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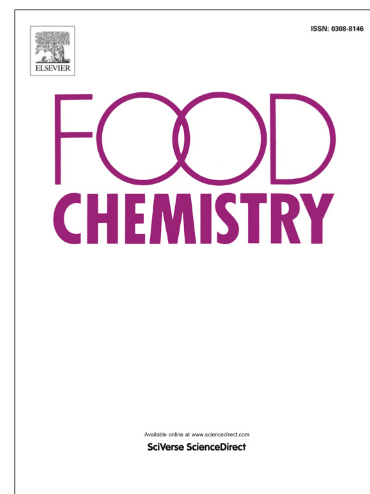

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Accounting for the effect of degree of milling on rice protein extraction in an industrial setting

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Abstract

The by-products of rice milling (BRM), which are predominately rice bran, are a potential source of soluble protein that has been underexploited due to difficulties in extraction. Significant advances have been made understanding how protein content changes with degree of milling (DOM) at the laboratory scale. However, these results cannot be compared due to the lack of information on how DOM affects protein extractability in industrially produced BRM. The colorimetry or particle size analysis may estimate milling degree in industrial scale, and protein extractability changes due to a series of abrasive milling passes. Both colorimetry and particle size could differentiate the industrial abrasive passes and correlated with the amount of bran/protein present. Both the 1st and 2nd pass of milling were suitable sources for the extraction. While the relative amount of protein extracted in each fraction changed, the protein profile of the major fractions was conserved between mill passes.

Key words: By-product of rice milling; rice bran protein; protein extraction; degree of milling; rice protein fractions

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1 Introduction

An estimated 481 million tons of rice will be produced worldwide in 2017/2018 (Childs, 2017), equating to approximately 96 million tons of by-product of rice milling (BRM) representing a substantial potential feedstock. The process was recently described by Pallas (2016), first, the rice paddy is passed through a dehulling machine removing the husk to produce brown rice. The brown rice is passed through multiple whiteners that apply abrasive forces removing the outer rice layers and embryo, which are collected via aspiration or airflow, revealing the white starchy endosperm. The milling waste is collected from the whiteners and mixed together. This by-product of rice milling consists of the rice bran including the pericarp, seat coat, nucellus, aleurone and subaleurone layers as well as the embryo, some starchy endosperm and any remaining hull (Friedman, 2013). In the rice industry, this by-product of rice milling (BRM) is known as rice bran (Prakash & Ramaswamy, 1996; Shih, 2012), however the term by-product of rice milling is used here to avoid any ambiguity with the biological definition of bran.

Rice proteins can be classified into four types: albumin, globulin, prolamin and glutelin (Betschart, Fong, & Saunders, 1977). Albumin is highly water soluble and particularly prized as a nutrient-rich protein fraction due to its high lysine content which provides superior nutritional value than other protein sources (Betschart, Fong, & Saunders, 1977; Mawal, Mawal, & Ranjekar, 1987). Although rice bran contains valuable protein, it is currently a low value product used predominately in animal feed as it is heavily contaminated by fibre, and the low yield of protein extracted from rice bran typically has poor solubility from the extraction processes that have been reported. This has limited the development of commercial processes to isolate pure, easily digested, rice bran protein for food industry applications (Hamada, 1997; Fabian & Ju, 2011). As highlighted in our recent review (Tran, Gidley, & Fitzgerald, 2016) the extractability of industrially produced BRM varies from 13% to 90%, one potential reason for this is differences in the DOM of BRM used in each of the studies. By quantifying the extraction of different protein fractions in BRM following a different number of abrasive whiteners in an industrial mill we can test the effect of DOM on the extractability and composition of protein.

