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IMPLICATION OF MICROWAVES ON THE EXTRACTION PROCESS OF RICE BRAN PROTEIN

Sidmara Bedin[1], Karine Zanella[1], Neura Bragagnolo[2] and Osvaldir P. Taranto[1]

[1] Universidade Estadual de Campinas, Faculdade de Engenharia Química, Campinas, SP, Brasil. E-mail: siddibedin@hotmail.com - ORCID: 0000-0003-0147-4956; ORCID: 0000-0001-5580-1233; ORCID: 000-0001-6334-6998
[2] Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos, Campinas, SP, Brasil. ORCID: 0000-0003-2847-7161

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Abstract - During the processing of rice grain, a wide range of by-products are generated, including Rice Bran (RB), which presents rich composition, offering great potential for application in the food and pharmaceutical industries. Thus, the objective of this work was to evaluate the best operating variables in the extraction of rice bran protein through alkaline extraction and Microwave-Assisted Extraction (MAE). It was found that MAE, in similar conditions, resulted in a higher yield of protein extraction and protein content in the extracted material in a relatively shorter process time, when compared to the values obtained in the alkaline extraction (MAE: yield: 22.04% higher, protein content: 6.19% higher, 30 times faster). In addition, the characterization of the extracts obtained showed that the use of microwaves did not affect the extracted rice bran proteins. Thus, it was possible to verify that MAE is potent and a strongly advisable technique for obtaining rice bran protein.
Keywords: Rice bran protein; Alkaline extraction; Microwave-assisted extraction.

INTRODUCTION

To obtain white rice, it is necessary to polish the grain, removing the husk and the film called bran, which represents around 10% of the grain (Orthoefer, 1996). Rice bran is a large-scale byproduct commonly used in animal feed composition, for oil extraction or as an organic fertilizer (Silva et al., 2006; Filardi et al., 2007; Soares et al., 2018).

Rice bran presents approximately 12 to 15% proteins (FAO, 2011; Oliveira et al., 2012), which have excellent nutritional, nutritive, nutraceutical and functional properties (Saunders, 1990; Tang et al., 2003). Besides, these proteins have high content of essential amino acids, especially the aromatic ones (9.46-11.41%), which act as strong antioxidants (Wang et al., 2017). Another important component of these proteins is lysine, since it is considered hypoallergenic (Parrado et al. 2006), has anticancer activity (Kawamura; Muramoto, 1993) and effects on cholesterol reduction (Yang et al., 2013). Therefore, the bran has been arousing the interest of researchers, who are looking for new ways to extract this protein.

The most commonly used method for obtaining rice bran protein is alkaline extraction followed by isoelectric point precipitation (Bandyopadhyay et al., 2008; Sereewatthanawut et al, 2008). However, under certain conditions, alkaline extraction may generate undesired protein denaturation, formation of secondary reactions, and present potential toxicity resulting from the formation of toxic compounds such as lysinoalanine (Wang et al., 1999; Sereewatthanawut et al, 2008). Due to this, other techniques have been used to obtain rice bran protein, such as physical extraction methods: highspeed blending, sonication, hydrothermal cooking, microfluidization, use of subcritical water, freeze-thaw, sonication (ultrasound-assisted extraction, UAE) (Tang et al., 2002; Amagliano et al., 2017) and microwave assisted extraction (MAE). The MAE technique, when compared to traditional techniques,

* Corresponding author: Sidmara Bedin - E-mail: siddibedin@hotmail.com

presents advantages such as high reproducibility in a short period of time, reduced solvent consumption, a faster process and lower energy consumption (Chemat et al., 2011). Some of the main parameters of MAE are the solid-liquid ratio, extraction process time, temperature, microwave power, sample nature, system agitation, solubility, dielectric constant and dissipation factor (Camel, 2000; Chan et al., 2011). In addition, finding the best conditions of operation of the process and the analysis of such parameters are fundamental for scale up (Chan et al., 2015). Furthermore, the use of MAE has shown good results in obtaining several compounds, such as phenolic compounds from *Scirpus holoschoenus* (Oussaid et al., 2018), and extraction and purification for polysaccharides from *Gentiana scabra* Bunge stems (Cheng et al., 2017). Extraction of flavonoids from *Phyllanthus emblica* (Krishnan; Rajan, 2017). The objective of this research was to evaluate the best operating variables in the extraction of rice bran protein through alkaline extraction and MAE. The values of extraction yield and protein content obtained in each of the methods were evaluated and compared through response surface methodology. The quality of the extracted protein obtained was also compared and evaluated through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), amino acid composition, nitrogen solubility index and *in vitro* digestibility.

EXPERIMENTAL

Material

The material was packed in airtight plastic bags and stored in a domestic freezer at -18 ºC. The RB (*Oriza sativa* L) was supplied by the company Broto Legal, located in the city of Campinas - São Paulo - Brazil, in July 2014. The material was degreased, and through the analysis of centesimal composition, the defatted rice bran (DRB) presented a protein content of 15.67 ± 0.38%, as described by Bedin and Taranto (2016).

Protein extraction

The parameters selected for alkaline extraction and MAE were chosen after intense literature review of different protein extraction processes (Choi et al., 2006; Zhu et al., 2013; Jarpa-Parra et al., 2014; Amponsah and Nayak, 2016). The ratio DRB:water 0.5:10 w/w was selected in order to generate a high driving force and concentration gradient between the solid and the solvent, thus allowing greater mass transfer from the solid to the medium, which is consistent with the principles of mass transfer (Bird et al., 1976).

