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Optimization of Rice Bran Protein Extraction: Green Approaches using Deep Eutectic Solvents and Microwave-Assisted Extraction Techniques

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Abstract:

A novel approach for green protein extraction was developed by combining deep eutectic solvents (DES) and microwave-assisted extraction (MAE) to optimize the extraction of protein from defatted rice bran (DRB). Rice bran, an agricultural byproduct, is rich in proteins and bioactive compounds; however, it remains largely underutilized due to the inefficiency of current extraction methodologies. Rice bran protein (RBP) is widely known for its hypoallergenic properties and nutritional benefits. The present study examined various DES types (choline chloride-based mixtures with different hydrogen bond donors: glycerol (neutral), oxalic acid (acid), and urea (base)) to extract protein from rice bran protein with microwave heating. The extraction efficiency was evaluated using different microwave power, extraction time, and sample-to-DES ratios. Key factors controlling the extraction and optimal operating conditions were optimized by response surface methodology to obtain the highest protein extracted from rice bran which was indicated by protein recovery. The extraction parameters that were determined to be optimal were as follows: a choline chloride-urea DES, a solvent-to-sample ratio of 5.79, microwave power of 296.97 W, and 44.75 s of extraction time. The optimization of protein extraction was characterized using a Box-Behnken Design of Experiment, with protein recovery as the response. The quality of the protein was then analyzed using SDS PAGE for molecular weight distribution. The selected DES-MAE method achieved a maximum protein recovery of 39.96 ± 0.5%. This combined DES-MAE technique offers a promising, environmentally friendly alternative for efficient RBP extraction.

Keywords: Deep Eutectic Solvent, Microwave-Assisted Extraction, Protein Extraction, Response surface methodology, Rice Bran Protein

INTRODUCTION

Rice bran is a byproduct of rice milling which represents a significant yet underutilized source of nutritional and functional proteins with considerable potential in the food, pharmaceutical, and biotechnology industries [1, 2]. Rice bran protein (RBP) has proven to be a potential ingredient to produce nutritious plant-based foods due to its nutraceutical and hypoallergenic properties [3]. RBP has gained significant interest due to its potential health benefits and essential amino acids [5]. Rice bran comprises 15-22% lipids, 34.1-52.3% carbohydrates, 7-11.4% fiber, 6.6-9.9% ash, 8-12% moisture, and 10-16% highly nutritional protein [1]. Research for methods of RBP extraction has been conducted before with traditional techniques such as alkali extraction yielding only 21-48% protein recovery [4], enzymatic alcalaze (endoprotease) [6] and even advanced solvent combinations like water-NaCl solution- ethanol-NaOH or AUC (acetic acid, urea, and cetyltrimethylammonium bromide) mixtures struggling to consistently achieve comprehensive protein isolation at economically viable scales due to the multiple step extraction [7].

The emergence of deep eutectic solvents (DES) has opened new horizons in green extraction technologies, offering a promising alternative to conventional protein isolation methods. Recent studies have highlighted the significant impact of DES composition on extraction efficiency. Bowen et al (2022) comprehensively reviewed the application of DES in protein extraction and purification, highlighting their potential for innovative bioprocessing [8]. Research by Sombutsuwan et al. (2024) demonstrated the critical role of acidity and alkalinity in biomolecule extraction and found that alkaline DES (K_2CO_3-glycerol; pH 11.21) showed the highest levels of protein content (12.81 mg/g) [9]. Notably, Ramlee et al (2024) explored optimum conditions for maximizing protein extraction using choline chloride-glycerol DES and obtained conditions at 80°C, 3 h, and 1:5 rice bran to DES ratio yielded the highest protein recovery at 24,42% [10]. Back extraction is recognized to be crucial for protein separation from the DES phase because of the strong interfacial mass transfer resistance. The attempt to adjust the pH and ion strength to an isoelectric point to induce precipitate of the protein [11]. The addition of alcohol and changing salt concentration were reported as a method for back extraction [12]. Research by Liu et al. found that the lower salt concentration was advantageous to the protein precipitation. The isoelectric point and four times volume ethanol precipitation (FTVEP) method showed the highest protein precipitation efficiency (97.97%) and lowest precipitation time of 4 min [13].

Conventional water bath heating was used to ensure an even temperature transfer and found around 80 ºC for 3 h extracted the highest protein recovery [10]. The long-time extraction could induce protein denaturation. Phongthai et al. overcame this long extraction time by using microwave-assisted extraction (MAE) of 90 sec. and obtained 1.54-fold protein recovery compared to conventional alkaline extraction [14]. The convergence of DES and MAE techniques presents an innovative approach to protein extraction that addresses several critical limitations of traditional methods. By leveraging the unique properties of DES and the rapid, energy-efficient nature of microwave irradiation, this research can potentially develop a more sustainable and efficient protein extraction strategy for rice bran.

This study investigates the optimization of protein extraction from defatted rice bran (DRB) using DES combined with MAE. The study focuses on optimizing key extraction parameters, including DES type (using different hydrogen bond donors), extraction time, microwave power, and DRB-to-DES ratio, to maximize RBP recovery while maintaining RBP quality. The effectiveness of this novel DES-MAE approach is evaluated by assessing RBP yield and characterizing the extracted RBP.

MATERIALS AND METHODS

1. Materials

Rice bran (RB) was obtained from a local farmer's mill in Sidoarjo, Indonesia. Choline chloride (ChCl) was obtained from HiMedia, India. Analytical grade of ethanol absolute (≥ 99.9 %), sodium hydroxide (NaOH), hydrochloric acid (HCl), glycerol anhydrous, oxalic acid, and urea were all obtained from Merck. Deionized water was obtained from Sumber Ilmiah Persada, Indonesia.