The whitening process that produces white rice and BRM is assessed by the degree of milling of the rice (DOM), which can be monitored in several ways. The most common method is to

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calculate the degree of milling based on the weight before and after milling with 2 to 12 % of the initial weight of rice commonly removed (Rosniyana, Hashifah, & Norin, 2007; Lamberts & Delcour, 2008). The DOM can be described simply by the length of total milling time, however this measurement is specific to each different mill making useful comparisons difficult (Schramm, Abadie, Hua, Xu, & Lima, 2007). Alternatively, the DOM can be determined by the appearance of milled rice based on sensory assessment and can be used to categorize into: under-milled rice, lightly milled rice, reasonably well-milled rice and well-milled rice (Perdon, Siebenmorgen, Mauromoustakos, Griffin, & Johnson, 2001; Lamberts & Delcour, 2008). Changes in the DOM result in a change in BRM chemical composition, as the proportion of bran and endosperm is altered. All previous reported research on the effect of DOM on BRM composition has been performed at the laboratory scale where the DOM can be controlled easily (Resurrection, Juliano, & Tanaka, 1979; Perdon, Siebenmorgen, Mauromoustakos, Griffin, & Johnson, 2001; Schramm, Abadie, Hua, Xu, & Lima, 2007; Lamberts & Delcour, 2008). This level of control is not common at an industrial scale where large volumes of rice are processed continually and simple visual appearance is used to qualitatively assess DOM. Developing physical measurements that can quantify the DOM of industrially produced BRM rapidly will allow current lab scale research to be more easily applied to industrially produced BRM. If in-line methods are accurate this will also allow industry to monitor the production of BRM to produce the highest yield of useful bran and milled rice.

This study was conducted to test whether three rapid methods to estimate DOM, colourimetry, microscopy and particle size analysis, can distinguish differences between BRM collected at first, second and third whitener stages in an industrial rice mill. The underlying chemical composition, including protein, lipid, carbohydrate components and ash from each of the passes will be related to the colour and particle size of BRM to establish if predictions of the DOM are possible based on simple measurements. The Osborne fractionation method is used to characterise the extractability of the protein fractions, and the composition of the extracted protein fractions is tested to determine if industrial milling effects the extractability, or quality, of the BRM proteins.

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2 Material and methods

2.1 Materials

All BRM is of the Reiziq variety and was collected directly from a commercial rice milling processing line by SunRice (Leeton, Australia) in April 2015. It is not possible to directly measure the milling time or weight of rice in the continuous process used. Instead, the BRM was collected from the 1st, 2nd and 3rd rice miller and referred to as 1st pass BRM, 2nd pass BRM and 3rd pass BRM respectively. Three samples were collected at different times and pooled to make a representative sample. BRM samples were stored at -20 ^{0}C in plastic packaging until used.

2.2 Methods

Protein content

Protein was estimated using a LECO TruSpec model CHN using the combustion method (Dumas method) as found in AOAC 997.09. Nitrogen in the sample was freed by combustion and measured using a thermal conductivity detector and the percent nitrogen content was used to calculate the protein content, with 5.95 used as a rice protein conversion factor (Resurrection, Juliano, & Tanaka, 1979)**.**

Lipid content

The lipid content was determined by placing 10g of ground BRM in a cellulose thimble with 200 ml of hexane and heating it for 6 hours using a Soxhlet apparatus. The hexane in the extracted solution was separated using a rotary condenser and the remaining oil was dried overnight at 40^{0}C then weighed to determine lipid content (DeVries, 2005).

The free fatty acid content was determined by mixing 1 g of extracted oil with 50 ml of 95% ethanol and 1 mL of 1% phenolphthalein (v/v) used as an indicator. The mixture was titrated with 0.005 M NaOH until a colour change was detected and the percentage of free fatty acids was calculated based on oleic acid using the AOCS official method Ca 5a-40 (American Oil Chemists, 2009).

Starch content

The starch content was determined by following the protocol of the total starch assay kit analysis from Megazyme according to the AOAC 996.11 method (McCleary, Solah, & Gibson, 1994). BRM was stirred with 2 M KOH for 20 minutes in an ice bath then treated

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with α-amylase and amyloglucosidase. 3 ml of glucose determination reagent was then added to the solution and the absorbance was read at 510 nm against a blank sample.

Moisture content

The moisture content was determined by measuring the weight loss after drying, as described in AOAC 925.10. Briefly, exactly 5g of sample was placed in a weighed container and dried in an oven at 105 ^{0}C then cooled to room temperature in a desiccator. The dried samples were weighed and dried until constant weight. Total drying time was about 60 hours.

Ash content

The ash content was determined as described in AOAC 923.03. Exactly 5 g of BRM was weighed into dried porcelain cups and heated at 550 ^{0}C for 8 hours. The cups and sample were allowed to cool, carefully transferred into a desiccator until they had cooled completely and were then weighed to determine the ash content.