For alkaline extraction:

1. Different samples of 20 g of DRB (d_{50} = 175 μm) were mixed with 400 mL of distilled water (ratio DRB:water 0.5:10 w/w);

2. The pH was adjusted (X_2: 9-11) with 1 M sodium hydroxide and the solution was homogenized with a magnetic stirrer for a predetermined time (X_1: 30-60 min), at controlled temperature (X_3: 25-55 ºC);

3. The solution was then centrifuged at 8000 g for 30 minutes and filtered through a nylon sieve, separating the solid residue from the supernatant containing the solubilized proteins;

4. The pH of the solution was adjusted to 4.7 and the solution was again centrifuged at 8000 g for 30 minutes;

5. The precipitate was washed with a small amount of distilled water, neutralized to pH 7, dried in a lyophilizer and stored at 4 °C for determination of proteins by the micro-Kjeldahl method, using the nitrogen conversion factor for rice protein of 5.95 (Merrill and Watt, 1973).

An experimental design 2^3 with three central points was used to analyse the effect of the variables on the RB protein extraction process through the alkaline method.

The MAE were carried out using a microwave extraction system (Start-E, Milestone, Sorisole, Itália), at a standard frequency of 50-60 Hz. The equipment consists of a metal box, equipped with a carousel of 8 Teflon tubes to place the sample, which are a closed-system.

1. Samples of 20 g of DRB (d_{50} = 175 μm) and 400 mL of distilled water (ratio DRB:water 0.5:10 w/w) were distributed in Teflon tubes that make up the carrousel of the microwave equipment;

2. The solution pH was adjusted with 1 M sodium hydroxide (X_2: 9-11), the temperature was varied (X_3: 30-55 °C) and the corresponding microwave power range generated was recorded (power range generated from 400-450 W) for pre-set time intervals (X_1: 60-120 s);

3. Afterwards, steps 3, 4 and 5 were performed, as described in alkaline extraction.

In order to evaluate the effect of the variables on the RB protein extraction process through MAE, an experimental design 2^3 with three central points was carried out.

The ranges of values used in the experimental design from alkaline extraction and from MAE are shown in Table 1.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

The composition of the protein extracts was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The protein extracts were mixed at the ratio of 1:1 (v/v) with a sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS and 20% glycerol). Fifteen grams of the samples were loaded onto 12% of

Table 1. Range of values of the variables used in the alkaline extraction and MAE.

Alkaline Extraction				
Variables	**Factors**	**Levels**		
		-1	**0**	**1**
Time (min)	X_1	30	45	60
pH	X_2	9	10	11
Temperature (ºC)	X_3	25	40	55
MAE				
Variable	**Factors**	**Levels**		
		-1	**0**	**1**
Time (s)	X_1	60	90	120
pH	X_2	9	10	11
Temperature (ºC)	X_3	30	42.5	55

the separation gels and 4% of the stacking gels were used. The samples were subjected to electrophoresis at a constant current of 120 V. Molecular weight markers (from 14.4 kDa to 97.4 kDa) purchased from Bio-Rad Co. were used. Then, the gels were stained overnight with a solution of Coomassie Brilliant Blue G-250 and de-stained with methanol-acetic acid before being washed and dried.

Amino acid composition

For determination of amino acids, the samples were hydrolyzed with 6 M hydrochloric acid for 24 hours. The amino acids released by the acid hydrolysis were reacted with phenylisothiocyanate (PITC), separated by reverse phase HPLC and detected by UV at 254 nm. The quantification was performed by internal multilevel calibration with the aid of alpha-aminobutyric acid (AAAB) as internal standard, as described by White et al. (1986) using a Luna C18 100 Å 5u 250x4.6 mm 00G-4252-EQ column. The total flow rate of the mobile phase used was 1.0 mL/min, column temperature 50 °C and UV detector wavelength 254 nm.

Nitrogen solubility index

The protein solubility was obtained according to the methodology described by Morr et al. (1985), in saline solution for induction of ionic interactions and aggregation of protein molecules. In a beaker, 100 mg of defatted rice bran protein and sufficient volume of 0.1 M NaCl were stirred to form a homogeneous paste. Then, 0.1 M NaCl was added until a volume of 8 mL was completed. The mixture was stirred with a magnetic stirrer, at a slower speed than vortex formation. The pH of each sample was adjusted with solutions of 0.1 N NaOH or 0.1 N HCl and varied in the range of 3 to 11. The dispersion was stirred for 1 hour with the pH controlled and then transferred to a 10 mL volumetric flask and the volume was quenched with 0.1 M NaCl solution. The mixture was then centrifuged at 8000 g for 30 minutes and filtered on filter paper. The value of soluble proteins in the supernatant was determined by the micro-Kjeldahl method. The percentage of protein solubility was calculated through Equation 1.

$$\text{Nitrogen solubility}(\%) = \frac{A \cdot 10}{m_{sample} \cdot \frac{S}{100}} \cdot 100 \quad (1)$$

where: A = protein concentration in the supernatant (mg/mL); S = concentration of protein in the sample (%); m_{sample} = sample mass (mg).

In vitro digestibility

The methodology applied for *in vitro* digestibility was based on Xia et al. (2012). RB protein (1%, w/v) was mixed with distilled water and the enzyme pepsin (enzyme: protein 1:100, w/w), the pH was adjusted to 1.5. The mixture was incubated at 37 ° C for 5 min under slight agitation. Thereafter, the mixture was neutralized with 1.0 M NaOH to stop the digestion reaction. The digestion was followed by the addition of trypsin (enzyme: protein 1:100, w/w). After incubation at 37 °C for 120 min, the mixture was heated at 95 °C for 10 min to terminate trypsin activity. An equal volume of 10% (w/v) trichloroacetic acid (TCA) was added to the mixture and centrifuged at 5500 g for 10 min. The TCA precipitate was collected and lyophilized, and the protein content was determined using the micro-Kjeldahl method. Casein was used as standard (Akeson; Stahmann, 1964). The digestibility value of the obtained casein was considered as 100%, and the digestibility value obtained for the rice bran protein was calculated as a function of the value obtained for casein. Protein digestibility was calculated using Equation 2.

$$\text{in vitro digestibility}(\%) = \frac{P_{sample} - P_{undigested}}{P_{sample}} \cdot 100 \quad (2)$$

where: P_{sample} = percentage of protein in the sample (%); $P_{undigested}$ = percentage of undigested protein (%).