2. Rice Bran Preparation

Pretreatment was performed according to Kim et al. (2014), where the rice bran was sieved with a 60-mesh screen to separate contaminants [15]. The rice bran fractions that successfully passed each sieving were collected and combined. They were then used in Soxhlet extraction experiments to separate crude rice bran oil (CRBO) from rice bran, and a by-product, defatted rice bran (DRB), was obtained. The preparation of DRB was performed according to Ribas et al. (2023), 25 g of rice bran and extracted with 250 mL of n-hexane solvent using the Soxhlet method for 3 h [16]. After extraction, the rice bran was then oven-dried at 50 ºC for 24 h to remove the remaining n-hexane in the sample. The DRB obtained was then collected and combined to be used in the RBP extraction process. The Soxhlet solution consisting of n-hexane and CRBO was separated using a rotary vacuum evaporator to recover the n-hexane.

3. Experimental design and optimization

For optimizing the RBP extraction, a four-variable and three-level Box-Behnken design (BBD, a response surface methodology (RSM) method) was applied to optimize the extraction parameters. There are four independent variables such sample to DES ratio (X1, mL g^{-1}), microwave power (X2, W), extraction time (X3, s), and DES type (X4, HBA: HBD) were studied by a fixed DRB sample weight of 5 g. The RBP recovery (%) was taken as the response (Y). RBP recovery (%) can be calculated from equation (1).

$$RBP\ recovery\ (\%) = \frac{C \times W}{C_0 \times W_0} \qquad (1)$$

Where C is protein content in RBP concentrated (RBPC) (%), W is the weight of RBPC (g), C_0 is protein content in DRB (%) and W_0 is the weight of DRB (g).

4. Preparation of deep eutectic solvent (DES)

The preparation of DES was described by Hewage et al. (2024) and Lin et al. (2022) with slight modifications [17, 18]. The DES consisted of the molar ratio of HBA: HBD (1:1 for ChCl-Oxalic Acid, 1:2 for ChCl-Glycerol, and ChCl-Urea). The DES was prepared by mixing, stirring, and heating HBA (ChCl) and HBD (Glycerol, urea, and oxalic acid) in a glass beaker. The mixture was continuously stirred and heated at 60 ◦C until it became a clear and homogeneous liquid for 30 minutes.

5. Extraction of rice bran protein (RBP)

The RBP extraction process followed the procedure by Ramlee., et al (2024) with slight modifications [10]. The procedure was to add 5 g DRB to 25 g DES (for a 1:5 ratio) and then manually stir until evenly distributed and heated by microwave at 200 W and 20 s. After the extraction was complete, the mixture was allowed to cool to room temperature and then centrifuged at 1100 g for 10 min. The remaining residue underwent re-centrifugation at 1100 g after being treated with 5 g of fresh DES three times. The next step was protein precipitation from DES. The steps were taken by Liu et al. (2017) with slight modifications [13]. The collected supernatant was added 4 times its volume by anhydrous ethanol and adjusted to pH 4.5 by 0.1 N HCl and 0.1 N NaOH for ChCl-Oxalic acid DES then incubated for 4 min. at room temperature. Then the solution was centrifuged for 1100g for 10 min to retrieve the precipitates. The precipitates were washed with 25 mL of deionized water to remove ethanol and re-centrifugation at 1100 g for 10 min. After this centrifuge, the precipitate was collected and freeze-dried to obtain the Rice Bran Protein Concentrate (RBPC).

6. Protein Content Determination by Kjeldahl Method.

Protein content was determined using the Kjeldahl method according to AOAC Official Method 2001.11 [23] with slight modifications. The analysis was performed using a Buchi Speed Digester K-439 and a Buchi KjelFlex K-360 automatic titration system (Buchi, Flawil, Switzerland). Rice bran or RBPC samples (0.125 g) were accurately weighed and transferred into digestion tubes. One Kjeldahl catalyst tablet (3.5 g K_2SO_4 and 0.4 g $CuSO_4 \cdot 5H_2O$) and 9 mL of concentrated H_2SO_4 were added to each tube. The samples were digested at 420°C for 2 h until a clear solution was obtained. After cooling, the digested samples were diluted with 20 mL of distilled water, and 50 mL of 40% (w/v) NaOH solution was added. The released ammonia was distilled into 25 mL of 4% boric acid solution containing methyl red-bromocresol green indicator. Titration was performed with standardized 0.05 N HCl until the color changed from green to pink. A conversion factor of 6.25 was used to calculate the protein content from the nitrogen content [14].

7. Particle Size and Zeta Potential Measurement by Dynamic Light Scattering.

The hydrodynamic diameter and zeta potential of RBPC were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). RBPC samples were dispersed in deionized water at a concentration of 0.1% (w/v) and adjusted to pH 7 using 0.1 M HCl or 0.1 M NaOH.

Dynamic light scattering (DLS) measurements were performed at 25°C. The Z-average hydrodynamic diameter was determined using the Stokes-Einstein equation, and the polydispersity index (PDI) was recorded as a measure of the width of particle size distribution. Zeta potential measurements were conducted using folded capillary cells (DTS1070). Each measurement was performed in triplicate, and the results were expressed as mean ± standard deviation.

8. Protein Profile Analysis by SDS-PAGE.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) with some modifications using a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, Hercules, CA, USA). Rice bran protein samples were dissolved in sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue) at a concentration of 2 mg/mL and heated at 80°C for 5 min.

