

Food and Applied Bioscience Journal



ISSN : 2286-8615
VOLUME 11 ISSUE 3
(SEPTEMBER - DECEMBER 2023)

Food and Applied Bioscience Journal

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Utilization of ultrasonic-assisted extraction for antioxidants isolation from black rice bran and its impact on the characteristics of chitosan films

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Submit: 13 September 2023, Received: 26 September 2023, Revised: 15 December 2023,

Accepted: 22 December 2023, Publish online: 25 December 2023

Abstract

Black rice bran is rich in anthocyanins and phenolic compounds, both renowned for their potent antioxidant properties. The use of ultrasound technology in combination with solvent extraction has the potential to increase the efficiency of extracting antioxidants from black rice bran. This study was conducted to investigate the optimal conditions for the extraction of antioxidants from black rice bran using ultrasonic-assisted extraction. Four key factors were examined: the rice bran-to-solvent ratio (1:13-1:27 w/v), extraction time (16-44 min), power intensity (25-95% amplitude), and pulse duration (1-9 seconds). The rice bran-to-solvent ratio and extraction duration exhibited a significant influence on DPPH radical scavenging activity, whereas power intensity and pulse duration notably impacted the total phenolic content and DPPH radical scavenging activity. The optimal extraction conditions were determined to be a 1:15 ratio of rice bran to solvent, a 20 min extraction duration, 66% amplitude, and a 3-second pulse. Under these conditions, the extraction yielded total phenolic and anthocyanin contents of 12.41 mg/g and 3.88 mg/g, respectively, with a DPPH radical scavenging activity (IC₅₀) of 63.19 mg/g. Chitosan films infused with the black rice bran extract exhibited reduced tensile strength compared to the control. The incorporation of glycerol, known to enhance film flexibility, resulted in increased elongation at break. Interestingly, the addition of the extract did not significantly alter the elongation at break but led to a decrease in L^* and b^* values and an increase in a^* value of the chitosan films. The findings of this study demonstrate the potential for enhancing the extraction efficiency of antioxidative compounds from black rice bran through ultrasound technology,

leading to the production of chitosan films incorporating black rice bran anthocyanins potentially to be utilized as a pH-sensitive indicator film.

Keywords: Anthocyanin; Extraction; Film; Chitosan; Black rice bran; Ultrasound

1. Introduction

Black rice is rich in antioxidants, comprising gamma-oryzanol, tocopherols, tocotrienols, phenolics, and notably, anthocyanin. The primary pigment in black rice is anthocyanin, predominantly featuring cyanidin-3-glucoside, renowned for its potent antioxidative properties. In a study by Ichikawa *et al.* (2001), it was noted that extracts obtained from black rice demonstrated significantly higher antioxidant activity when compared to extracts from other rice varieties. Currently, antioxidants have garnered significant attention and interest across various domains due to their crucial role in counteracting the detrimental effects of free radicals, which are primary contributors to bodily deterioration. Furthermore, anthocyanins find extensive utility as a natural colorant in pH-sensitive indicator films, owing to their capacity to undergo color transitions in response to variations in pH levels (Kanha *et al.*, 2022).

Ultrasonic waves, characterized by a frequency exceeding the audible range for humans (greater than 16 kHz), are directional waves designed to precisely target specific areas. These waves facilitate enhanced solvent penetration into the raw materials earmarked for extraction, thereby augmenting mass transfer efficiency. Furthermore, ultrasound waves disrupt both the surface of the cell wall and intracellular structures. Many studies have confirmed the efficacy of Ultrasonic-Assisted Extraction (UAE) as a highly efficient method for isolating antioxidant compounds from diverse raw materials. Huang *et al.* (2009) investigated the extraction of flavonoids from *Folium eucommiae*, employing a comparative analysis of UAE, conventional heating, microwave-assisted extraction, and enzymatic extraction with ethanol as the solvent. Their findings revealed that UAE yielded a notably higher quantity of flavonoids compared to the other methods, demonstrating its superior efficiency. These results align with the research conducted by Khan *et al.* (2010), who examined the extraction of phenolic substances from orange peels using a solvent (ethanol-water mixture) and UAE. Their study demonstrated that UAE not only produced higher quantities of phenols but also exhibited enhanced antioxidant activity, as indicated by greater oxygen radical absorbance capacity (ORAC), in contrast to solvent extraction alone. Additionally, Chen *et al.* (2007) undertook a comparative analysis of anthocyanin extraction from red raspberries, employing conventional methods involving heating with a 1.5 M hydrochloric acid solution and 95% ethanol, and UAE utilizing ultrasound at 22 kHz. Their investigation revealed that UAE reduced the required solvent-to-time ratio for extraction and significantly enhanced the efficiency of anthocyanin extraction.