RESULTS AND DISCUSSION

Protein extractions

Tables 2 and 3 shows the experimental conditions of 11 treatments originated from the experimental design as well as percent protein and percent yield of protein extract for alkaline extraction and MAE, respectively.

The results contained in Tables 2 and 3 show that the highest extraction yield and higher protein content occurred at the higher planning level (alkaline extraction: X_1: 60 min; X_2: 11; X_3: 55 ºC; MAE: X_1: 120 s; X_2: 11; X_3: 55 ºC). To verify the statistical significance of the system variables, analyses of the main effects and their interactions were performed for a 95% confidence limit ($p<0.05$). The extraction

Table 2. Matrix of experimental design and values of extraction yield and protein content for alkaline extraction.

Treatments	Variable values			Response variables	
	Time (min)	pH	Temperature (°C)	Yield (%)	Protein content (%)
Al1	30 (-1)	9 (-1)	25 (-1)	10.25	66.31
Al2	60 (+1)	9 (-1)	25 (-1)	10.21	72.50
Al3	30 (-1)	11 (+1)	25 (-1)	12.04	75.30
Al4	60 (+1)	11 (+1)	25 (-1)	12.04	74.59
Al5	30 (-1)	9 (-1)	55 (+1)	10.84	72.92
Al6	60 (+1)	9 (-1)	55 (+1)	11.13	74.03
Al7	30 (-1)	11 (+1)	55 (+1)	12.78	74.87
Al8	60 (+1)	11 (+1)	55 (+1)	12.85	75.32
9(PC)	45 (0)	10 (0)	40 (0)	11.35	73.14
10(PC)	45 (0)	10 (0)	40 (0)	11.36	73.93
11(PC)	45 (0)	10 (0)	40 (0)	11.37	74.27

Table 3. Matrix of experimental design and values of extraction yield and protein content for MAE.

Treatments	Variable values			Response variables	
	Time (s)	pH	Temperature (°C)	Yield (%)	Protein content (%)
M1	60 (-1)	9 (-1)	30 (-1)	11.57	75.67
M2	120 (+1)	9 (-1)	30 (-1)	12.46	76.84
M3	60 (-1)	11 (+1)	30 (-1)	15.01	75.95
M4	120 (+1)	11 (+1)	30 (-1)	15.41	77.54
M5	60 (-1)	9 (-1)	55 (+1)	12.20	76.18
M6	120 (+1)	9 (-1)	55 (+1)	14.72	76.39
M7	60 (-1)	11 (+1)	55 (+1)	15.08	78.66
M8	120 (+1)	11 (+1)	55 (+1)	15.68	79.98
9(PC)	90 (0)	10 (0)	42.5 (0)	14.20	77.49
10(PC)	90 (0)	10 (0)	42.5 (0)	13.82	77.10
11(PC)	90 (0)	10 (0)	42.5 (0)	13.38	76.97

yield and protein content were the response variable, varying the time, pH and temperature for alkaline extraction MAE (data not shown).

Empirical models were proposed to estimate the extraction yield and protein content for each extraction method. The statistical analysis of the model equations and the significance of each coefficient were determined through analysis of variance (ANOVA), using the F-test and p-value. For the construction of the models, all factors were first considered, and then, if necessary, factors were eliminated from the least significant to the most, by evaluating the adjusted R^2 value. The model is significant when the adjusted R^2 value reaches its maximum value with the elimination of non-significant factors.

For alkaline extraction, in the extraction yield, it was verified that the independent variables, time, pH and temperature, and the interactions (1 and 2) and (1 and 3) presented statistical significance ($p<0.05$). For the protein content, pH and temperature, and the interactions (1 and 2) and (2 and 3) presented statistical significance. For MAE, it was found that time, pH and temperature presented statistical significance for the extraction yield and for the protein content.

The empirical models, in coded variables, proposed to estimate the extraction yield ($Y_{Al,Y}$) and protein content ($Y_{Al,PC}$, with R^2: 0.9027) for alkaline extraction and the extraction yield ($Y_{M,Y}$, with R^2: 0.9899) and the protein content ($Y_{M,PC}$, with R^2: 0.9870) for MAE, as a function of the statistically significant parameters ($p<0.05$), in coded variables, were generated. Initially, the models were constructed considering all the factors, and then, if necessary, factors were eliminated from the least significant to the most, by evaluating the adjusted R^2 value. However, the $Y_{Al,Y}$ model was significant, but not predictive, thus, only the model $Y_{Al,PC}$ was presented, according to Equation 3. The other models are presented in equations 4 and 5.

$$Y_{Al,PC} = 73.380 + 0.881X_1 + 1.790X_2 + + 1.053X_3 - 0.947X_1X_2 - 0.491X_1X_3 - 0.981X_2X_3 \quad (3)$$

$$Y_{M,Y} = 13.964 + 0.445X_1 + 1.383X_2 + 0.298X_3 - - 0.196X_1X_2 + 0.122X_1X_3 - 0.213X_2X_3 \quad (4)$$

$$Y_{M,PC} = 77.160 + 0.537X_1 + 0.881X_2 + 0.650X_3 + + 0.192X_1X_2 - 0.154X_1X_3 + 0.636X_2X_3 \quad (5)$$

The obtained models $Y_{Al,PC}$, $Y_{M,Y}$ and $Y_{M,PC}$ were significant and predictive ($p<0.05$) for fitting the response, indicating that these models could explain suitably the relationship between the response and the independent variables.

Then, the response surfaces corresponding to the obtained models were generated. Although the empirical model proposed to estimate the extraction yield was significant and not predictive, the response surfaces were generated. The intention was to indicate a trend of the response variable as a function of the dependent variables. The response surfaces representing the alkaline extraction and the presented models were generated and are shown in Figures 1 and 2.

