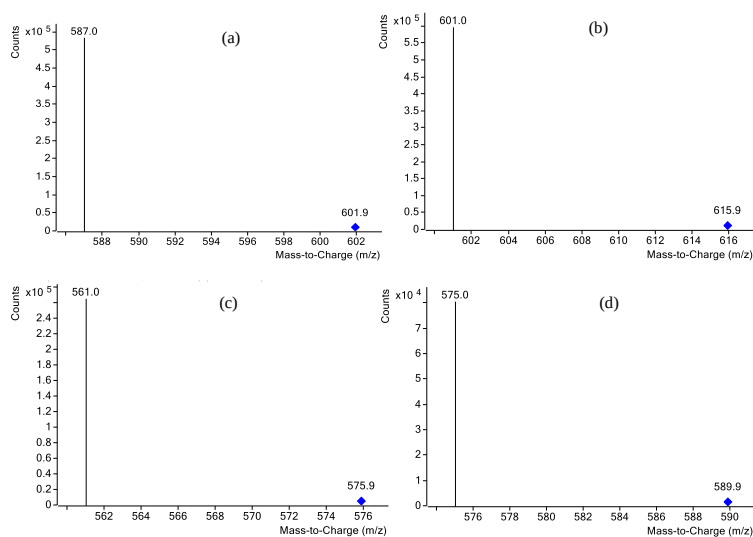


Figure 4. Mass spectra of isolated gamma oryzanol: (a) cycloartenyl ferulate, (b) 24-methylene cycloartenyl ferulate, (c) campesteryl ferulate, and (d) β -sitosteryl ferulate

mixture, the optimal first-crystallization solvent to ORF ratio, crystallization temperature, and time were 7.5:1 (v/v), -15°C, and 7.5 h, respectively. The liquid phase derived from the first crystallization was subsequently subjected to a second crystallization for the gamma oryzanol crystal. The optimal second-crystallization temperature and time were 5°C and 6 h, with gamma oryzanol recovery and purity of 58.03-61.17% and 87.10-90.29%, respectively. Furthermore, the physicochemical properties of the gamma oryzanol crystal were characterized and the results indicated good agreement between the isolated gamma oryzanol and the gamma oryzanol standard. Essentially, the findings revealed that an ethyl acetate/ethanol mixture, together with two-step crystallization, is operationally viable for gamma oryzanol extraction from rice bran soapstock.

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