Chitosan, a linear biopolymer composed of glucosamine and N-acetyl glucosamine units linked via β -(1-4) glycosidic bonds, is derived from chitin found in various sources such as insects, shellfish, shrimp, crab, lobster, and fungi. This versatile compound has found extensive application as a film-forming agent in the production of indicator films, known for its exceptional properties. For the creation of pH-sensitive indicator films capable of color modulation in response to pH variations, film-forming agents can incorporate both synthetic and natural colorants. Nevertheless, natural pigments, exemplified by anthocyanins, hold a heightened appeal in the development of indicator films owing to their safety profile in relation to human health (Kanha *et al.*, 2022). The introduction of anthocyanins into a chitosan film is anticipated to exert an influence on the film's physical characteristics. Yoshida *et al.* (2014) demonstrated that a chitosan film incorporating anthocyanins exhibited increased thickness, a denser structural composition, and decreased elongation at break compared to a film lacking pigment. Additionally, Zhang *et al.* (2019) observed that the combination of chitosan with titanium dioxide (TiO₂) nanoparticles and anthocyanin extract from black plum peel enhanced tensile strength while concurrently diminishing elongation at break.

The objectives of this study were twofold: firstly, to establish the optimal parameters for ultrasonic-assisted extraction of antioxidants, particularly anthocyanins, from black rice bran employing Response Surface Methodology (RSM); and secondly, to investigate the impact of incorporating black rice bran extract into a chitosan-based film. These endeavors aim to yield crucial insights for potential future applications as a pH-sensitive indicator film.

2. Materials and Methods

2.1 Materials

The paddy rice of the Kam Doi Saket variety (black rice) was subjected to milling (NW250, Natrawee Technology, Thailand), after which the resulting bran was collected, ground, and sieved through a 300-mesh sieve. The processed bran was subsequently stored in aluminum foil at a temperature of -18°C. Chitosan, acquired from Union Science Company Limited (Thailand), exhibited an acetyl group removal percentage of 91.50%.

2.2 Ultrasonic-assisted extraction of black rice bran

2.2.1 Optimization of rice bran-to-solvent ratio and extraction time

The experimental design utilized for this investigation was the Central Composite Design (CCD). In this study, a solvent comprising 1% acetic acid in a 70% ethanol solution was employed. The variables under scrutiny included the rice bran-to-solvent ratio, ranging from 1:13 to 1:27 (W/V) (designated as X_1), and the extraction duration, spanning 16 to 44 min (designated as X_2) (as detailed in Table 1). The power intensity was set at a constant value of 55% amplitude, while the pulse duration remained fixed at 5 seconds, using a low frequency (20 kHz) ultrasonic generator (VCX500, Sonics & Materials, Inc., Newtown, CT, USA). Following extraction, the resultant mixture underwent centrifugation at 3000×g for 10 minutes (Magafuge 1.0R, Heraeus, Germany) and subsequent filtration using Whatman No. 1 filter paper. The filtered extract was then subjected to evaporation in a rotary evaporator (Buchi, Labortechnik AG, Switzerland) at 45°C until its volume was

reduced to 50 mL. The resulting extract was subsequently stored in a sealed amber bottle at a 4°C until the analytical analysis. The response surface models of the plot between the dependent (Y) and independent (X) variables were described by the following quadratic polynomial equation (Eq. 1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2 \quad (1)$$

2.2.2 Optimization of power intensity and pulse duration

In this section, a Central Composite Design (CCD) experimental approach was implemented to optimize two key factors: power intensity, varying from 25% to 95% amplitude (designated as X_3), and pulse duration, ranging from 1 to 9 seconds (designated as X_4) (as elaborated in Table 4). The optimal rice bran-to-solvent ratio and extraction time determined in the prior experiment were applied in this phase. Following the extraction process, the resultant extracts underwent the same procedures of centrifugation, filtration, evaporation, and storage as detailed in the previous experiment. The experimental results were plotted as a contour plot and described by the quadratic polynomial equation (Eq. 2):

$$Y = \beta_0 + \beta_1 X_3 + \beta_2 X_4 + \beta_3 X_3^2 + \beta_4 X_4^2 + \beta_5 X_3 X_4 \quad (2)$$

2.3 Analysis of antioxidant contents and activity

2.3.1 Total phenolics content

The total phenolic content (TPC) was quantified following the procedure outlined by Miliuskas *et al.* (2004), with some adaptations. A mixture was prepared by combining 50 μ L of the sample with 3 mL of distilled water, 250 μ L of Folin-Ciocalteu's reagents, and 750 μ L of 7.5% NaCO_3 , and this mixture was incubated for 10 minutes at room temperature. Subsequently, 950 μ L of distilled water was added, followed by thorough mixing. The resulting mixture was then allowed to incubate in a dark environment at room temperature for 2 h before its absorbance was measured at 765 nm using a UV-visible spectrophotometer (Genesys 10, Thermo Scientific, NY, USA) against a blank. The TPC was expressed as mg gallic acid equivalent (GAE) per gram of dry weight (DW), determined through calibration with a standard linear plot of gallic acid, which exhibited a linearity range of 50 to 1,000 μ g/mL ($R^2 > 0.99$).

