

Obtaining concentrated rice bran protein by alkaline extraction and stirring – Optimization of conditions

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Abstract

Defatted rice bran has 15% (w/w) of protein content, which is considered a high nutritional value protein, although it is hard to extract pure protein compounds from grains, such as rice. These work aim is to optimize concentrated rice bran protein (CRBP) extraction from defatted rice bran. This study was split into two steps: 1) Fractional Factorial Design 2^{4-1} (independent variables: X_1 (pH: 8, 9, 10), X_2 (temperature: 25, 35, 45°C), X_3 (stirring speed: 80, 100, 120 rpm) and X_4 (stirring time: 60, 120, 180 min), and 2): Central Composite Rotatable Design with variables X_1 (temperature: 35, 38, 45, 52, 55°C) and X_2 (stirring time: 120, 146, 210, 274, 300 min). Maximum protein content in CRBP was 48.53% at pH 10.0, 80 rpm, 300 min of stirring time and 52°C, with an extraction yield of 34.51%. CRBP obtained looked like a fine velvety powder with colour parameters, L^* 78.29 ± 0.95 , a^* -4.97 ± 0.05 and b^* 16.51 ± 0.10 (CIELAB).

Keywords

By-product

Alkaline extraction

Response surface

methodology

Yield

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Introduction

According to Food and Agriculture Organization of the United Nations (FAO, 2014), the global forecast for rice crop production in 2014/2015 is 744.6 million tons (496.6 million tons, benefited base). In rice processing, rice bran is a by-product, which represents 8%–11% (w/w) of whole rice grain (Parrado *et al.*, 2006). Therefore, a generation of 59.6–81.9 million tons of rice bran is expected in the same period all over the world.

In rice processing industry, rice bran is used to extract oil with organic solvent and solid residue is used to formulate animal foods (Silva *et al.*, 2006). It presents farinaceous, fibrous and smooth aspects to the touch (Lakkakula *et al.*, 2004). Rice bran consists of pericarp, aleurone and germ with a high protein content of about 15% (Hoogenkamp, 2012). Therefore, defatted rice bran has a protein content higher than 15% (Hamada, 2000), reaching to 17% (Gupta *et al.*, 2008).

Rice bran proteins are hypoallergenic and contain appreciable amounts of lysine. Its amino acid profile makes it a suitable ingredient for hypoallergenic baby food formulations (Wang *et al.*, 1999) and hypocholesterolemics (Chrastil, 1992). Anticarcinogenic (Shoji *et al.*, 2001) and antioxidants (Chanput *et al.*, 2009) properties have also been reported.

Some similarities were found between amino

acid sequences of rice globulin and wheat gluten, but rice proteins do not contain the epitope part of an antigen that combines with the products of a specific immune response in charge for coeliac disease. This fact is especially important from a nutritional point of view (Oszvald *et al.*, 2008). Rice bran protein digestibility exceeds 90% (Zhang *et al.*, 2012).

Hettiarachchv (2011) claimed in a patent that purified, unmodified and modified bioactive pentapeptide from heat stabilized defatted rice bran has anti-cancer, anti-obesity, anti-Alzheimer and other health-promoting activities. In addition, the bioactive pentapeptide may be an effective anti-hypertensive, anti-mutagenic and anti-microbial agent. Due to its excellent health-promoting activities *in vitro*, the bioactive pentapeptide can be incorporated as an active ingredient into pharmaceutical, nutraceutical and food compositions.

Tang *et al.* (2003) demonstrated that the extracted rice bran protein has potential as an ingredient in many foods for presenting thermal properties, hydrophobicity, nitrogen solubility, emulsifying and foaming properties, which is desirable in ingredients for many foods formulation. Rice bran needs a previous treatment to be used as a food ingredient for human consumption (Pan *et al.*, 2005). In rice, the protein is found as encapsulated protein bodies throughout the endosperm, which may have different sizes. Protein bodies are insoluble and remain intact during cooking (Demana, 1999).

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Rice protein must be released from cells, in order to be available for industrial applications, so cell walls need to be broken (Voet and Voet, 2003). This step has a great influence not only in protein extraction yield, but also in its biological activity. Protein may associate with other cellular components, as well as the possible presence of compounds from proteolytic degradation and contaminants, that can influence the subsequent purification process (Becerra *et al.*, 2001).