Phytic acid content

Phytic acid content was determined following the protocol of the phytic acid total phosphorus assay kit from Megazyme (Bray, Ireland). One gram of sample was extract with 20 mL HCl 0.66 M overnight. The extracted solutions were then treated with phytase and reacted with alkaline phosphatase and H_2SO_4/ascorbic acid solution. The absorbance was read at 655nm in a spectrophotometer (Pharmacis Ultrospec III) and compared with a standard solution.

Particle size distribution

The particle size distribution of the BRMs was determined using laser light scattering on a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). The particle size of samples was calculated based on Mie theory that assumes the particles are spherical (Cornell, Hoveling, Chryss, & Rogers, 1994). The refractive index used for rice bran was 1.5 and for water was 1.33. Five gram of BRM was dispersed in 70 ml of water and slowly added to distilled water within the Mastersizer system until an obscuration rate of 15% was reached. The suspensions were stirred continuously then measured at 25^{0}C.

Colour index

Five gram of BRM was ground in a ball grinder (Geno Grinder Spex) for 3 minutes at 1500 rpm with three steel balls, then 2 g of ground BRM was used to measure colour indices including L^*, a^* and b^* using a Konica Minolta CR-400 Colorimeter.

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Confocal scanning laser microscopy

Exactly 0.1 g of BRM was stained with 1ml of Calcofluor white M2R 0.05 % (w/V) for 10 minutes (Hemery, Mabille, Martelli, & Rouau, 2010) to highlight any plant cell walls. The stained BRMs were then washed with deionised water and centrifuged at 10000 *x g* for 10 minutes, this washing step was repeated 6 times. The samples were then observed promptly using a Zeiss LSM700 confocal microscope. The cell wall structure was observed at 405 nm.

Fractionation of protein by Osborne's method

The extractable BRM proteins were fractionated based on the Osborne method with the modification of performing the ethanol extraction last (Adebiyi, Adebiyi, Hasegawa, Ogawa, & Muramoto, 2009). This was due to our preliminary testing showed that the extractability of BRM protein reduced dramatically after soaking in 70% ethanol. Five gram of each BRM was extracted sequentially at 25 ^{0}C in 35 ml of distilled water, 5 % w/w NaCl, 0.1 M NaOH and 70 % w/w ethanol. For each extraction, the mixture was shaken in a suspension mixer (Ratek) for 1 hour at 50 rpm then centrifuged at 5000 *x g* for 20 minutes. The supernatants were collected and the residues were extracted one more time with 25 ml of the same solvent. The second supernatant was mixed with the first supernatant then freeze dried for 72 hours and stored at -20 ^{0}C for further analysis. The mixture of supernatants collected from 0.1 M NaOH was neutralised to pH 7 with 0.1 M HCl before freeze drying.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The freeze-dried extracted protein fractions from the different passes of BRM were characterised using the SDS-PAGE method of Laemmli et al. (Laemmli, 1970) with minor modifications. The amount of protein loaded into each well of a 4 - 20% BioRad precast gel was 15 µg in 15 µL of 1x Laemmli buffer. The mini vertical electrophoresis system from Bio Rad was used to run the gel at a constant voltage of 200 V for 40 minutes. After electrophoresis the gel was stained with Coomassie blue R-250 then de-stained with glacial acetic acid and methanol overnight. The precision plus protein Kaleidoscope standard (Bio rad Catalog 1610375) was used to determine the molecular weight of the protein. The prolamin fraction was not analysed by SDS-PAGE due to its low extraction yield, approximately 100 mg, which was used for the protein concentration experiments.

Non-starch carbohydrate

The non-starch carbohydrate level was determined by subtraction of the protein content, lipid content, starch content and ash content from the total weight.

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Statistical analysis

All data were replicated in at least two independent experiments and analysed by one-way analysis of variance (ANOVA) with Minitab statistical software. The results are reported as the mean ± standard deviation. Sigma plot was used to create the graphs.

3 Results and discussion

3.1 Rapids method for classifying BRM materials

The easy selection of an appropriate source material for protein extraction makes it desirable to have a rapid method to determine the approximate composition of BRM from industrial sources without having to undergo the laborious process of determining the lipid, protein, starch, fibre and ash content sequentially. The differences in colour and physical properties of the bran and embryo layer compared to the endosperm make colorimetry and particle size potentially useful to estimate the DOM and composition of the BRMs in the industrial process.