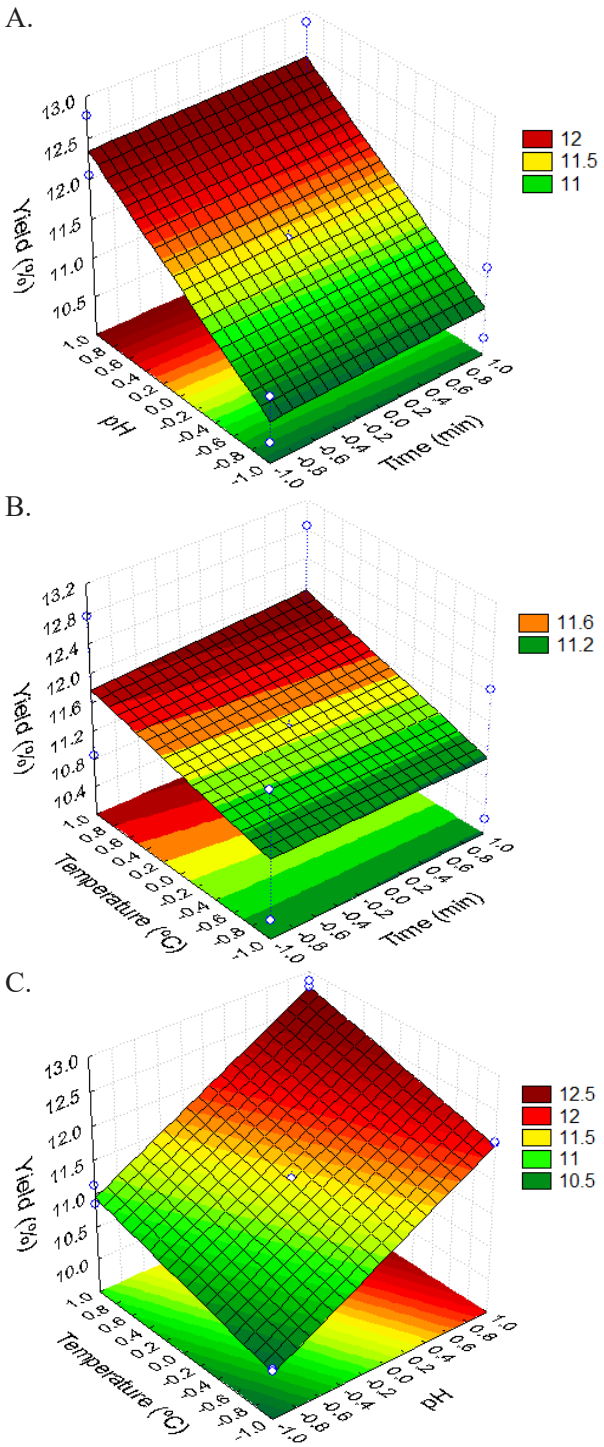

Figure 1. Response surfaces for alkaline extraction yield.

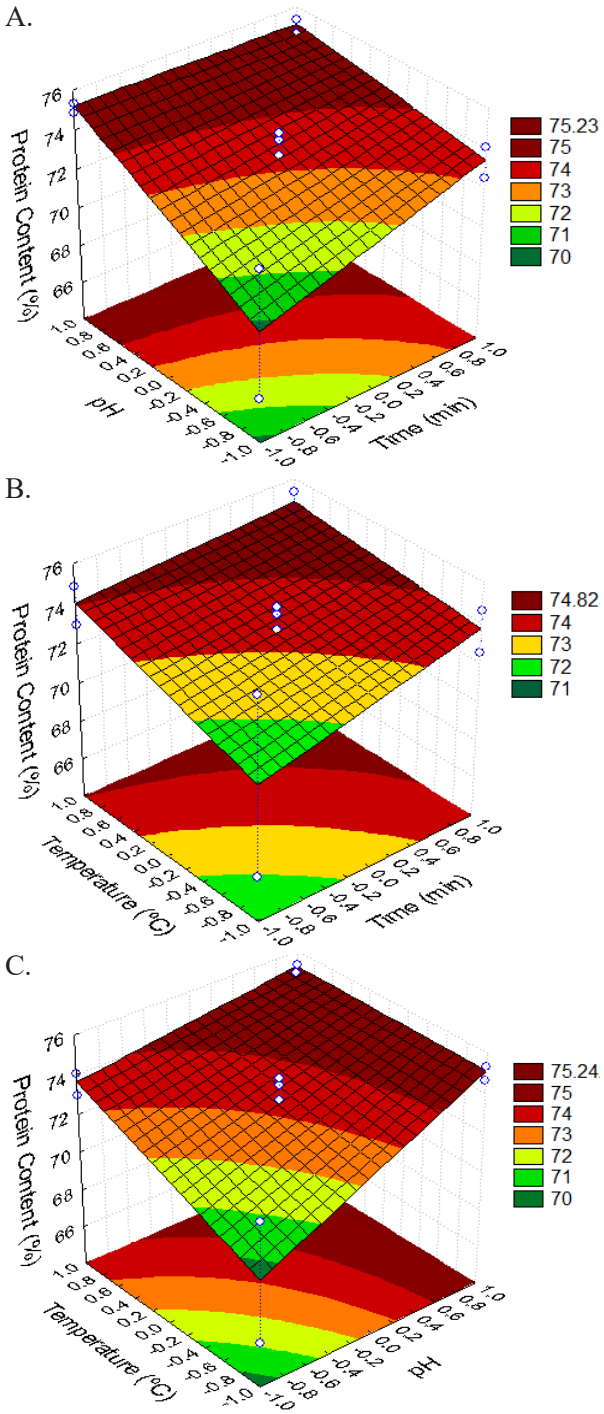

Figure 2. Response surfaces for protein content in alkaline extraction.