The separating gel (12% acrylamide) was prepared using 1.5 M Tris-HCl buffer (pH 8.8) containing 0.4% SDS, while the stacking gel (4% acrylamide) was prepared using 0.5 M Tris-HCl buffer (pH 6.8) containing 0.4% SDS. Aliquots of 10 μL of each sample were loaded onto the gel along with a molecular weight marker (PageRuler Plus Prestained Protein Ladder, ThermoFisher). Electrophoresis was conducted at a constant voltage of 120 V until the tracking dye reached the bottom of the gel.

After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 staining solution (0.1% Coomassie Brilliant Blue R-250, 40% methanol, and 10% acetic acid) for 1 h with gentle agitation. Destaining was performed using a solution containing 40% methanol and 10% acetic acid until clear protein bands were visible against a clear background.

9. Data analysis.

Response Surface Methodology (RSM) with a Box Behnken Design (BBD) was used to investigate the interactive effects of key factors on protein recovery percentage. Protein recovery was measured precisely using established methods, and the resulting data were analyzed using Minitab software. Analysis of variance (ANOVA) was performed to determine the statistical significance ($p < 0.05$) of the factors and their interactions. Minitab version 2017 was used for modeling and regression analysis. As shown in Table 2. A total of 45 experiments were designed in random order and carried out. The variables and levels are shown in Table 1. Multiple regression analysis was conducted to establish an empirical second-order polynomial model shown in equation (2)

$$Y=\beta_0+\sum_{i=1}^{4}\beta_i X_i+\sum_{i=1}^{4}\beta_{ii} X_i^2+\sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij} X_i X_j \qquad (2)$$

Where β0 is defined as a constant, βi is the linear coefficient, βii is the quadratic coefficient, and βij is the cross-product coefficient; Xi and Xj are independent variables.

Table 1. General parameters level and range

Factors	**Variables**	**Level and range**		
		Minimum level	**Central point**	**Maximum level**
		-1	**0**	**1**
Weight ratio [Sample: DES]	*X1*	1:3	1:5	1:7
Microwave Power [W]	*X2*	200	300	400
Extraction Time [s]	*X3*	20	45	70
DES Type [HBA: HBD]	*X4*	ChCl: Glycerol	ChCl: Urea	ChCl: Oxalic Acid

RESULT AND DISCUSSION

1. Optimization of RBP extraction.

To obtain the optimal solvent extraction of rice bran protein, Choline Chloride as a hydrogen bond acceptor (HBA) was mixed with three types of hydrogen bond donor (HBD) such as glycerol (neutral), urea (basic) and oxalic acid (acid). The molar ratio of HBA and HBD used is 1:2 for ChCl: glycerol and ChCl: urea and 1:1 for ChCl: oxalic acid [19, 20, 21]. During recent years, RSM has been extensively used to develop, improve, and optimize chemical processes and extraction of effective substances [2, 13]. The analysis of variance is shown in Table 3. The significance of the model was analyzed using an F test. An F value of 9.57 and a p value of <0,05 indicated that the model was statistically significance. The "lack of fit F value" (2.85) indicates that the lack of fit is not significant relative to pure error.

The Response Surface Methodology (RSM) coupled with Box-Behnken Design (BBD) effectively modelled the microwave-assisted extraction (MAE) of rice bran protein using various choline chloride-based deep eutectic solvents (DESs). The model shown in Table 2 exhibited good fitting with a statistically significant F-value (9.57, $p < 0.001$) and an acceptable coefficient of determination ($R^2 = 85.77\%$), indicating that the model adequately explained the variability in protein recovery. The high R^2-adjusted value (76.81%) suggests that the model accounts for a substantial portion of the variability when adjusted for the number of predictors. The non-significant lack-of-fit ($p = 0.099$) further validates the model's adequacy for predicting protein recovery under various extraction conditions [24]

$$\begin{aligned} Y = & -243.1 + 33.08X_1 + 0.931X_2 + 2.132X_3 - 1.971X_1X_1 \\ & - 0.001195X_2X_2 - 0.0177X_3X_3 - 0.02935X_1X_2 - 0.0631X_1X_3 \\ & - 0.000708X_2 \end{aligned} \tag{3}$$

The p-value of lack of Fit is greater than 0.05 (0.099), this suggests the lack of fit is not significant. The coefficient of determination (R2) for the model was 0.857 showing that the model adequately represented the relationship between the chosen parameters. A final equation (3) was generated and could be used to make predictions about the response for given levels of each factor. The protein recovery (%) is shown in Fig 1 (b). The optimum condition has the maximum protein recovery (%) obtained from ChCl: urea DES with 5.79 solvent to solid ratio, 296.97 W microwave power, and 44.75 s extraction time.

Table 2. Design Layout for Box-Behnken Model

No	X1	X2	X3	X4	Protein Recovery [%]
1	1	-1	0	ChCl-OA	20.11
2	0	1	1	ChCl-OA	6.09
3	0	0	0	ChCl-Gly	29.78
4	0	1	1	ChCl-Gly	10.65
5	-1	1	0	ChCl-Gly	23.99
6	0	1	-1	ChCl-Gly	13.80
7	-1	0	-1	ChCl-Ur	4.40
8	-1	0	1	ChCl-OA	3.01
9	-1	-1	0	ChCl-Gly	6.10
10	1	-1	0	ChCl-Ur	25.99
11	0	1	-1	ChCl-OA	7.61
12	-1	0	1	ChCl-Ur	16.30
13	1	0	1	ChCl-Ur	14.94
14	0	0	0	ChCl-Ur	33.50
15	1	0	1	ChCl-Gly	20.47
16	0	1	1	ChCl-Ur	16.13