3.1.1 Colour properties

Table 1 shows the colorimetry results for BRM from the three passes. The lightness (L*) of the 3rd pass was 60.55 which was significantly ($P<0.05$) higher than the 2nd pass and the 1st pass which were 48.22 and 43.44 respectively. The yellowness (b*) of passes BRM reduced significantly from 22.6 to 19.25 and 9.41 respectively for the 1st pass, 2nd pass and 3rd pass. The redness (a*) was reduced significantly from 1.97 to 0.48 and -0.5 in the 1st pass BRM, 2nd pass BRM and 3rd pass BRM respectively. These clear differences are presumably due to differences in the rice components present, as observed in a previous study linking increases in (L*) with the amount of barley endosperm present and a similar decrease in b* due to a decrease in the proportion of outer layers (Klamczynski, Baik, & Czuchajowska, 1998). As this trend is observed in our results it can be expected that BRM from the 3rd pass will contain more starchy endosperm while the outer layers and embryo are present in greater proportions in the 1st and 2nd passes. From these results the use of either L* or b* can be proposed to distinguish the passes as they can readily differentiate the samples from the three different passes in milling. Studies with other rice varieties and mills should be undertaken to generalise and further validate this finding.

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3.2 Particle size distribution

The BRM is a mixture of different components with different physical properties that will comminute differently when abrasive processes are applied to produce a diverse range of particle sizes. The particle size of rice starchy endosperm has been reported to be 9.4 µm (D[3,4]), much smaller than rice bran which is 62 µm (D[3,4]) under similar milling conditions (Jeong, Ji, Kang, Jung, Dong, Kang, et al., 2008). The volume distribution of particle sizes for the three passes BRM are shown in Figure 1, which shows that they are not uniform in particle size and have a wide distribution from 2-1000 µm which includes three peaks/shoulders.

The BRM from the 1st pass contains a peak and shoulder below 100 µm and a larger peak centred around ~900 µm. The BRM from the 2nd pass has a greater volume percent of the peak below 100 µm while the large peak has a maximum at a greater size than in the 1st pass. The BRM from the 3rd pass shows a particle size distribution that continues this trend with the large peak now greater than 1000 µm and the peak below 100 µm representing a significantly greater volume, and a third minor peak now apparent at approximately 300 µm. The increasing volume of small particles from the 1st pass to 3rd pass and the increase of L* from the 1st pass to 3rd pass are both consistent with the presence of increasing amounts of starchy endosperm.

Microscopy was undertaken to confirm whether the small particles are related to the starchy endosperm or the bran; Figure 2 contains the images from BRM for each of the three passes. The images of the 1st pass and 2nd pass of BRM both contain multicellular particles, with cell walls stained blue by Calcofluor, together with smaller particles. The 3rd pass shows a large number of small particles with a smaller number of the cell wall structures when compared to BRM from the 1st and 2nd passes. These particles are globular and approximately 2-5 µm in size which is similar in shape and size to that of rice starch granules shown by Dhital et al (2015). This provides additional evidence that the small peaks in the particle size results are likely to be of endosperm origin rather than from the rice bran.

Based on the results described above, changes in the DOM clearly change the colour properties and particle size distribution of the three types of BRM. The lightness (L*) increases with increased numbers of smaller particles while the yellowness (b*) is higher in the 1st pass and 2nd pass and these have more large BRM particles.

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3.1.3 Chemical components of BRM after each of three passes

To confirm that the changes in colour and particle size observed due to DOM also affect the composition of BRM, the protein, lipid, starch, water, ash, phytic acid and non-starch carbohydrate concentrations in the three types of BRM were analysed and the results are shown in Table 2. There is a significant difference ($P<0.05$) between each of the pass BRM for all components with the only similarities in the lipid, water and phytic acid concentrations in the 1st and 2nd passes. The BRM from 2nd pass contains the highest level of protein at 13.47 % followed by the BRM from the 1st pass at 12.7 % of protein, while the 3rd pass contains only 8.88 % protein. The protein content of the embryo is reported to be 17.3-26.4 % and 11.7-15.2 % in the aleurone layer and sub-aleurone layer respectively while the starchy endosperm, pericarp layer, seed coat and nucleus layer contains 0.5-8 % protein (Kulp & Lorenz, 1991; Luh, Barber, & Barber, 1991; Champagne, Wood, Juliano, & Bechtel, 2004). Therefore the 2nd pass should contain the highest ratio of proteinaceous tissues such as the embryo, aleurone and sub aleurone layers followed by the 1st, while the 3rd pass should contain the highest proportion of low protein components including the starchy endosperm.