Rice bran proteins extraction by alkaline solutions is frequently used (Gupta *et al.*, 2008; Paraman *et al.*, 2008; Hyun-Jun *et al.*, 2009; Yadav *et al.*, 2011; Zhang *et al.*, 2012; Han *et al.*, 2015), due to the maximum solubility of almost all food proteins occurs at an alkaline pH (Damodaran, 1996). Food cell walls rupture is assisted by mechanical and non-mechanical methods (Geciova *et al.*, 2002). In addition to organic solvents, many plant cells need to be ruptured using mechanical action. The crude lysate can be centrifuged or filtered to remove cell fragments, leaving the protein of interest in the supernatant solution (Voet and Voet, 2003). Plant cell wall is difficult to break because it is formed by cellulosic contents.

Due the important nutritional and functional properties of rice bran protein (Zhang *et al.*, 2012; Han *et al.*, 2015), as well as the variability of the yield obtained in the processes discussed in the literature about the extraction and purification of this protein (Tang *et al.*, 2002; Paraman *et al.*, 2008; Yadav *et al.*, 2011; Zhang *et al.*, 2012; Watanabe *et al.*, 2015), studies to improve the process are necessary. Therefore, the objectives of the study were to evaluate the effects of pH, temperature, stirring speed and agitation time on the extraction yield of protein from defatted rice bran to optimize the extraction conditions of rice bran protein to obtain a concentrated rice bran protein (CRBP).

Material and Methods

Preparation of the sample

The defatted rice bran (DRB) in pellet form was provided by Industry Riograndense de Óleos Vegetais (IRGOVEL–Pelotas/RS) – crop 2013/2014, and was previously grounded in a Wiley mill (Solab, SL 31, Type Willye, Brazil) with a mean particle size of 70 mesh, packed in plastic bags and frozen (-20°C) until experiments moment. The protein content of the rice bran was 16.75 ± 0.33 (dry base). For extraction of rice bran proteins, an alkaline method involving the traditional monitoring of mechanical agitation with the aid of an Ultra-turrax stirrer (Fisatom, 713D,

Brazil) with propeller shaft paddle (Fisatom, 200 380, Brazil) to assist in cell disruption. For each test, 60 g of DRB was suspended in 340 ml of ultra-pure water (MS 2 000, Gehaka, Brazil) and the pH was adjusted (pH 21 pH/mV, Hanna Instruments, Brazil) with 2 mol.L⁻¹ NaOH. A water bath (Quimis, Brazil) was used to control samples temperature during experiments. The conditions (pH, temperature, time and stirring speed) for each test was controlled following experimental planning design.

Planning for determining the best experimental condition for the extraction of rice bran protein

The first step involved the Fractional Factorial Design (FFD) 2⁴⁻¹ (two levels, three repetitions at the central point), corresponding to 11 runs, conducted in a randomised order. Four independent variables were studied: X_1 (pH: 8, 9, 10), X_2 (temperature: 25, 35, 45°C), X_3 (stirring speed g: 80, 100, 120 rpm) and X_4 (stirring time: 60, 120, 180 min). The independent variables and their range of work were selected based on preliminary studies and protein extraction conditions described in literature (Gnanasambandam and Hettiarachchy, 1995; Paraman *et al.*, 2006; Paraman *et al.*, 2008; Gupta *et al.*, 2008; Zhang *et al.*, 2012).

After extraction, samples were centrifuged at 11979 g for 15 min (25°C) (Cientec, CT5000R, Brazil). In each test, the supernatant (S_1) was collected and the total nitrogen content was determined using the Kjeldahl method (n=3) and was multiplied by a nitrogen-to-protein conversion factor value of 5.95 (AOAC, 2005). It was considered the best extraction conditions, when a higher protein contents was achieved (g protein.100 ml⁻¹ aqueous extract).

Significant variables ($p < 0.05$) for protein extraction was found though statistical analysis of FFD, and a second step was performed: Central Composite Rotatable Design (CCRD) 2² (four axial points and three repetitions at the central point). Eleven runs in randomised order with variables X_1 (temperature: 35, 38, 45, 52, 55°C) and X_2 (stirring time: 120, 146, 210, 274, 300 min) were performed. The pH was set at 10 and the stirring speed at 80 rpm. After extraction, the 11 samples were subjected to centrifugation and protein content determination was done as described before.