17	1	0	-1	ChCl-Ur	18.75
18	1	1	0	ChCl-OA	12.26
19	0	-1	1	ChCl-Gly	12.81
20	0	-1	-1	ChCl-Gly	3.40
21	-1	-1	0	ChCl-Ur	5.17
22	0	0	0	ChCl-Ur	33.02
23	0	-1	1	ChCl-OA	4.46
24	0	1	-1	ChCl-Ur	19.14
25	1	1	0	ChCl-Gly	8.74
26	-1	0	-1	ChCl-Gly	6.00
27	0	0	0	ChCl-OA	21.49
28	0	0	0	ChCl-Gly	30.87
29	-1	-1	0	ChCl-OA	11.05
30	0	0	0	ChCl-OA	25.13
31	1	1	0	ChCl-Ur	13.25
32	1	-1	0	ChCl-Gly	11.14
33	-1	1	0	ChCl-Ur	10.43
34	1	0	-1	ChCl-OA	8.02
35	0	-1	-1	ChCl-OA	6.81
36	0	-1	1	ChCl-Ur	9.96
37	1	0	1	ChCl-OA	22.59
38	0	-1	-1	ChCl-Ur	15.16
39	-1	1	0	ChCl-OA	13.82
40	0	0	0	ChCl-OA	20.44
41	1	0	-1	ChCl-Gly	30.74
42	0	0	0	ChCl-Ur	34.12
43	-1	0	-1	ChCl-OA	2.13
44	-1	0	1	ChCl-Gly	10.40
45	0	0	0	ChCl-Gly	29.47

2. Influence of process variables on RBP recovery

2.1 Effect of solvent-to-solid ratio

The linear effect of the solvent-to-solid ratio demonstrated the strongest positive influence on protein recovery (coefficient = 4.15, $p = 0.001$) which indicates that higher solvent volumes relative to rice bran mass significantly enhanced protein extraction. This can be attributed to increased mass transfer efficiency and reduced viscosity at higher solvent ratios, which facilitate better penetration of DES into the rice bran matrix [25]. However, the significant negative quadratic term (ratio*ratio, coefficient = -7.88, $p < 0.001$) indicates that protein recovery reaches a maximum value and then decreases at excessive solvent ratios, likely due to dilution effects that reduce the extraction efficiency [26].

2.2 Effect of microwave power

Microwave power showed a positive linear effect (coefficient = 1.42), although not statistically significant at $\alpha = 0.05$ ($p = 0.199$). The highly significant negative quadratic effect (coefficient = -11.95, $p < 0.001$) reveals that protein recovery increases with microwave power up to an optimal level, after which further increases lead to decreased recovery. This phenomenon can be explained by the thermal degradation of proteins at excessive microwave power, which disrupts their native structure and reduces extractability [27]. Additionally, localized superheating at high microwave power may cause protein denaturation and aggregation, preventing efficient extraction [28].

2.3 Effect of extraction time

Extraction time exhibited the weakest positive linear effect (coefficient = 0.38, $p = 0.727$) among the process variables, suggesting that time alone does not significantly influence protein recovery within the studied range. However, the significant negative quadratic effect (coefficient = -11.06, $p < 0.001$) indicates that prolonged extraction beyond the optimal time adversely affects protein recovery. Extended microwave exposure may lead to thermal degradation of proteins and potential re-adsorption phenomena, where initially extracted proteins reattach to the rice bran matrix [29].

2.4 Effect of DES types

The type of hydrogen bond donor (HBD) in the DES significantly influenced protein recovery ($p = 0.035$). Urea-based DES demonstrated superior protein extraction capability (coefficient = 2.32, $p = 0.046$) compared to glycerol-based DES (coefficient = 0.57, $p = 0.611$) and oxalic acid-based DES. This superiority can be attributed to urea's protein denaturing properties, which disrupt protein-protein interactions and enhance solubilization [30]. Choline chloride: urea likely forms stronger hydrogen bonds with protein molecules, facilitating their dissociation from the rice bran matrix [31].

2.5 Interaction effects between process variables

The significant interaction between solvent ratio and microwave power (coefficient = -5.87, $p = 0.001$) indicates that the effect of solvent ratio on protein recovery depends on the applied microwave power. At lower power levels, increasing the solvent ratio substantially improves extraction efficiency, while at higher power levels, this effect is less pronounced. This interaction likely occurs because higher microwave power enhances solvent penetration and reduces the dependence on solvent volume [32]. Similarly, the interaction between solvent ratio and extraction time (coefficient = -3.16, p = 0.048) suggests that the positive effect of increased solvent ratio diminishes with longer extraction times. This interaction may be explained by the saturation of the solvent with extracted proteins over time, reducing the advantage of additional solvent volume at extended extraction durations [33]. The non-significant interactions between process variables and DES type suggest that the optimal conditions for microwave parameters remain relatively consistent across different DES formulations, although the absolute protein recovery values differ.

2.6 Optimal extraction conditions

Based on the regression equations, the optimal conditions for maximum protein recovery were determined to be: solvent-to-solid ratio of 8.4-8.9 mL/g, microwave power of 390-410 W, and extraction time of 60-65 seconds, with choline chloride: urea as the preferred DES. Under these conditions, the model predicts a maximum protein recovery of approximately 39-42%. These findings align with previous studies on MAE of plant proteins, where intermediate microwave power and moderate extraction times typically yield optimal results [14]. The relatively short optimal extraction time (50-65 seconds) highlights the efficiency of microwave-assisted extraction compared to conventional methods, which typically require 30-60 minutes for comparable protein recovery [34].