The lipid content in the 1st and 2nd pass is nearly 3 times higher than in the 3rd pass, consistent with the reported lipid contents of 21 – 39 % and 16.6 % in rice embryo and rice aleurone layers respectively, but less than 0.5 % in the starchy endosperm (Kulp & Lorenz, 1991; Luh, Barber, & Barber, 1991). The free fatty acids within the lipids decreases from 12.43 % in the 1st pass to 10.31 % in the 2nd and 4.68 % in the 3rd pass. This variation indicates that the oil present in the outer layers of the rice bran either has a different composition, or is more susceptible to rancidity, than the oil within the starchy endosperm. This trend also exists for ash, which has been reported to be present at 7.9% in the embryo and 9.6 % in the bran while the starchy endosperm contains only 0.6% (Kulp & Lorenz, 1991). Based on the analysed results, the amount of embryo and outer layers including aleurone and sub aleurone in the 1st and 2nd pass must be higher than they are in the 3rd pass. By this logic, phytic acid in BRM from the 1st and 2nd passes is nearly double that in the 3rd pass, therefore it could be concluded that phytic acid occurs mostly in the outer layers and embryo of rice grain. While containing reduced protein, lipid and ash, the 3rd pass contains the largest amount of starchy endosperm with starch content 5.5 times higher than the 1st pass and 2.5 times higher than the 2nd pass. The dramatic increase in the starch content of the 3rd pass is due to the majority of the outer layers and embryo being removed in the 1st and 2nd passes as observed by Schramm (2007). The starch content of up to 55 % in the 3rd pass BRM is above the upper end of the

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range of 25 % - 45 % starch contained in commercial rice bran (Prakash & Ramaswamy, 1996) which presumably reflects a mixture of mill pass treatments used commercially. These results agree with the particle size, confocal microscopy and colour property estimations of BRM composition from different passes and further validates the measuring of colour or particle size distributions as potential methods to quickly distinguish the DOM of BRM. The colorimetry method along with an appropriate calibration is likely to be the most convenient method as it is quick and able to be performed continuously. One consequence of the strong effect of DOM on the concentrations of components (Table 2) is that BRM from early passes are likely to be more valuable for protein extraction.

3.2 Effect of DOM on BRM protein properties

The value of the protein fraction in BRM will depend largely on what proteins are extracted and how easily extractable they are. The Osborne method categorises protein into 4 types based on their solubility, and was used to determine whether the DOM changes the nature of the BRM protein in the different passes. The 4 protein fractions obtained by the Osborne classification are: water soluble albumin, salt-water soluble globulin, alkali soluble glutelin/oryzenin and ethanol soluble prolamin (Betschart, Fong, & Saunders, 1977; Lasztity, 1996). Table 3 shows the results of the Osborne sequential extraction process that in combination extracted 69-77 % of the total protein present in the BRM (as estimated from N content) with the ratio of the protein fractions differing depending on the DOM.

BRM from the 1st and 2nd pass contain significantly more of the albumin fraction than the other extracted protein fractions and the albumin fraction in the 3rd pass (Table 3). In contrast, 53% of protein extracted in the 3rd pass is glutelin, 4-5 times higher than the proportion of glutelin in the 1st pass or 2nd pass. The high proportion of albumin in BRM from the 1st pass and the 2nd pass suggest that most albumin is concentrated in the outer layers and embryos of rice grain while the glutelin is found mostly in the starchy endosperm as evidenced by the high amount in the 3rd pass. This is in agreement with a previous study which reported that 63.8 – 73.4 % of protein in the starchy endosperm of 6 varieties of rice is glutelin (Basak, Tyagi, & Srivastava, 2002). Furthermore, the ratio of the protein fractions in BRM could be rapidly estimated using the colour properties or particle size distribution. Increases in the lightness (L*) and increases in the number of small particles in BRM both correlate with an increase in the relative concentration of glutelin. While BRM with higher yellowness (b*) and fewer small particles, contains a higher relative concentration of the albumin protein

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fraction. This is due largely to the higher proportion of smaller starch rich particles, which are whiter than the bran particles, and high in glutelin.