Statistical analysis

All FFD and CCRD experiments were conducted randomly and data were analysed through Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA). Results were expressed as mean \pm standard error (SE) and were considered significantly different when $p \leq$

0.05. The adjustment to the second order equation model was expressed through R^2 coefficient and statistical significance was determined by F test (analysis of variance, ANOVA). The best extraction condition was considered the one with the highest protein content and thus, the obtained concentrate. The surface response model obtained in the second step was expressed in Eq. (1) (Brereton, 2003).

$$\hat{Y} = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

Where: \hat{Y} = predict response, X_1, X_2 = coded variables, β 's = estimated coefficients in surface response model.

For quadratic model proposed, the critical point was calculated by the first derivate of the mathematical function, which describes the response surface and equates it to zero (Bezerra *et al.*, 2008). In reason of the many differences between computer experiments and real experiments conform discussed in Simpson *et al.* (2004), performing a confirmation experiment is important for confiability of the optimum conditions. Therefore, it was conducting an assay (in triplicate) under the optimum conditions obtained (pH 10, 80 rpm, 300 min, 52.3°C).

Obtaining and characterising CRBP

The steps of extraction, purification and drying to obtain the CRBP are shown in Figure 1. Aqueous extract (S_1) with the highest protein content obtained had its pH adjusted to 4.5 with 3 mol.L⁻¹ HCl (pH 21 pH/mV, Hanna Instruments, Brazil), which is rice protein isoelectric point (Gupta *et al.*, 2008). The solution was kept resting for 30 min at 10°C for protein precipitation, followed by centrifugation at 11979 g (15 min, 25°C). The supernatant (S_2) was discarded and the precipitate containing the protein was washed with purified water and stored in the centrifuge tubes, followed by centrifugation at 11979 g (15 min, 25°C). After the washing procedure was performed three times, the sample was neutralised (pH 7 with 2 mol.L⁻¹ NaOH), followed by drying in a Spray Dryer (MSDi 1.0, Labmaq, Brazil), with a drying chamber of 500 mm × 150 mm and a double-fluid nozzle atomiser type of 1.2 mm diameter orifice. The aqueous extract was fed by peristaltic pump 0.7 (L.h⁻¹) and air inlet temperature at 90°C (3.5 Nm³.h⁻¹).

The total nitrogen content (dry basis) of DRB, the aqueous extract (S_1) and CRBP was determined using the Kjeldahl method (n=3) and result was multiplied by a nitrogen-to-protein conversion factor of 5.95 and moisture analysis was used to evaluate protein content in dry base, both were determined

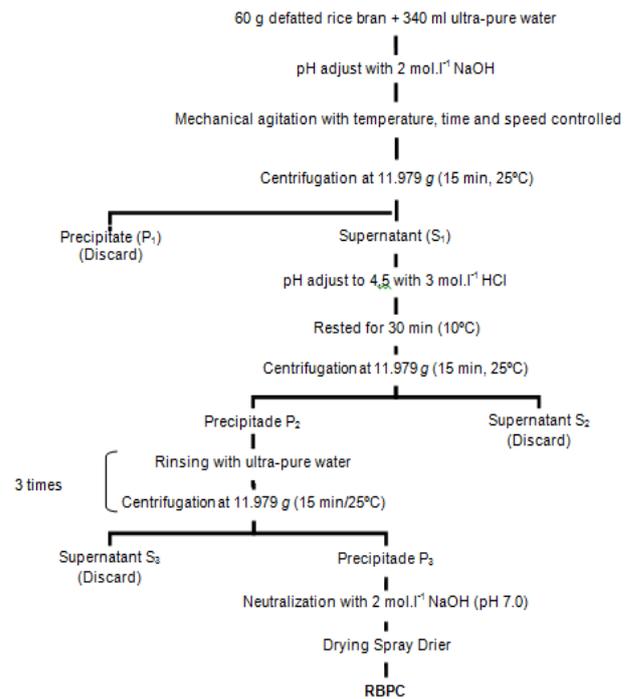


Figure 1. Flowchart of the process of protein extraction and obtaining the rice brain protein concentrate

as described in Association of Official Analytical Chemists (AOAC, 2005).