2.7 Predictive capability and model limitations

While the model demonstrates good predictive capability (R^2-predicted = 56.63%), certain observations (runs 32 and 41) showed large residuals, indicating potential variability in the extraction process under specific conditions. These outliers may result from localized overheating or inconsistent microwave field distribution in certain parameter combinations [38]. The relatively lower R^2-predicted value compared to R^2-adjusted suggests that while the model fits the experimental data well, its predictive performance for new observations may be more limited. This is a common characteristic in complex extraction systems where multiple factors and their interactions influence the response [39].

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	17	4537.93	266.94	9.57	0.000
Linear	**5**	**676.35**	**135.27**	**4.85**	**0.003**
Ratio	1	412.47	412.47	14.79	0.001
Power (W)	1	48.40	48.40	1.74	0.199
Time (s)	1	3.47	3.47	0.12	0.727
HBD	2	212.01	106.01	3.80	0.035
Square	**3**	**3163.29**	**1054.43**	**37.82**	**0.000**
Ratio*Ratio	1	688.50	688.50	24.69	0.000

Power (W)*Power (W)	1	1581.32	1581.32	56.72	0.000
Time (s)*Time (s)	1	1354.85	1354.85	48.60	0.000
2-Way Interaction	**9**	**698.30**	**77.59**	**2.78**	**0.019**
Ratio*Power (W)	1	413.38	413.38	14.83	0.001
Ratio*Time (s)	1	119.61	119.61	4.29	0.048
Ratio*HBD	2	46.14	23.07	0.83	0.448
Power (W)*Time (s)	1	37.59	37.59	1.35	0.256
Power (W)*HBD	2	77.48	38.74	1.39	0.266
Time (s)*HBD	2	4.10	2.05	0.07	0.929
Error	**27**	**752.77**	**27.88**		
Lack-of-Fit	21	684.19	32.58	2.85	0.099
Pure Error	6	68.58	11.43		
Total	**44**	**5290.70**			

3. Extraction of RBP

The extraction efficiency of rice bran protein depended on the mass transfer and equilibrium distribution processes of rice bran protein from the rice bran cells and tissues into the solvent. The extraction efficiency of rice bran protein increases with increasing the solid-to-DES ratio across a reasonable range, and the larger DES ratio results in a slight decrease. The extraction process can be described as follows. Firstly, thermal and bubble cavitation effects produced by the microwave led to an increase in the extractive capacity of DES for the extraction of rice bran protein. Moreover, extended microwave effects led to penetration through the cell walls, enhancing the proteins out into the DES mixture. Because the protein and DES contain polar components, sufficient thermal transfer, and bubble cavitation can be obtained under microwave irradiation. Higher extraction times above 45 s were inefficient because they may result in the overheating of the extraction mixture, denaturation of protein, and energy losses. According to the volume exclusion effects [22], the mechanism of precipitation of proteins can be explained as follows: the protein molecules are sterically excluded from the regions of the solvent occupied by ChCl-based DES molecules when added by ethanol in isoelectric point (pl). As a result, protein gets concentrated and precipitates out when its solubility limit is exceeded. Since steric exclusion of ChCl-based DES also results in the preferential hydration of the protein structure. At an isoelectric point, the net charge of protein becomes zero leading to reduced electrostatic repulsion between protein molecules. This condition also promotes aggregation and precipitation as attractive forces between proteins become more dominant. As proteins aggregate, they may be serially excluded from solvent regions occupied by DES molecules, leading to further concentration and eventual precipitation. The addition of ethanol decreases the solvation layer around proteins by displacing existing hydrogen bonds within the

DES matrix. This reduction in hydration can lead to increased protein-protein interactions, as hydrophobic regions of protein become more exposed.

The optimized MAE-DES method demonstrated superior efficiency compared to conventional alkaline extraction methods reported in the literature for rice bran protein, which typically achieve 25-35% protein recovery with extraction times of 60-120 minutes [35]. The enhanced performance can be attributed to the synergistic effects of DES and microwave irradiation. DESs effectively disrupt the cell wall structure and protein-carbohydrate/protein-lipid interactions, while microwave energy causes rapid internal heating, creating pressure gradients that accelerate mass transfer [36]. Moreover, the process avoids the use of harsh chemicals and extreme pH conditions typically employed in conventional alkaline extraction, potentially preserving the native structure and functionality of the extracted proteins [37]. The shorter processing time also reduces energy consumption, aligning with green chemistry principles.

4. The molecular weight of RBPC

SDS-PAGE is a widely used technique for analyzing the subunit composition and molecular weight distribution of proteins. The band reveals the overall protein composition in defatted rice bran (DRB) and RBPC extracted with each DES provides insights into the subunit composition of the rice bran protein. In Fig.1 (a) the electrophoretic profiles revealed that RBPC extracted using the four methods had similar subunit compositions. These results correspond to the MWs of prolamin (10, 13, and 16 kDa), albumin (18-20 kDa), globulin (25,5-26), and glutein (22-23 and 37-39 kDa). A similar result was also obtained by another researcher for the protein of rice bran where glutelin was composed of acidic subunits (30-39 kDa) and basic subunits (19-25) which came from a 54 kDa polypeptide precursor. Two subunits were linked to each other by an intermolecular disulfide bond resulting in glutelin molecules with MWs ranging above 54 to 100 kDa [14].