2.3.2 Total anthocyanin content

The determination of total anthocyanin content (TAC) followed the pH differential method outlined by Giusti and Wrolstad (2001). The sample was appropriately diluted using two buffer solutions: potassium chloride solution (pH 1.0) and sodium acetate solution (pH 4.5). The absorbance of these mixtures was measured at 510 nm (A_{510}) and 700 nm (A_{700}) using a UV-visible spectrophotometer (Genesys 10, Thermo Scientific, NY, USA) for the calculation of the absorbance difference (A_{diff} ; Eq. 3). The TAC was quantified in terms of mg cyanidin-3-O-glucoside equivalent (C3G) per liter (L) using the following equation (Eq. 4) and reported as mg C3G per gram (DW).

$$A_{\text{diff}} = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}} \quad (3)$$

$$\text{Total anthocyanin content (mgC3G/L)} = \frac{A_{\text{diff}} \times \text{MW} \times \text{DF} \times 1000}{\epsilon \times l} \quad (4)$$

Where MW = molecular weight of C3G (449.39 g/mol)
 DF = dilution factor
 E = molar absorptivity (26,900 L/mol·cm)
 L = path length (1.0 cm)

2.3.3 DPPH free radical scavenging activity

The DPPH free radical scavenging activity was assessed following the methodology outlined by Brand-Williams *et al.* (1995). Briefly, 1.0 mL of the sample at five different dilutions was combined with 3.0 mL of 0.1 mM DPPH solution dissolved in 99% ethanol. This mixture was vigorously vortexed for 30 seconds and then allowed to incubate in the dark for 30 min at room temperature. Subsequently, the absorbance of the sample (A_{sample}) and the control (A_{control} ; distilled water) at 517 nm was measured against a blank using a UV-visible spectrophotometer (Genesys 10, Thermo Scientific, NY, USA). The radical scavenging ability was determined using the following equation (Eq. 5):

$$\% \text{ Radical scavenging} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100 \quad (5)$$

The scavenging activity of black rice bran extracts was expressed as the half maximal inhibitory concentration, IC_{50} (mg/g), which was determined through interpolation from linear regression analysis. A lower IC_{50} value signifies a higher degree of antiradical activity.

2.4 Preparation of chitosan film incorporated with black rice bran extract

Chitosan (2.0 g) was solubilized in a 100 mL solution containing 1% acetic acid, and the dissolution process was allowed to proceed for a duration of 3-4 h with continuous stirring until complete dissolution was achieved. Subsequently, black rice bran extract was introduced into the solution at varying concentrations of 1%, 2.5%, and 5% w/w, while 25% w/w of glycerol was also incorporated. Following thorough mixing, the resultant blends were cast into film forms by pouring them into trays and subjected to drying in a hot air dryer (UN110, Memmert GmbH + Co. KG, Bavaria, Germany) at a temperature of 45°C. These film specimens were subsequently stored within a desiccator until they underwent testing to evaluate their material properties.

2.5 Analysis of film properties

2.5.1 Mechanical properties

The tensile strength (MPa) and elongation at break (%) of the film were assessed following the American Society for Testing and Materials (ASTM) method ASTM D882-10. A universal testing machine was employed for the evaluation, with the film being trimmed into a rectangular shape measuring 2.5 cm × 10.0 cm. Two sets of grips were utilized, one fixed in position while the other was mobilized at a pulling rate of 50 mm/min.

2.5.2 Film thickness

Film thickness was determined utilizing a needle or dial-type thickness gauge (Gotech Testing Machines, Inc) with a precision of 0.01 mm. The thickness measurements were conducted at five random locations on each film.

2.5.3 Film color

Film color was quantified using a colorimeter (Hunter Lab, ColorQuest XE) within the CIELAB system, where L^* values spanned from 0 (representing darkness) to 100 (representing lightness), $-a^*$ value indicates greenness, and $+a^*$ value indicates redness, while $-b^*$ value represents blueness and $+b^*$ value represents yellowness.

2.6 Statistical analysis

Design-Expert software version 13.0.5.0 (Stat-Ease Inc., Minneapolis, MN, USA) was employed for designing the CCD experiments, calculating the statistically relevant parameters, and generating three-dimensional graphs. All other analyses were conducted in triplicate. The analysis of variance (one-way ANOVA) and determination of significant differences among means using Duncan's multiple range test (DMRT) were performed utilizing the Statistical Package for the Social Sciences (Version 24, SPSS Inc., Chicago, IL, USA) with a significance level set at $P \leq 0.05$.

3. Results and Discussion

3.1 Effect of rice bran-to-solvent ratio and extraction time

The impact of variations in the rice bran-to-solvent ratio and extraction time on the total phenolics content (ranging from 12.64 to 17.12 mg GAE/g), total anthocyanin content (varying between 4.76 and 5.78 mg C3G/g), and DPPH free radical scavenging activity (expressed as IC_{50} values, ranging from 40.04 to 167.81 mg/g) of the black rice bran extracts obtained through the CCD for the UAE method is summarized in Table 1. The response value for DPPH free radical scavenging activity exhibited a significant fit to a linear model with an R^2 value of 0.68 ($P \leq 0.05$), while no significant model was observed for the other responses ($P > 0.05$) (Table 2). This significant model suggests that it effectively elucidates the relationship between independent and dependent variables.