In Figure 1 (a) it was possible to observe the relationship between independent variables and response value through three-dimensional surface plots. Two variables were plotted, time and pH, while the third variable was maintained. It was observed that the time and pH had a linear effect on the protein yield. A possible reason could be that the proteins extracted at alkaline pH have a preponderance of negatively charged species due to the ionization of the carboxylic groups and the deprotonation of the amine

groups. This fact makes the protein-solvent interaction increase, since the electrostatic repulsion resulting between the proteins facilitates their separation, as well as encourages their interaction with the solvent, thus facilitating high pH extraction (Kinsella; Phyllips, 1989). Similar results were obtained by Bora and Ribeiro (2004), who obtained higher extraction yields of macadamia protein extracts (*Macadamia integrofolia*) for higher pH; Gadalkar et al., (2017) when evaluating the recovery of protein from rice mill industry waste (RB) using alkaline extraction for a range of 0 to 180 min, also obtained higher yields of extraction for the longer times.

For Figure 1 (b), the time and temperature variables were evaluated. It was noted that an increase in temperature generated an increase in extraction yield, with lower influence of the independent time variable. A justification would be that, with increasing temperature, the molecules began to move faster and the mass transfer rate of the interface between solid and liquid increased. Due to this, a greater mass transfer and solubility were promoted, reducing the viscosity of the solution and thus increasing the extraction rate (Bird et al., 1976). However, very high temperatures have been avoided since they can reduce protein activity and cause thermal denaturation of proteins, which is in the range of 75 to 95 °C (Sgarbieri, 1996).

The influence of temperature and pH on the extraction yield is directly proportional to each other, since both the increase in temperature and the increase in pH generate a higher extraction yield. A similar result was observed by Jarpa-Parra et al. (2014), who obtained higher extraction yields when evaluating different alkaline pH values in lentil protein extraction.

In Figure 2 (a) it was found that the protein content of the extracts increased as the pH increased, without great influence of the independent time variable. This may be justified by a greater solubilization of the glutelin fraction, which is soluble in alkali and corresponds to the second most abundant of the rice bran proteins, being smaller only than the albumin fraction, which is soluble in water (Juliano, 1993; Sgarbieri, 1996). Similar results were reported by Rhee et al. (1973), when extracting protein isolates and peanut oil at higher pH.

In Figure 2 (b), it is observed that the increase in temperature resulted in an increase in protein content in the extracted material. This fact may be associated with intrinsic properties or environmental conditions of the bran, such as the cellular location of the protein or its binding to other components, which is more easily reached with higher temperatures (Chen and Houston, 1970).

Figure 2 (c) confirms the positive influence of temperature and pH on the extracted protein content, since both the increase in temperature and the increase in pH generate a higher protein content in the extract.

In Figure 3 (a) it was possible to perceive the positive influence of pH and extraction time. It was observed that the time applied in the MAE is significantly lower than that applied in the alkaline extraction, however, it generated even higher yields of extraction. One probable explanation is that, in conventional heating methods, convection and conduction are used. Thus, only the surface of the material is directly heated, with subsequent heating of the material being generated by conduction from the surface. However, in the case

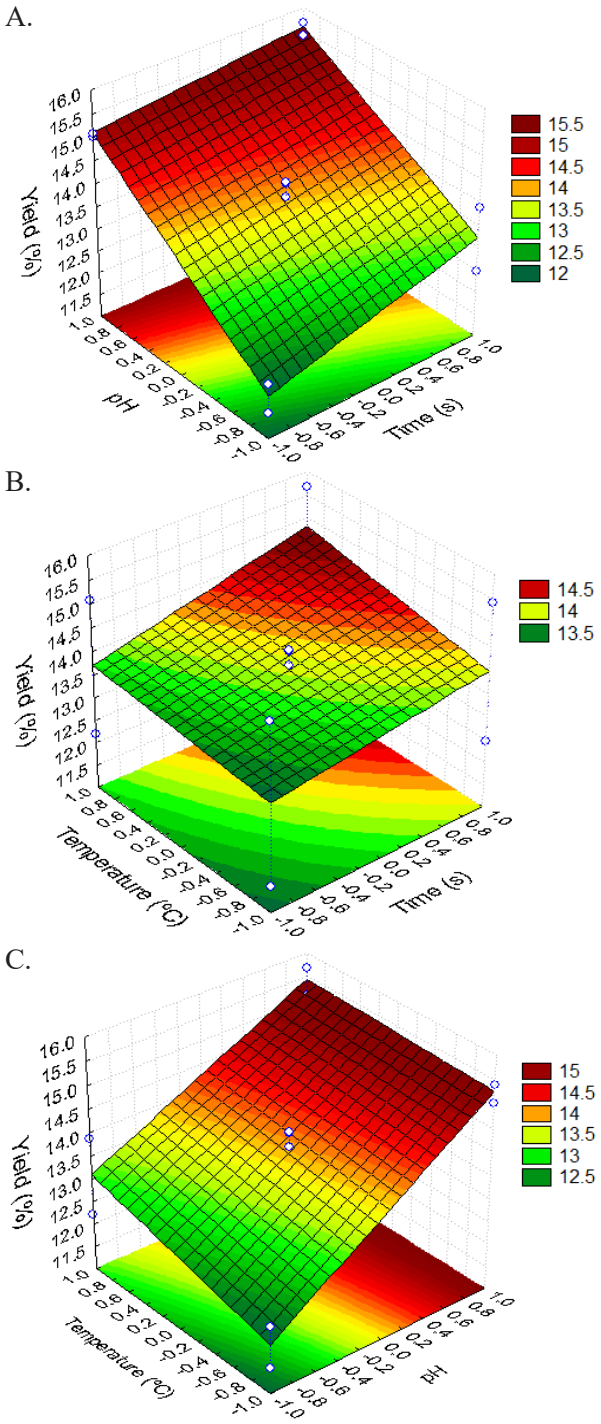

Figure 3. Response surfaces for MAE yield.

of microwave heating, there is a direct generation of heat inside the material. This is caused by molecular oscillation in a rapidly alternating electric field (Bouraoui et al., 1993), facilitating the breakdown of cell walls and release of the protein, which are enhanced with longer processing times. Similar results were observed by Choi et al. (2006), who obtained higher yields of soluble soy protein by MAE by increasing the process time.