CRBP colour was determined by instrumental colour measurement using a Minolta® colorimeter (Chroma Meter, CR410, Japan) with integrating sphere and a viewing angle of 45°. It also had lighting d/45 and illuminating D and luminance values L^* , a^* (red-green component), b^* (yellow-blue component) they were expressed to the colour system CIELAB (Commission International for Illumination). Using a^* characterises the standards determined by the Committee. Colour measurements were performed on CRBP samples surface conditioned in Petri plates, measuring three different points of each sample.

Results and Discussion

Extraction of rice bran protein

In the first step (FFD), studied variables, experimental response (Y) and predicted response (\hat{Y}) are shown in Table 1. When the pH (X_1), temperature (X_2) and stirring time (X_3) passed from lower level (-1) to the highest level (+1), that means, the pH increased from 8 to 10, temperature from 25 to 45°C and the time from 60 to 180 min, it had a positive contribution significantly (p<0.05) in extraction yield that increased from 0.276 to 0.459 (g protein.100 ml⁻¹ aqueous extract). Stirring speed had no significant effect on response (p>0.05). Variance percentage (R^2) explained by the model was 0.93 (Table 2).

Therefore, in the second step, for CCRD runs,

Table 1. Matrix of fractional factorial design 2^{4-1} and coded with actual values (in parenthesis) of the variables in protein extraction of rice bran by alkaline treatment and cell disruption by mechanical agitation

Runs	X_1^a	X_2^b	X_3^c	X_4^d	Y^e	\hat{Y}^f
1	-1 (8.0)	-1 (25)	-1 (80)	-1 (60)	0.276 ± 0.006	0.270
2	+1 (10.0)	-1 (25)	-1 (80)	+1 (180)	0.400 ± 0.008	0.396
3	-1 (8.0)	+1 (45)	-1 (80)	+1 (180)	0.403 ± 0.008	0.393
4	+1 (10.0)	+1 (45)	-1 (80)	-1 (60)	0.382 ± 0.006	0.403
5	-1 (8.0)	-1 (25)	+1 (120)	+1 (180)	0.299 ± 0.004	0.320
6	+1 (10.0)	-1 (25)	+1 (120)	-1 (60)	0.341 ± 0.002	0.331
7	-1 (8.0)	+1 (45)	+1 (120)	-1 (60)	0.330 ± 0.008	0.327
8	+1 (10.0)	+1 (45)	+1 (120)	+1 (180)	0.459 ± 0.024	0.453
9	0 (9.0)	0 (35)	0 (100)	0 (120)	0.360 ± 0.002	0.362
10	0 (9.0)	0 (35)	0 (100)	0 (120)	0.345 ± 0.002	0.362
11	0 (9.0)	0 (35)	0 (100)	0 (120)	0.383 ± 0.009	0.362

^a pH; ^b Temperature ($^{\circ}\text{C}$); ^c Stirring speed (rpm); ^d Stirring time (min); ^{e,f} Experimental responses (mean \pm standard error) and Predicted response for the content of protein extracted ($\text{g protein} \cdot 100 \text{ ml}^{-1}$ aqueous extract), respectively

the stirring speed was fixed at 80 rpm and the pH value was fixed at the highest level (10), despite the significant effect observed in FFD. Chen and Houston (1970) showed that at values above 10, there is a higher yield of rice bran protein extraction. It was observed that a pH higher than 10 demands a huge volume of $2 \text{ mol} \cdot \text{L}^{-1}$ NaOH and aqueous solution colour changes from light yellow to a greenish tone. It is known that in a higher the pH, protein unfolding can occur, in addition partial hydrolysis of peptide bonds, deamination of Asn and Gln, and destruction or aggregation of sulfhydryl groups, which can lead to irreversible denaturation of protein structure (Damodaran, 1996). Undesirable reactions involving amino acid racemisation, formation of toxic compounds, such as lysinoalanine, reduced digestibility, loss of essential amino acids and reduced nutritional value can also occur (Martínez-Maqueda *et al.*, 2013). Therefore, in order to avoid possible undesirable change, a pH level over 10 was avoided.

Anderson and Guraya (2001) studied rice bran protein extraction without the use of chemical solvents. Instead, water was used as a diluent, with 30 minutes of stirring at speed of 7500 rpm ($38\text{--}39^{\circ}\text{C}$). The researchers concluded that the physical processes alone are not able to break through the disulfide bonds and extensive network of aggregation of proteins, being extremely important for the extraction of proteins. With the alkalisation of the medium by adding NaOH, that causes rupture of disulphide bonds, improving protein solubility, emulsification properties and foaming ability (Damodaran, 1996).