Table 1 Actual level of independent variables (X_1 and X_2) along with the observed value for response variables

Treatment	Factors		Response		
	X_1	X_2	TPC (mgGAE/g)	TAC (mgC3G/g)	DPPH (IC ₅₀) (mg/g)
1	20	30	16.31 ± 2.57	5.30 ± 2.55	89.29 ± 0.86
2	15	40	14.88 ± 3.97	4.99 ± 2.68	43.76 ± 0.74
3	20	30	16.66 ± 1.73	5.42 ± 1.26	55.44 ± 0.86
4	27	30	17.12 ± 1.67	5.54 ± 2.09	118.59 ± 1.13
5	15	20	15.79 ± 2.32	5.03 ± 1.09	54.45 ± 1.02
6	20	30	15.66 ± 2.99	5.67 ± 1.84	96.09 ± 0.56
7	20	30	16.66 ± 3.81	5.55 ± 2.20	65.19 ± 0.67
8	13	30	13.05 ± 3.22	5.78 ± 3.55	40.04 ± 0.55
9	20	30	14.54 ± 2.71	5.21 ± 2.92	56.12 ± 0.79
10	20	44	12.64 ± 3.16	5.06 ± 3.87	111.43 ± 1.21
11	25	20	13.14 ± 1.36	4.76 ± 2.37	123.06 ± 0.36
12	25	40	15.61 ± 2.80	5.59 ± 1.66	167.81 ± 1.39
13	20	16	15.94 ± 1.39	5.56 ± 3.38	102.79 ± 0.73

Note: X_1 : rice bran-to-solvent ratio, X_2 : extraction time (min), Mean±SD from triplicate determinations ($n=3$)

Table 2 Equation models and R^2 of each response

No.	Responses	Equation models	R^2
1	Total phenolics content	<i>n.s.</i>	-
2	Total anthocyanin content	<i>n.s.</i>	-
3	DPPH radical scavenging activity (IC ₅₀)	$Y = -82.82 + 7.59X_1 + 0.58X_2$	0.68

Note: Y = responses, X_1 : rice bran-to-solvent ratio, X_2 : extraction time (min), *n.s.* = not significant

Fig 1 illustrates the influence of the rice bran-to-solvent ratio and extraction time on the DPPH radical scavenging activity of the black rice bran extract. The figure clearly demonstrates that an increase in the rice bran-to-solvent ratio led to an increase in the IC₅₀ value, indicating a reduction in the DPPH radical scavenging activity of the extract. This decline may be attributed to a dilution effect. Conversely, a longer extraction period resulted in a decrease in DPPH radical scavenging activity, which could be attributed to factors such as the generation of heat during ultrasonication (Tiwari *et al.*, 2010) and the occurrence of undesirable reactions such as enzymatic degradation and oxidation (Vilkhu *et al.*, 2008).

The optimization of extraction conditions aimed to identify the ideal values for the independent variables, namely the rice bran-to-solvent ratio and extraction time, that would yield an extract with the highest DPPH radical scavenging activity. The optimized condition was achieved with a rice bran-to-solvent ratio of 1: 15 and an extraction time of 20 min, resulting in a predicted DPPH radical scavenging activity (IC₅₀) of 42.69 mg/g (Table 3). To verify the accuracy and reliability of this optimal condition, an experimental validation was conducted, yielding an IC₅₀ value of 56.06 mg/g, along with total phenolic content (TPC) and total anthocyanin content

(TAC) values of 13.13 mgGAE/g and 5.27 mgC3G/g, respectively (Table 3). These optimal conditions were subsequently employed in the subsequent experiment.

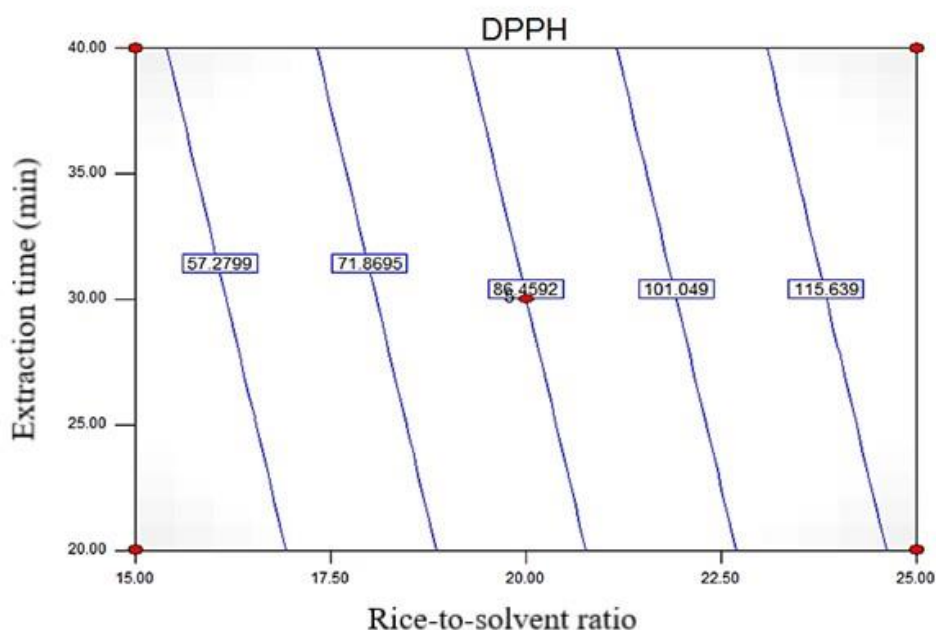


Fig 1 Contour plot of rice bran-to-solvent ratio and extraction time (min) on DPPH radical scavenging activity (IC_{50})