In Figure 3 (b) it was found that the increase in temperature and time generated higher extraction yields. This fact can be explained by the softening of the bran tissue caused by the higher temperatures, improving the diffusion rate (Maran and Priya, 2016), as well as by the higher dissolution reaction facilitated by the high temperatures, providing energy to break the bonds in the solid (Lefsih et al., 2017). Thus, there is an increase in the effective diffusion coefficient of the soluble protein, due to the rupture of the rice bran cells, and an increase in the extraction efficiency (Choi et al., 2006). A similar result was observed by Phongthai et al. (2016) when extracting raw organic rice bran protein from Thailand by increasing the extraction time from 60 to 120 s.

Figure 3 (c) confirmed the positive influence of temperature and time on the extraction process evaluated. However, care should be taken with very long microwave emission times. Bandyopadhyay et al. (2012), when extracting proteins from Indian defatted rice bran meal by MAE, evaluated the extraction times of 20, 30, 40, 60 and 90 seconds, and the best results were achieved with 40 seconds, indicating that long extraction times are not always beneficial as they can damage the desired extract.

In Figure 4 (a) it was possible to observe that the increase in pH and time generated higher levels of protein in the extracted material. It is known that most of the proteins contained in rice bran have limited solubility in water, since they are combined with other components of the bran, such as starch and fiber. However, many of these bonds are pH sensitive (Shih et al., 1999) and can be cleaved under alkaline pH conditions. Thus, alkaline pH achieves a higher protein purity of the extracts. Similar fact was observed by Yeom et al. (2010), when investigating rice bran protein isolates prepared with autoclave and enzymatic hydrolysis, obtaining higher protein levels for alkaline pH.

Figure 4 (b) shows that increasing the time and temperature generates higher levels of protein. This can be attributed to the fact that the microwaves associated with greater process times generate the rupture of weak hydrogen bonds. Another possibility may be the migration of ions that increases the penetration of the solvent into the matrix, facilitating the release of the intracellular extract by interrupting the cell wall, improving protein extraction (Li et al., 2010).

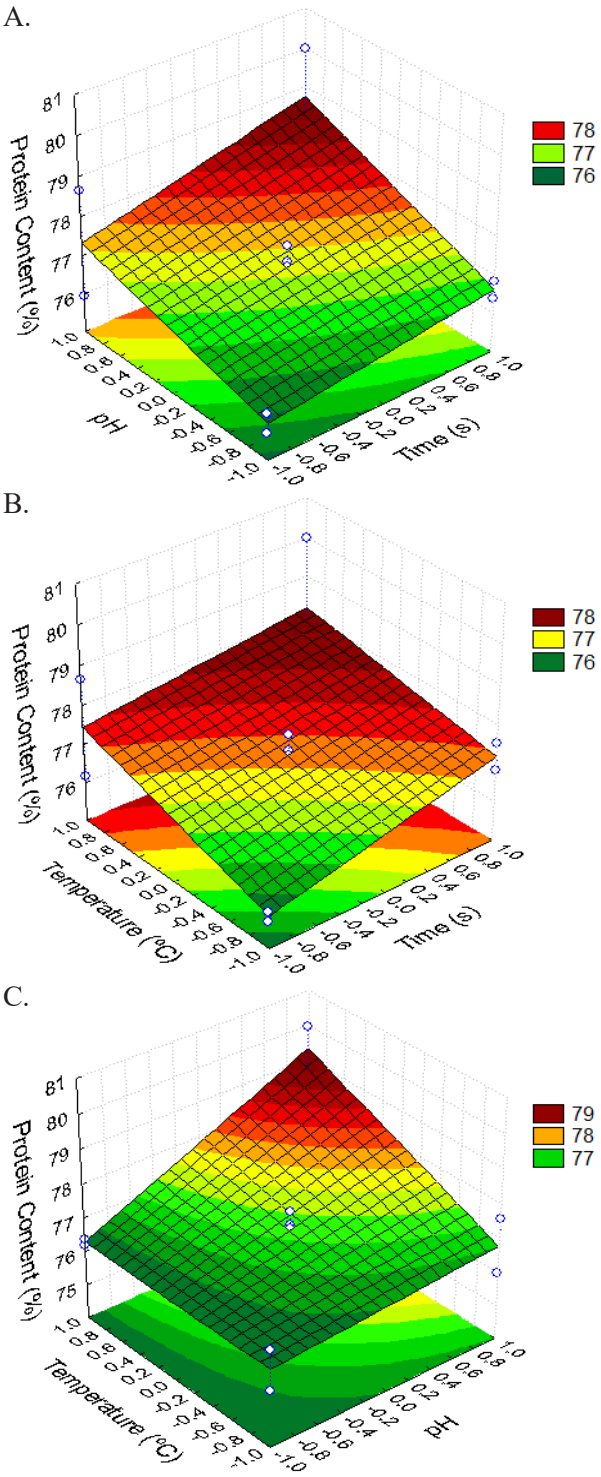

Figure 4. Response surfaces for protein content in MAE.

In Figure 4 (c) the positive influence of pH and temperature was confirmed. However, caution should be taken with high microwave-generated temperatures as well as long exposure times. Such conditions may cause gradual protein denaturation, protein unfolding, leading to surface exposure of hydrophobic and sulfhydryl groups located in the inside of the molecule. These effects may result in irreversible

protein aggregation and in the formation of covalent complexes, leading to a decrease in protein solubility and extractability (Renkema et al., 2000).

To evaluate the protein efficiency, purity of the extracts obtained and the process time, a comparison was made between the best conditions of each of the extraction methods. The results are shown in Table 4.