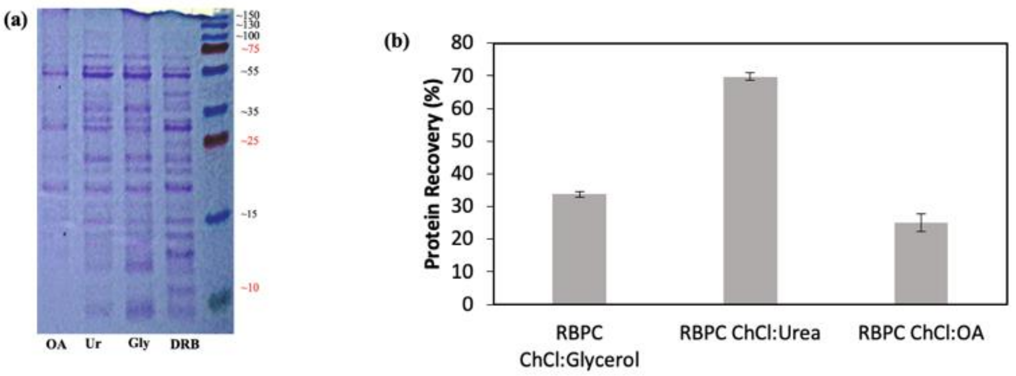


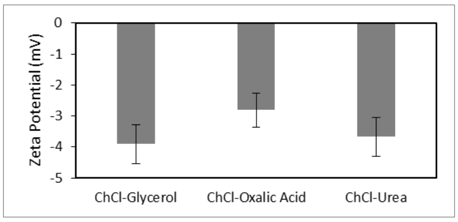
Fig. 1 SDS-PAGE profile of DRB (Defatted Rice Bran), RBPC extracted from OA (ChCl: Oxalic Acid), Ur (ChCl: Urea) and Gly (ChCl: Glycerol) DES (a); Protein recovery in optimum condition (b)

5. Particle Size Distribution and Zeta Potential Analysis

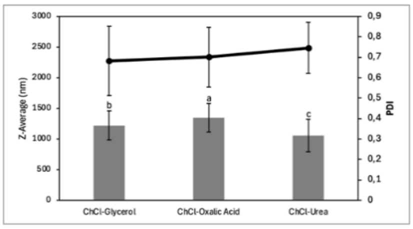
The ChCl: Oxalic Acid extract produced the largest protein aggregates (1320 ± 235 nm), followed by ChCl: Glycerol (1160±325 nm), while ChCl: Urea yielded significantly smaller particles (980 ± 170 nm, $p < 0.05$). These differences in particle size distribution can be attributed to variations in protein-protein interactions and aggregation behavior influenced by the extraction medium [42].

The polydispersity index (PDI) values, represented by the black line in Figure 4.9b, were relatively high (0.65-0.75) for all samples, indicating heterogeneous particle size distributions. This heterogeneity is expected considering the complex mixture of different protein fractions in rice bran, as confirmed by the SDS-PAGE profiles. The slightly lower PDI value for ChCl: Urea extract (0.65±0.08) compared to ChCl: Glycerol (0.70±0.09) and ChCl: Oxalic Acid (0.73±0.11) suggests that urea-based DES produces more homogeneous protein particles, potentially due to its stronger and more uniform denaturing effect on different protein fractions [43].

The smaller particle size and relatively lower PDI of proteins extracted with ChCl: Urea align with its superior extraction performance observed in the RSM analysis. Smaller protein aggregates typically exhibit better solubility and functional properties, which could have important implications for the potential applications of the extracted rice bran proteins in food formulations [44].



(a)



(b)

Figure 2. Zeta Potential (mV) of RBPC extracted with each DES (a); Hydrodynamic Particle Size and PDI for each RBPC

The significant differences in both zeta potential and particle size distribution among the three DES extracts demonstrate that the choice of hydrogen bond donor in DES formulation not only affects extraction efficiency but also influences the physicochemical properties of the extracted proteins. These findings provide valuable insights for tailoring DES compositions to obtain rice bran protein concentrates with desired colloidal properties for specific applications.

CONCLUSION

This study successfully demonstrated the application of deep eutectic solvents (DESs) combined with microwave-assisted extraction (MAE) as an efficient and environmentally friendly approach for rice bran protein recovery. The systematic investigation using Response Surface Methodology with Box-Behnken Design revealed that the nature of hydrogen bond donor in DES formulations, solvent-to-solid ratio, microwave power, and their interactions significantly influence the extraction efficiency of rice bran proteins. Choline chloride: urea demonstrated superior extraction capability compared to choline chloride: glycerol and choline chloride: oxalic acid, achieving approximately 39-42% protein recovery under optimized conditions (5.79 solvent to solid ratio, 296.97 W microwave power and 44.75 s extraction time). The quadratic model developed in this study exhibited good predictive capability ($R^2 = 85.77\%$, $p < 0.001$) which provides a reliable tool for optimizing extraction parameters. SDS-PAGE analysis demonstrated that the protein profiles of extracts from all DES formulations were consistent with that of the native protein, indicating that the DES extractions maintained protein integrity. Dynamic light scattering analysis demonstrated that proteins extracted with ChCl: Urea possessed smaller particle sizes (980±170 nm) and more uniform size distribution (PDI = 0.65±0.08) compared to those extracted with ChCl: Glycerol and ChCl: Oxalic Acid. All protein extracts exhibited negative zeta potentials, with ChCl: Glycerol and ChCl: Urea extracts showing more negative values (-4.0±0.6 mV and -3.6±0.5 mV, respectively) than ChCl: Oxalic Acid extract (-2.8±0.4 mV), indicating differences in surface charge properties that could impact functional characteristics.