Table 3 The predicted and actual values of extraction of black rice bran antioxidant at optimum rice bran-to-solvent ratio and extraction time

	TPC (mgGAE/g)	TAC (mgC3G/g)	DPPH (IC_{50}) (mg/g)
Predicted	-	-	42.69
Experimental	13.31	5.27	56.06
% Error	-	-	31.32

Note: Rice bran-to-solvent ratio of 1: 15 and extraction time of 20 minutes

3.2 Effects of power intensity and pulse duration

Table 4 illustrates the impact of power intensity and pulse duration on various attributes of black rice bran extracts obtained through the CCD method for UAE. These attributes include total phenolics content (ranging from 10.07 to 14.03 mg GAE/g), total anthocyanin content (varying between 3.94 and 6.88 mg C3G/g), and DPPH free radical scavenging activity (expressed as IC_{50} values, ranging from 46.43 to 74.59 mg/g). The response values for total phenolics content and DPPH free radical scavenging activity exhibit a significant fit to a quadratic model with R^2 values of 0.88 and 0.77, respectively ($P \leq 0.05$). Conversely, no significant model was observed for total anthocyanin content ($P > 0.05$) as presented in Table 5. This significance in the model suggests its effectiveness in elucidating the relationship between independent and dependent variables.

Table 4 Actual level of independent variables (X_3 and X_4) along with the observed value for response variables

Treatment	Factors		Response		
	X_3	X_4	TPC (mgGAE/g)	TAC (mgC3G/g)	DPPH (IC ₅₀) (mg/g)
1	60	5	13.13 ± 1.16	6.88 ± 1.81	50.74 ± 0.31
2	35	8	11.93 ± 3.14	4.44 ± 3.54	65.89 ± 0.54
3	60	5	13.03 ± 2.51	4.78 ± 1.18	50.96 ± 0.91
4	85	2	13.48 ± 2.59	4.18 ± 3.08	60.56 ± 1.34
5	60	5	13.78 ± 2.36	4.70 ± 1.48	55.58 ± 0.38
6	35	2	12.13 ± 3.14	5.03 ± 2.36	61.50 ± 0.57
7	25	5	10.07 ± 2.01	3.94 ± 2.99	67.91 ± 0.35
8	60	5	14.03 ± 1.08	5.73 ± 1.18	51.45 ± 0.65
9	60	5	13.11 ± 1.80	4.88 ± 3.63	46.43 ± 0.46
10	95	5	12.33 ± 1.28	4.40 ± 1.82	59.59 ± 0.93
11	85	8	12.53 ± 0.90	4.57 ± 1.83	58.61 ± 0.76
12	60	1	13.07 ± 2.51	4.49 ± 2.91	48.98 ± 1.23
13	60	9	13.33 ± 1.54	5.23 ± 1.10	74.59 ± 0.30

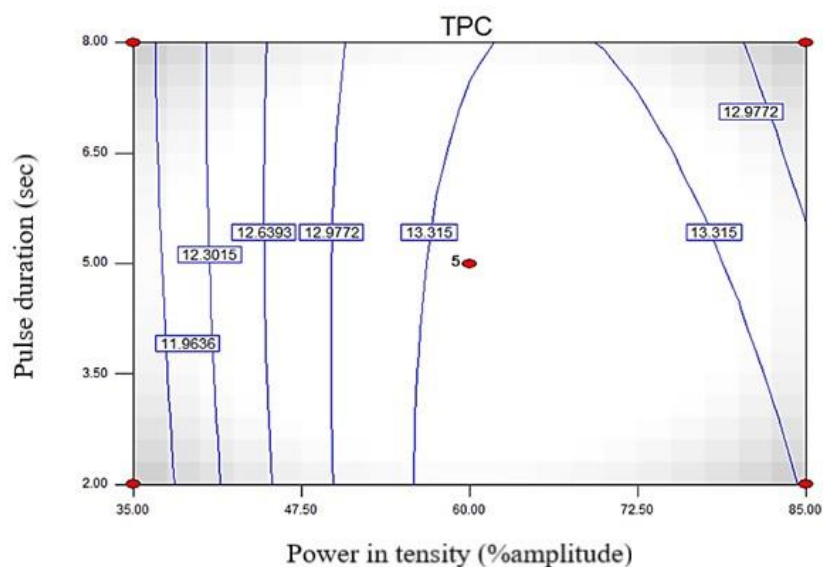
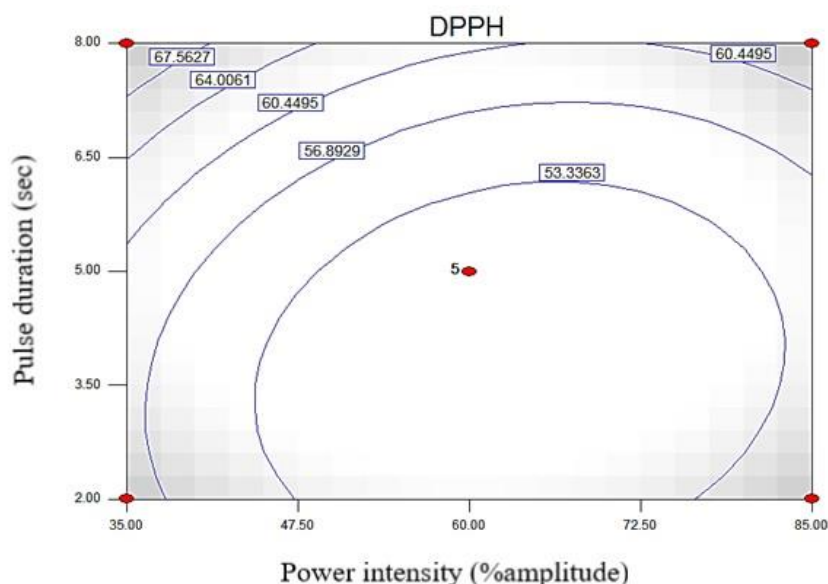
Note: X_3 : power intensity (% amplitude), X_4 : pulse duration (sec), Mean±SD from triplicate determinations ($n=3$)