Evaluating the values contained in Table 4, it was verified that, for MAE, 22.04% higher extraction yield and a 6.19% higher protein content were observed in a time 30 times shorter than for alkaline extraction. In this way, it can be concluded that MAE is an excellent option to obtain rice bran protein, being a highly recommended environmentally friendly technique. Among the benefits associated with the use of the MAE are the great reduction in the process time, improved selectivity of the desired extracts, higher yields of the extracts, high reproducibility in a short period of time, besides presenting simplified manipulation (Kaufmann et al., 2002). Tang et al. (2002) evaluated other different physical processes to extract protein from heat-stabilized defatted rice bran. As a control, 10 g of heat-stabilized defatted rice bran (passed through 40 mesh) were mixed with 400 mL of deionized water, in a ratio of 1:10, at pH 6.5, reaching a protein extraction yield of 11.7%. In similar conditions, adding freeze-thaw treatment, gave a protein extraction yield of 12.0%. The addition of different sonication levels was also evaluated (sonication at 0, 20, 40, 80, and 100% from a sonicator with capacity of 750 W) for 5 minutes, extracting 9.5, 9.9, 10.0, 11.4, and 13.5% of protein, respectively. Phongthai et al. (2017) extracted rice bran protein through conventional alkaline extraction (ratio DRB:water 1:10 (w/v), pH 10), temperature 25 °C, time 1 h) and obtained an extraction yield of 2.92%. Using UAE and the best process conditions (ratio DRB:water 0.99:10 (w/v), pH 10, power sonication of 76% - sonicator with capacity of 750 W - and time of 18 minutes), the researchers achieved an extraction yield of 4.73%. Thus, it was observed that the physical methods evaluated increased the extraction yield, but MAE resulted in even greater amounts of protein. Improved efficiency in the extraction processes was also observed by Amponsah and Nayak (2016), when investigating MAE and UAE to extract and improve the recovery of allergens from various soybean matrices (soybean meal, soy protein isolate and extract of soybean), using conventional alkaline extraction as a control. The authors obtained higher protein recoveries in the MAE samples than the recoveries of the controls in all soybean matrices evaluated. Bagherian et al. (2011), when extracting grapefruit pectin by a conventional method, UAE and MAE, obtained a yield increase of 45.15% for MAE after 5 minutes.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

To evaluate and compare the quality of the protein extracts obtained in each of the extraction methods, the samples referring to the lower and upper levels of each of the three experimental designs (Al1, Al8, M1 and M8) were characterized. The electrophoretic profile of the samples is shown in Figure 5.

From Figure 5 it can be observed that all the analyzed samples had small bands of polypeptides at 16 kDa and below 14.4 kDa, related to prolamin, a band at 26 kDa, related to globulin (Kumagai et al., 2006), and an intense band at 40 kDa, related to glutelin, superimposed with albumin (Romero et al., 2012). A strong band was verified at 57 kDa, related to proglutelin (Kumagai et al., 2006). Similar profiles were observed by Silva (2012) when analyzing rice bran protein isolate obtained by alkaline and enzymatic extraction, by Romero et al. (2012), when analyzing the properties of protein concentrate of rice, and by Xia et al. (2012) when evaluating rice protein prepared by enzyme-assisted micro fluidization and also obtained by alkaline extraction.

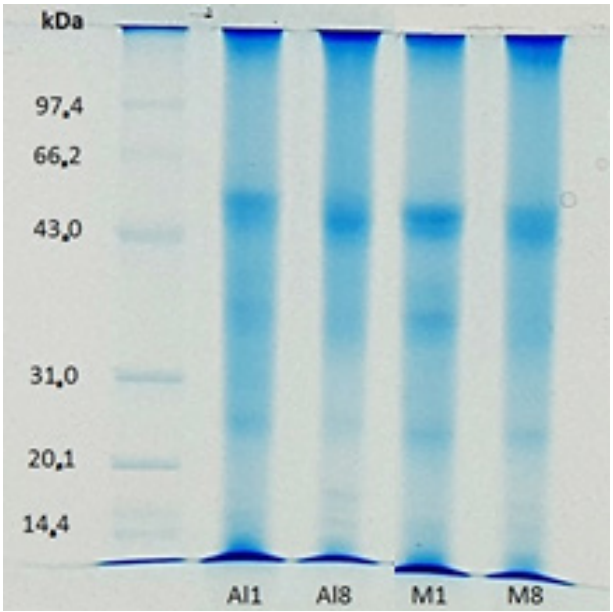

Figure 5. Electrophoretic profile of molecular weight of rice bran protein extract.

Table 4. Extraction yield, protein content and process time for alkaline extraction and MAE.

Method	Treatment	Yield (%)	Protein content (%)	Time (min)
Alkaline	Al8	12.85 ± 0.01	75.32 ± 0.58	60
MAE	M8	15.68 ± 0.41	79.98 ± 0.27	2

Amino acid composition

The amino acid composition of the protein extracts is shown in Table 5.

The data in Table 5 showed high levels of histidine, threonine, valine, leucine, isoleucine, lysine, phenylalanine and methionine in all samples, which are essential amino acids for infants (Cho et al., 1984). There are good levels of arginine and cystine, which are essential amino acids for low birth weight babies, since they have severe nutritional requirements due to the rapid growth and immaturity of gastrointestinal function (Behrman and Vaughan, 1983). Similar amino acid profiles were observed by Gnanasambandam and Hettiarachchy (1995) when evaluating stabilized and stabilized rice bran protein concentrates. The lysine content of the samples ranged from 4.74 to 5.76 g_{lysin}/100 $g_{protein}$, being higher than that found by Parrado et al. (2006), with 3.46 g_{lysin}/100 $g_{protein}$ for the rice bran enzyme extract. Wang et al. (1999) obtained 4.7 g_{lysin}/100 $g_{protein}$ content when analyzed for rice bran protein isolate. The variation in the lysine content may be associated with the variety of rice used, as well as to the way of obtaining the rice bran protein.

To identify the first limiting amino acid of the extracts obtained, the essential amino acid score of the samples was evaluated. The values are shown in Table 6.

From the data in Table 6, lysine was identified as the first limiting amino acid of rice bran protein, since it has essential amino acid score < 1. It was further observed that the essential amino acids were present in suitable values (values close to 1).

Table 5. Amino acid composition of rice bran protein extract (g/100 $g_{protein}$).