Paraman *et al.* (2008) reported that the maximum rice endosperm protein yield (43.1%) was obtained under the extraction conditions of solvent pH 11.0 at 40°C for 3 h with 8:1 solvent-solid ratio (v/w). Tang *et al.* (2002) used the alkaline and enzymatic protein extraction from heat-stabilized defatted rice bran (HDRB) and concluded that blending of amylase and protease extracted 5.0% more protein than amylase treatment, achieving a yield of 66%. Yadav *et al.* (2011) reported a yield of 13.2% and protein content in the CRBP of 37.6%, extracting proteins from the DRB at pH 11, shaking for 60 min (60°C). Zhang *et al.* (2012) reported to obtain the protein content of 32.9% by treating rice bran at pH 9.5, shaking for 2 h (50°C). Watanabe *et al.* (2015) studied the combinations of the recovery of phosphorus compounds and the IP-EWT method (technique of isoelectric precipitation combined with electrolyzed-water treatment) for protein recovery and purification. In this study was obtained 52.3% of protein from defatted rice bran under pH 12.8. The CRBP obtained in the conditions showed a browned colour.

In the second step (CCRD), variables studied, experimental response (Y) and predicted response (\hat{Y}) are shown in Table 3.

Results show significant positive contribution for both variables (temperature ($p = 0.040$) and stirring time ($p = 0.027$)) in extraction yield for linear terms. The quadratic terms have positive contribution to stirring time and negative for temperature, although not significant ($p > 0.05$), they were important for

Table 2. Effects of independent variables on the performance of the extraction of rice bran protein by mechanical agitation obtained by fractional factorial design 2^{4-1}

Factors	Effect	Standard error	t	p – value
Media	0.362	0.005	65.926	0.000*
(X ₁) pH	0.068	0.013	5.312	0.002*
(X ₂) Temperature (°C)	0.065	0.013	5.029	0.002*
(X ₃) Stirring speed (rpm)	-0.008	0.013	-0.590	0.577
(X ₄) Stirring time (min)	0.058	0.013	4.509	0.004*

* $p \leq 0.05$; $R^2 = 0.93$

Table 3. Matrix of Design Central Composite Rotational with real and coded values (in parentheses) of the variables studied in the extraction of rice bran protein by alkaline treatment and cell disruption by mechanical agitation

Assay	X ₁ ^a	X ₂ ^b	Y ^c	\hat{Y} ^d
1	-1 (38)	-1 (146)	0.386 ± 0.005	0.392
2	1 (52)	-1 (146)	0.449 ± 0.009	0.440
3	-1 (38)	1 (274)	0.418 ± 0.005	0.446
4	1 (52)	1 (274)	0.488 ± 0.006	0.500
5	-1.41 (35)	0 (210)	0.409 ± 0.002	0.388
6	1.41 (55)	0 (210)	0.458 ± 0.010	0.460
7	0 (45)	-1.41 (120)	0.418 ± 0.022	0.425
8	0 (45)	1.41 (300)	0.531 ± 0.007	0.505
9	0 (45)	0 (210)	0.473 ± 0.005	0.447
10	0 (45)	0 (210)	0.423 ± 0.003	0.447
11	0 (45)	0 (210)	0.445 ± 0.005	0.447

^a Temperature (°C); ^bTime (min); ^{c,d}Experimental responses (mean ± standard error, humid base) and Predicted responses for the content of protein extracted (g protein. 100 ml⁻¹ aqueous extract), respectively

model adjustment, and therefore, were kept into the model (Equation (2)). It was found that an increasing in stirring time variable (X_2) from the lower (-1.41, 120 min) to the highest level (1.41, 300 min) and an increasing in the variable temperature (X_1) from the lowest level (-1.41, 35°C) to level 1 (52°C) promoted an increasing in the amount of extracted protein.

$$\hat{Y} = 0.447 + 0.025 X_1 + 0.029 X_2 - 0.012 X_1^2 + 0.009 X_2^2 \quad (2)$$

In quality of the model's adjustment analysis, the regression was significant ($p = 0.0289$), with an explained variation of 80%. The interaction parameter of the variables (temperature x time), despite of not significant ($p > 0.05$), it was incorporated into the residual error to perform variance analysis (ANOVA).