The equation presented in Table 5 emphasizes the differential impact of power intensity and pulse duration on total phenolics content, as indicated by the notably higher coefficient for X_3 in comparison to X_4 . Visualized in Fig 2, the contour plot illustrates the relationship between power intensity and pulse duration concerning total phenolics content, revealing that an elevation in both power intensity and pulse duration corresponds to an increase in total phenolics content. Furthermore, it is worth noting that vegetal cell walls exhibit heightened vulnerability to damage when subjected to ultrasound at elevated amplitude levels, ultimately resulting in the amplified release of phenolic compounds and anthocyanins into the solvent (Muangrat *et al.*, 2018; Hossain *et al.*, 2012). Additionally, the considerable shear forces generated during the UAE process facilitate the infiltration of the solvent into vegetal cells, thereby expediting the hydration process. This augmented solvent penetration subsequently leads to cell swelling and concurrent fragmentation of the vegetal tissue matrix, effectively accelerating these processes (Muangrat *et al.*, 2018; Toma *et al.*, 2001). However, it is crucial to acknowledge that this enhancement reaches a plateau when power intensity and pulse duration exceed excessively high levels. This phenomenon may be attributed to the heat generated during UAE, which can result in the thermal degradation of phenolic compounds in black rice bran. This finding aligns with the research conducted by Fabian *et al.* (2010), who reported thermal degradation of phenolic compounds in rice bran during high-temperature extraction. Numerous contemporary ultrasonic generators are equipped with a pulse mode to mitigate sample heating during sonication. Nevertheless, this study has observed that an extension of the pulse duration resulted in a reduction of DPPH radical scavenging activity (Fig 3). This phenomenon may be attributed to the prolonged duration of heat exposure to the sample.

Table 5 Equatin models and R^2 of each response

No.	Responses	Equation models	R^2
1	Total phenolics content	$Y=5.28 + 0.24X_3 + 0.15X_4 - 1.65(10^{-3})X_3^2 - 3.18(10^{-3})X_4^2 - 2.50(10^{-3})X_3X_4$	0.88
2	Total anthocyanin content	<i>n.s.</i>	-
3	DPPH radical scavenging activity (IC_{50})	$Y=91.79 - 1.16X_3 - 2.78X_4 + 9.72(10^{-3})X_3^2 + 0.57X_4^2 - 0.02X_3X_4$	0.77

Note: Y = responses, X_3 : power intensity (% amplitude), X_4 : pulse duration (sec), *n.s.* = not significant

**Fig 2** Contour plot of power intensity and pulse duration on total phenolics content (mgGAE/g)**Fig 3** Contour plot of power intensity and pulse duration on DPPH radical scavenging activity (IC_{50} ; mg/g)

The optimization of UAE conditions for black rice bran resulted in the attainment of the highest levels of total phenolics content and DPPH radical scavenging activity, which were achieved at an amplitude of power intensity of 66% and a pulse duration of 3 seconds. These established conditions were employed to predict the maximum total phenolics content and DPPH radical scavenging activity (minimum IC₅₀), yielding values of 13.58 mgGAE/g and 49.87 mg/g, respectively. Subsequent experimental validation under fixed conditions of a rice bran -to-solvent ratio of 1:15 and an extraction time of 20 min confirmed these predictions, with observed values of 12.41 mgC3G/g for total phenolics content and 63.19 mg/g for DPPH radical scavenging activity (Table 6).