Amino acid	Al1	Al8	M1	M8
Aspartate	9.79	9.20	7.81	9.08
Glutamate	13.41	12.45	12.75	12.04
Hydroxyproline	0.06	0.07	0.03	0.05
Serina	5.07	4.54	4.86	4.23
Glycine	6.12	5.47	5.81	5.22
Histidine	3.09	2.67	3.03	2.46
Arginine	11.48	9.81	10.69	9.39
Threonine	4.37	4.04	4.07	3.85
Alanine	6.83	6.08	6.28	5.95
Proline	4.43	4.00	4.20	3.84
Tyrosine	3.97	3.51	3.70	3.46
Valine	6.86	5.97	6.42	5.75
Methionine	2.16	1.73	2.02	1.79
Cystine	1.96	1.67	1.92	1.58
Isoleucine	4.16	3.53	3.78	3.38
Leucine	6.89	6.17	6.37	6.04
Phenylalanine	5.01	4.30	4.80	4.19
Lysine	5.76	4.74	5.76	4.94

*Tryptophan was not determined.

Nitrogen solubility index

To evaluate the attractive and repulsive interactions between the solvent and solute molecules, the solubility graphs were generated, according to Figure 6.

Table 6. Essential amino acids score.

Amino acid	Sample (g/100 $g_{protein}$)				FAO/WHO[a] (g/100 $g_{protein}$)
	Al1	Al8	M1	M8	
Histidine	1.63	1.40	1.59	1.30	1.9
Threonine	1.29	1.19	1.20	1.13	3.4
Valine	1.96	1.71	1.83	1.64	3.5
Methionine[b]	1.63	1.35	1.56	1.33	2.52
Isoleucine	1.49	1.26	1.35	1.21	2.8
Leucine	1.04	0.94	0.96	0.92	6.6
Phenylalanine[c]	1.42	1.24	1.35	1.21	6.3
Lysine	0.99	0.82	0.99	0.85	5.8

[a]Reference FAO/WHO (1991) pattern (essential amino acid for a child of 2 to 5 years). [b]Methionine + cysteine; [c]Phenylalanine + tyrosine.

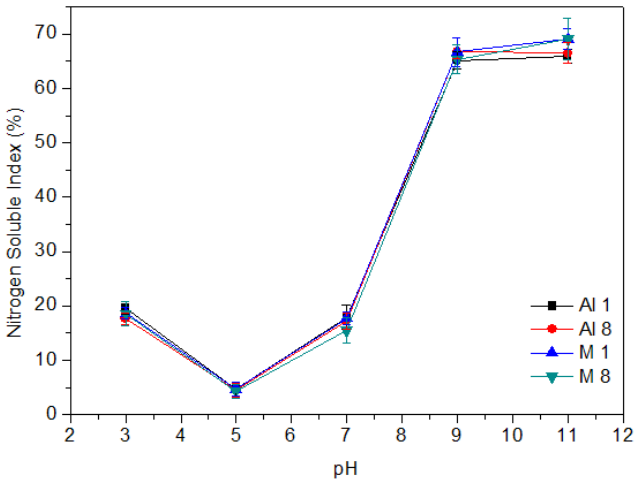

Figure 6. Nitrogen solubility index of the DRB protein obtained by alkaline method for Al1 and Al8 assays and MAE for M1 and M8 assays.

The minimum solubility occurs at pH 5, a fact expected since for most proteins the lowest solubility occurs at the isoelectric point (4.7). At the isoelectric point, the number of electric charges of the proteins are equivalent (number of deprotonated COO^- equal to the number of protonated NH_3^+) and no longer repel each other. Hence, the system has net charge equal to zero, with the maximum hydrophobic interaction with water and electrostatic repulsion and ionic hydration being minimal, favoring the formation of aggregates and precipitation of the molecules (Damodaran, 1996; Sgarbieri, 1996). At alkaline pH, solubility increases as the electrostatic repulsion forces between negatively charged proteins help to keep them separate and increase the ionic interactions between the protein and the solvent, presenting solubility values higher than 64% for all samples (Mao and Hua, 2012).

Similar results were observed by Silva (2012), when evaluating the solubility of FAD protein isolate, obtaining a maximum value between 60 and 70%. Wang et al. (2015), when evaluating the solubility of rice bran protein concentrate, obtaining a maximum solubility value of 80%. The difference in solubility values in the above reports can be attributed to intrinsic

characteristics of the bran used, such as, for example, the variety and form of rice cultivation.

In vitro digestibility

The digestibility values obtained for samples Al1, Al8, M1 and M8 were 62.34 ± 1.70, 62.10 ± 1.09, 62.67 ± 1.71 e 64.08 ± 1.14, respectively. It was found that the results do not differ statistically ($p>0.05$) according to Tukey's test. It was verified that the analyzed samples have protein digestibility superior to 60%, and do not present statistical difference between them. Similar results were observed by Xia et al. (2012), when evaluating the digestibility of rice protein obtained by alkaline extraction that reached values of 60.94%.

Comparing the digestibility of rice bran protein with other materials, it is observed that it presents a higher value than that of sorghum, which has a digestibility of 54.56% (Restle et al., 2002), and higher than soy protein concentrate, which had a digestibility of 51.1±1.28% (Phongthai et al., 2016). A possible justification for this difference in digestibility values is due to the variation in the amino acid composition of the materials (Wang et al., 1999).

CONCLUSIONS

The use of MAE proved to be a very efficient environmentally friendly extraction process for rice bran protein, when compared to alkaline extraction (yield: 12.85%, protein content: 75.32% in 60 minutes). MAE reached extraction yield and protein content 22.04 and 6.19% higher (yield: 15.68 and protein content: 79.98%), 30 times faster. The characterization of the extracts showed that the use of microwave did not affect negatively their characteristics, since the electrophoretic profile, ammonia composition, solubility and protein digestibility of the extracts of MAE were very similar to those of the alkaline extraction. Thus, it can be concluded that MAE is an efficient and highly recommended technique for obtaining rice bran protein.

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