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REFERENCE

[1]. Fabian, C., & Ju, Y. H. (2011). A review on rice bran protein: Its properties and extraction methods. Critical Reviews in Food Science and Nutrition, 51(9), 816–827.

[2]. Zheng, Y., Gao, N., Wu, J., & Yin, B. (2019). Rice bran protein: extraction, nutraceutical properties, and potential applications. In Elsevier eBooks (pp. 271–293).

[3]. Sapna, I., & Jayadeep, A. (2021). Influence of enzyme concentrations in enzymatic bioprocessing of red rice bran: A detailed study on nutraceutical compositions, antioxidant and human LDL oxidation inhibition properties. Food Chemistry, 351.

[4]. Gupta, S., Chandi, G.K., and Sogi, D.S. (2008). Effect of extraction temperature on functional properties of rice bran protein concentrates. Int J Food Eng. 4: 2–19.

[5]. Desai, M., Parikh, J., & Parikh, P. A. (2010). Extraction of Natural Products Using Microwaves as a Heat Source. Separation and Purification Reviews, 39(1–2), 1–32.

[6]. Hamada, J. (2000). Characterization and Functional Properties of Rice Bran Proteins Modified by Commercial Exoproteases and Endoproteases. Journal of Food Science, 65(2), 305–310.

[7]. Adebiyi, A. P., Adebiyi, A. O., Jin, D., Ogawa, T., & Muramoto, K. (2008). Rice bran protein-based edible films. International Journal of Food Science & Technology, 43(3), 476–483.

[8]. Bowen, H., Durrani, R., Delavault, A., Durand, E., Chenyu, J., Yiyang, L., Lili, S., Jian, S., Weiwei, H., & Fei, G. (2022). Application of deep eutectic solvents in protein extraction and purification. Frontiers in Chemistry, 10.

[9]. Sombutsuwan, P., Durand, E., & Aryusuk, K. (2024). Effect of acidity/alkalinity of deep eutectic solvents on the extraction profiles of phenolics and biomolecules in defatted rice bran extract. PeerJ Analytical Chemistry, 6,e29.

[10]. Ramlee, N. K. a. F. K., Daud, N. N. M. a. N., Sarip, N. M. S. M., Bakar, N. a. R. A., & Zainudin, N. M. a. M. (2024). A Three-Level Factorial Model for Maximising Protein Extraction from Rice Bran with Choline Chloride: Glycerol. Journal of Advanced Research in Micro and Nano Engieering, 19(1), 63–77.

[11]. Ge X-L, Shi T, Wang H, Zhang J, Zhang Z-Q (2016) Development of an aqueous polyethylene glycol-based extraction and recovery method for almond (Prunus armeniaca L.) protein. Food Anal Methods.

[12]. Li, N., Wang, Y., Xu, K., Huang, Y., Wen, Q., & Ding, X. (2016). Development of green betaine-based deep eutectic solvent aqueous two-phase system for the extraction of protein. Talanta, 152, 23–32.

[13]. Liu, R., Yu, P., Ge, X., Bai, X., Li, X., & Fu, Q. (2016). Establishment of an Aqueous PEG 200-Based Deep Eutectic Solvent Extraction and Enrichment Method for Pumpkin (Cucurbita moschata) Seed Protein. Food Analytical Methods, 10(6), 1669–1680.

[14]. Phongthai, S., Lim, S., & Rawdkuen, S. (2016). Optimization of microwave-assisted extraction of rice bran protein and its hydrolysates properties. Journal of Cereal Science, 70, 146–154.

[15]. Kim, S., Chung, H., & Lim, S. (2014). Effect of various heat treatments on rancidity and some bioactive compounds of rice bran. Journal of Cereal Science, 60(1), 243–248.

[16]. Ribas, F. B. T., Gasparetto, H., & Salau, N. P. G. (2023). Sustainable extraction of rice bran Oil: Assessing renewable solvents, kinetics, and thermodynamics. Process Safety and Environmental Protection, 197, 342–354.

[17]. Hewage, A., Olatunde, O. O., Nimalaratne, C., House, J. D., Aluko, R. E., & Bandara, N. (2023). Improved protein extraction technology using deep eutectic solvent system for producing high purity fava bean protein isolates at mild conditions. Food Hydrocolloids, 147, 109283.

[18]. Liu, C., Hong, W., Yang, B., Lin, C., Wang, L., & Chen, C. (2022). Switchable deep eutectic solvents as efficient and sustainable recycling media for carbon fiber reinforced polymer composite waste. Journal of Cleaner Production, 378, 134334.

[19]. Faraone, A., Wagle, D., Baker, G., Novak, E., Ohl, M., Reuter, D., Lunkenheimer, P., Loidl, A., & Mamontov, E. (2018). Glycerol Hydrogen-Bonding Network Dominates Structure and Collective Dynamics in a Deep Eutectic Solvent. The journal of physical chemistry. B, 122 3, 1261-1267.

[20]. Monteiro, H., Paiva, A., Duarte, A., & Galamba, N. (2022). On the not so anomalous water-induced structural transformations of choline chloride-urea (reline) deep eutectic system. Physical chemistry chemical physics: PCCP, 25 1, 439-454.

[21]. Gontrani, L., Bonomo, M., Plechkova, N., Dini, D., & Caminiti, R. (2018). X-Ray structure and ionic conductivity studies of anhydrous and hydrated choline chloride and oxalic acid deep eutectic solvents. Physical chemistry chemical physics: PCCP, 20 48, 30120-30124.