Table 6 The predicted and actual values of extraction of black rice bran antioxidant at optimum conditions

	TPC (mgGAE/g)	TAC (mgC3G/g)	DPPH (IC ₅₀) (mg/g)
Predicted	13.58	-	49.87
Experimental	12.41	3.88	63.19
% Error	8.62	-	26.71

Note: optimal conditions were rice bran-to-solvent ratio of 1: 15, an extraction time of 20 minutes, power intensity of 66% amplitude, and pulse duration of 3 seconds.

3.3 Effect of incorporation of black rice extract on chitosan film properties

3.3.1 Mechanical properties of film samples

The impact of incorporating black rice bran extract and glycerol on the mechanical properties of chitosan films is summarized in Table 7. This table reveals that the addition of glycerol exerted a profound influence on the mechanical characteristics of the chitosan films. Specifically, samples containing glycerol displayed lower tensile strength and significantly higher elongation at the point of rupture compared to samples without glycerol ($P \leq 0.05$). Glycerol, being a plasticizer, contributes significantly to enhancing the flexibility of chitosan films by reducing intermolecular forces within the polymer. This reduction promotes greater mobility of polymer chains, rendering the films more pliable and lowering the glass transition temperature, ultimately resulting in reduced tensile stress and increased elongation at break (Laohakunjit and Noomhorm, 2004). Notably, the incorporation of glycerol did not yield a significant impact on film thickness ($P > 0.05$).

In contrast, the introduction of black rice bran extract led to a slight reduction in the tensile strength of the film, with a more pronounced effect observed at a 5% extract concentration. Tensile strength serves as a representation of the film's maximum strength. It is noteworthy that chitosan composition, film microstructure, and intermolecular forces all play pivotal roles in determining the mechanical properties of chitosan films. Moreover, the crystallinity of the film is a key factor influencing its mechanical behavior. The decrease in tensile strength could be attributed to a reduction in the crystalline structure within the chitosan matrix. Existing literature suggests that the incorporation of polyphenols into chitosan films can impede the formation of crystalline structures within the chitosan matrix. This, in turn, weakens intermolecular hydrogen bonds and hinders interactions between polymer chains, resulting in diminished mechanical properties (Ahmed *et al.*,

2016; Wan *et al.*, 2003). However, based on our findings, the incorporation of black rice bran extract did not exert any significant influence on elongation at break or film thickness ($P>0.05$).

Table 7 Mechanical properties of chitosan films incorporated with black rice bran extract

Film sample	TS (MPa)	EB (%)	Thickness ^{n.s.} (mm.)
CH (control 1)	121.81 ± 6.77 ^a	5.24 ± 0.76 ^b	0.13 ± 0.01
CH-1.0% BRE	116.96 ± 7.97 ^a	3.83 ± 0.95 ^b	0.16 ± 0.04
CH-2.5 BRE	111.90 ± 10.83 ^{ab}	4.10 ± 0.16 ^b	0.16 ± 0.06
CH-5.0% BRE	105.20 ± 12.88 ^b	4.16 ± 0.41 ^b	0.16 ± 0.06
CH-GLY (control 2)	43.80 ± 5.19 ^c	48.12 ± 3.76 ^a	0.12 ± 0.01
CH-GLY-1.0% BRE	44.2 ± 4.23 ^c	49.74 ± 2.95 ^a	0.10 ± 0.01
CH-GLY-2.5% BRE	42.5 ± 6.26 ^c	48.20 ± 3.84 ^a	0.11 ± 0.03
CH-GLY-5.0% BRE	31.8 ± 5.10 ^d	49.96 ± 7.70 ^a	0.12 ± 0.01

Note: Determinations and the different letter indicate significant different at $P\leq 0.05$, *n.s.* = not significant, Mean ± SD from triplicate determinations ($n=3$), TS= tensile strength, EB=elongation at break, CH=chitosan, GLY=glycerol, BRE=black rice bran extract

3.3.2 Film color

The visual appearance and color characteristics of the chitosan film samples are presented in Fig 4. Notably, the chitosan films without the incorporation of black rice bran extract (designated as control 1 and control 2) exhibited a vibrant yellow hue. The addition of glycerol had negligible impact on the color of the majority of film samples, with similar color parameters observed in both control films. Specifically, the L^* , a^* , and b^* values exhibited no statistically significant differences ($P>0.05$; Table 8), except for CH-GLY-5.0% BRE, which showed a slightly higher b^* value compared to CH-5.0% BRE. This observation aligns with the findings of Dick *et al.* (2015), where the introduction of glycerol induced a color shift in edible film production from chia seed mucilage, resulting in a slight increase in the b^* value.

Conversely, the incorporation of black rice bran extract markedly impacted the color of the chitosan film. Visually, it is evident that the CH-1.0% BRE and CH-GLY-1.0% BRE samples displayed analogous black hues. However, when black rice bran extract was introduced at higher concentrations, the resultant films exhibited a more pronounced magenta tint, a characteristic that was corroborated by the measured color values (Fig 4). Notably, the addition of black rice bran extract led to a reduction in L^* and b^* values, while concurrently elevating the a^* value, in direct proportion to the concentration of the extract (Table 8). These shifts in color parameters signify that the chitosan film adopted a darker appearance, with reduced yellowness and increased redness, upon the addition of black rice bran extract.