From these results, it was possible to draw a

surface response and contour curve for protein content obtained in the process (alkaline extraction with cell disruption by mechanical agitation) (Figure 2(A) and 2(B)). It was verified, that runs subjected to shorter agitation time (120 and 146 min) and lower temperatures (35 at 38°C), produced a lower protein extraction yield. The opposite was observed in higher agitation times and temperatures, providing high efficiency in protein extraction of rice bran, which shows an optimal point to the process.

Using Statistica 8.0 software for Response Surface Methodology analysis, the optimized condition of the experiment was found at coded temperature 1.0417 (i.e. 52.3°C) and stirring time 1.41 (i.e. 300 min). After confirming optimum condition (pH 10, 80 rpm, 300 min, 52.3°C) for rice bran protein extraction. Extractions in triplicate was performed in order to assure that Eq. (2) is a valid model. It

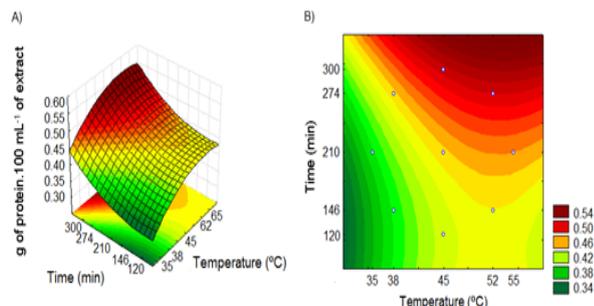


Figure 2. Protein extracted from rice bran (g protein.100 ml⁻¹ aqueous extract) as a function of time (min) and temperature (°C). A) Surface response B) Contour curves

was obtained value of 0.528 g protein.100 ml⁻¹ of aqueous extract. Compared to the theoretical value (0.519 g protein.100 ml⁻¹ aqueous extract), an error of 1.7% was found. Results confirm predictive model validity and show that data were properly adjusted to experimental results.

Obtaining concentrated protein

CRBP was obtained in the form of a fine powder with a velvety texture, and instrumental measurement of the colour values were $L^* 78.29 \pm 0.95$, $a^* -4.97 \pm 0.05$ and $b^* 16.51 \pm 0.10$, according to the values obtained in Kaewka, Therakulkait and Cadwallader's (2009) study. Authors cited the colour of CRBP hydrolyzed, obtained by alkaline extraction and freezing or spray drying, followed by acid hydrolysis, neutralisation, and freezing or spray drying again. The values obtained were for L^* in a range of 64.28 ± 0.18 to 86.25 ± 0.03 , for a^* in a range of 4.57 ± 0.14 to 0.61 ± 0.02 and for b^* in a range of 23.59 ± 0.13 to 14.59 ± 0.06 . The authors concluded that the method of drying directly influences the CRBP colour. Drying temperature can promote pigment darkening due to Maillard reaction. Moisture content in rice bran, supernatant S₁ and CPRB were 11.09%, 97.67% and 4.65%, respectively.

Another studies got 33.5% of protein in CRBP by extraction at a pH of 9.5, without agitation, and milling rice brain particles smaller than 100 mesh (Gnanasambandam and Hettiarachchy, 1995), in addition to CRBP with 42.6% of protein content at pH 9.0 with two hours of extraction, stirring and addition of α -amylase for carbohydrates hydrolyse (Yeom *et al.*, 2010). CRBP with 32.9% of protein was also found using alkaline extraction, pH 9.5, agitation of 300 rpm and a temperature of 50°C (Zhang *et al.*, 2012). In this study, CRBP was produced with a protein content of 48.53 ± 2.08 (dry basis), which was higher than those obtained by other authors using the following conditions: pH 10.0; stirring speed, 80

rpm; stirring time, 300 min and temperature, 52.3°C.

Conclusion

It was possible to establish an improved method for the CRBP production with high content of protein. Variables pH, temperature and stirring time promoted positive effects on protein extraction techniques, while stirring speed had no significant effect. The response surface methodology graphically generated a set of operational variables and appropriate levels to process. It was possible to obtain concentrated rice bran protein with 48.53% of protein (dry basis), a value that is higher than obtained in other works.

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