[22]. Kumar, V., Sharma, V. K., & Kalonia, D. S. (2008). Effect of polyols on polyethylene glycol (PEG)-induced precipitation of proteins: Impact on solubility, stability and conformation. International Journal of Pharmaceutics, 366(1–2), 38–43.

[23]. AOAC. (2005). Official Methods of Analysis of AOAC International, 18th ed. Association of Official Analytical Chemists International, Gaithersburg, MD.

[24]. Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., & Escaleira, L.A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76(5), 965-977.

[25]. Xu, D.P., Zheng, J., Zhou, Y., Li, Y., Li, S., & Li, H.B. (2017). Ultrasound-assisted extraction of natural antioxidants from the flower of Limonium sinuatum: Optimization and comparison with conventional methods. Food Chemistry, 217, 552-559.

[26]. Jiang, Z., Thuy, I.N., Aacharya, S., & Wei, J. (2018). Optimization extraction of protein from the Phaeodactylum tricornutum using response surface methodology. Journal of Food Process Engineering, 41(4), e12689.

[27]. Tiwari, B.K. (2015). Ultrasound: A clean, green extraction technology. TrAC Trends in Analytical Chemistry, 71, 100-109.

[28]. Picot, L.P.A., & Lacroix, M. (2003). Effects of gamma irradiation on the microbiological and nutritional qualities of ready-to-use vegetables. Radiation Physics and Chemistry, 67(2), 169-173.

[29]. Routray, W., & Orsat, V. (2012). Microwave-assisted extraction of flavonoids: A review. Food and Bioprocess Technology, 5(2), 409-424.

[30]. Dai, Y., van Spronsen, J., Witkamp, G.J., Verpoorte, R., & Choi, Y.H. (2013). Natural deep eutectic solvents as new potential media for green technology. Analytica Chimica Acta, 766, 61-68.

[31]. Li, G., & Row, K.H. (2016). Utilization of deep eutectic solvents in dispersive liquid-liquid micro-extraction. Journal of Chromatography A, 1472, 21-34.

[32]. Alara, O.R., Abdurahman, N.H., & Olalere, O.A. (2018). Optimization of microwave-assisted extraction of flavonoids and antioxidants from Vernonia amygdalina leaf using response surface methodology. Food and Bioproducts Processing, 107, 36-48.

[33]. Xu, D.P., Zheng, J., Zhou, Y., Li, Y., Li, S., & Li, H.B. (2017). Ultrasound-assisted extraction of natural antioxidants from the flower of Limonium sinuatum: Optimization and comparison with conventional methods. Food Chemistry, 217, 552-559.

[34]. Hadidi, M., Khaksar, F.B., Pagan, J., & Ibarz, A. (2020). Application of ultrasound-microwave assisted technique for extraction of pigments and phenolic compounds from black tea leaves. Journal of Food Engineering, 271, 109697.

[35]. Fasolin, L.H., Pereira, R.N., Pinheiro, A.C., Martins, J.T., Andrade, C.C.P., Ramos, O.L., & Vicente, A.A. (2019). Emergent food proteins – Towards sustainability, health and innovation. Food Research International, 125, 108586.

[36]. Aryee, A. N. A., Agyei, D., & Udenigwe, C. C. (2018). Impact of processing on the chemistry and functionality of food proteins. In Proteins in food processing (2nd ed., pp. 27-45). Woodhead Publishing.

[37]. Chanioti, S., & Tzia, C. (2018). Extraction of phenolic compounds from olive pomace by using natural deep eutectic solvents and innovative extraction techniques. Innovative Food Science & Emerging Technologies, 48, 228-239.

[38]. Kalagatur, N.K., Kang, H.Y., Ullah, A., Kommalapati, V., Rawat, S., & Haq, Q.M.R. (2018). Response surface methodology approach for optimization of plant extraction conditions and extract-mediated synthesis of silver nanoparticles. Artificial Cells, Nanomedicine, and Biotechnology, 46(3), S800-S815.

[39]. Chan, C.H., Yusoff, R., & Ngoh, G.C. (2016). Modeling and prediction of extraction profile for microwave-assisted extraction based on absorbed microwave energy. Food Chemistry, 201, 25-30.

[40]. Myers, R.H., Montgomery, D.C., & Anderson-Cook, C.M. (2016). Response Surface Methodology: Process and Product Optimization Using Designed Experiments (4th ed.). John Wiley & Sons, New Jersey.

[41]. Avramenko, N. A., Low, N. H., & Nickerson, M. T. (2016). The effects of limited enzymatic hydrolysis on the physicochemical and emulsifying properties of a lentil protein isolate. Food Research International, 89, 171-177.

[42]. Du, Z., Zheng, T., Wang, P., Hao, Y., & Wang, Y. (2020). Microwave-assisted extraction of proteins from rice bran by deep eutectic solvents. ACS Sustainable Chemistry & Engineering, 8(18), 7033-7042.

[43]. Resendiz-Vazquez, J. A., Ulloa, J. A., Urías-Silvas, J. E., Bautista-Rosales, P. U., Ramírez-Ramírez, J. C., Rosas-Ulloa, P., & González-Torres, L. (2017). Effect of high-intensity ultrasound on the technofunctional properties and structure of jackfruit (Artocarpus heterophyllus) seed protein isolate. Ultrasonics Sonochemistry, 37, 436-444.

[44]. Cardoso, V. M. O., Campani, G., Parente, M. E. M., Hubinger, M. D., & Picone, C. S. F. (2020). Study of the effectiveness of deep eutectic solvents as a tool for extraction of rice bran proteins. Food Hydrocolloids, 108, 105998.