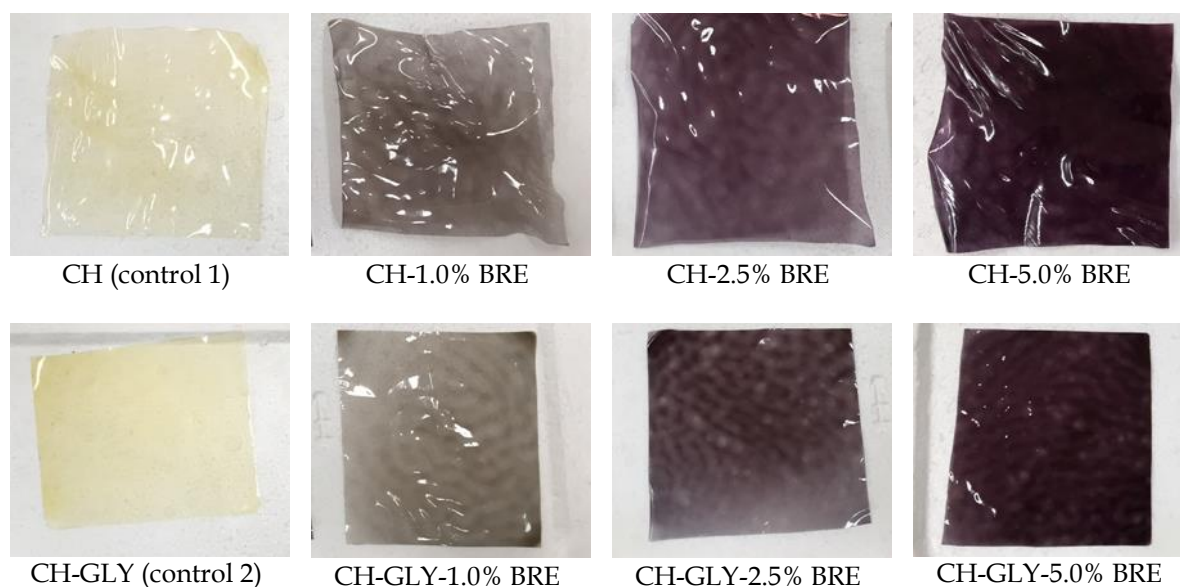


Fig 4 Chitosan film incorporated with black rice bran extract (CH=chitosan, GLY=glycerol, BRE=black rice bran extract)

Table 8 Color parameters of chitosan films incorporated with black rice bran extract

Film sample	L^*	a^*	b^*
CH (control 1)	94.63 ± 0.40^a	-1.33 ± 0.16^d	9.14 ± 1.68^a
CH-1.0% BRE	78.56 ± 1.75^b	2.10 ± 0.20^c	3.58 ± 0.30^b
CH-2.5% BRE	63.67 ± 1.22^c	6.67 ± 1.39^b	-2.10 ± 0.35^c
CH-5.0% BRE	42.77 ± 1.93^d	15.58 ± 0.48^a	-5.76 ± 0.24^d
CH-GLY (control 2)	95.19 ± 0.25^a	-1.40 ± 0.14^d	8.45 ± 0.10^a
CH-GLY-1.0% BRE	81.83 ± 1.93^b	0.52 ± 0.07^c	5.04 ± 0.34^b
CH-GLY-2.5% BRE	61.61 ± 1.29^c	5.34 ± 0.87^b	-1.19 ± 0.32^c
CH-GLY-5.0% BRE	44.78 ± 4.30^d	13.83 ± 1.74^a	-2.37 ± 0.53^c

Note: Determinations and the different letter indicate significant different at $P \leq 0.05$, *n.s.* = not significant, Mean $\pm$ SD from triplicate determinations ($n=3$), CH=chitosan, GLY=glycerol, BRE=black rice bran extract

The alteration in the color of the chitosan film induced by the incorporation of black rice bran extract can be attributed to the presence of anthocyanin, a red-purple pigment naturally occurring in black rice bran. Notably, anthocyanin pigments are known to be pH-sensitive and capable of undergoing color changes in response to fluctuations in pH levels. Therefore, the insights gleaned from this study hold potential for the future development of pH-sensitive indicator films.

4. Conclusion

This study highlights the efficacy of Response Surface Methodology (RSM) as a valuable tool for determining the optimal conditions for extracting antioxidants from black rice bran using the ultrasonic-assisted extraction method. The key process parameters essential for extracting antioxidants from black rice bran were successfully optimized. The optimal extraction conditions were determined to be a 1:15% ratio of rice bran to solvent, an extraction duration of 20 min, an amplitude of 66, and a pulse duration of 3 seconds. When black rice bran extract was incorporated into chitosan films at a higher concentration (5%), it led to a reduction in the tensile strength of the film. However, this higher concentration imparted a more pronounced and expressive color to the film. Consequently, the insights gained from this study serve as a valuable guideline for the extraction of antioxidants from black rice bran and the potential development of pH-sensitive indicator films in the future.

Acknowledgement

This research work was supported by the Faculty of Agro-Industry, Chiang Mai University.

Conflict of interest statement

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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