In short, the majority of the high nutritional value, easily extractable, albumin protein fraction exists in BRM from the 1st and 2nd pass while the 3rd pass contains a majority of the difficult to extract, the glutelin. Globulin is a minor fraction of the extractable protein with a relatively consistent concentration of about 10 % in the 3 different passes. Prolamin has a low concentration of 3% in the BRM from the 1st and 2nd pass and double that amount in the 3rd pass that is rich in starchy endosperm. The variation observed in the extractability show that the degree of milling of BRM clearly affects on the rations of protein fractions extracted by Osborne method, therefore DOM could be a main factor that could alter the BRM protein extractabilities

The differences in extractability in the different passes may be due to differences in the population of proteins that are present in the rice endosperm and rice bran, we test this by characterising the molecular weight of proteins within each of the extracted fractions. For protein characterisation, figure 3 shows the SDS-PAGE gel patterns of albumin, globulin and glutelin protein fractions from each pass BRM. All of the BRM protein fractions are mixtures of many proteins with molecular weights in the range of 8 kDa - 100 kDa. This result agrees with earlier studies by Adebiyi et al (2009) with rice bran protein and Agboola, Ng & Mills (2005) with rice flour protein. Each protein fraction of the three BRM passes have very similar gel patterns showing that the extracted protein fractions are very consistent, this suggests that the protein fractions come from the same tissue sources and that the three passes of BRM are each a mixture of the same rice grain components. The albumin fractions have the most intense band at 50 kDa, as well as four clear bands in the range of 100 - 75 kDa, 75 – 50 kDa, around 25 kDa, and 17 -10 kDa. Adebiyi et al (2009) reported that the SDS-PAGE gel of extractable albumin protein of rice bran has clear bands in the range of 66 – 45 kDa with the most intense band at 53 kDa, however the tested samples were from commercial rice bran which is likely to have been pre-processed by heat and from a mixture of many rice varieties. Mawal reported the purified rice albumin of rice Basmati 370 seed using Con A Sepharose column chromatography to have one band at 60 kDa. The globulin fractions in all three passes BRM are all predominately low molecular weights of 10 kDa and 14 kDa. However, there are weak globulin protein bands at 60-62 kDa in the 1st and and 2nd and weak bands at 22-23 kDa in the 2nd and 3rd pass. This indicates that there were some large molecular weight globulins occurring in the outer layers and germ and different smaller

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molecular weight globulins closer to, or amongst, the endosperm of the rice grain. Rice flour globulin contains proteins of molecular weight from 13.9 kDa to 54.8 kDa according to Agboola, Ng & Mills (2005). Glutelin fractions in three passes BRM all have prominent bands in the low molecular weight region with the most intense at 30 – 32 kDa, and other clear bands at 17 – 18 kDa and at 12 kDa. This is consistent with a previous study that reported rice glutelin to be distributed in the range from 38.5 kDa to 15.8 kDa (Agboola, Ng, & Mills, 2005). The 1st pass BRM glutelin has another intense band at 10 KDa, this may come from the outer layers which are separated early in the milling process. This pass also has some weak bands at 35 kDa, 27 kDa and 21 kDa in the prolamin fraction which are not present in the 2nd or 3rd pass that contain 10 -15 kDa prolamin fractions. In short, the molecular weight of BRM protein is less than 100 kD and the albumin, globulin glutelin and prolamin extractions show very clear differences in molecular weight pattern, but the protein fractions, except for prolamin, from the different passes have similar SDS-PAGE patterns.

3.5 Conclusions

The by-products of rice milling (BRM) from three consecutive mill passes display clear differences in their physical properties, chemical composition, and protein solubility related to the changes in their degree of milling (DOM). Both the colour and the particle size distributions of the BRM correlate with the changes in protein composition which results from a different DOM. The BRM from 1st and 2nd pass were rich in protein, oil and non-starch carbohydrates, and displayed a high yellowness (b*) and a low proportion of starch granules. In contrast starch was the major component in the 3rd pass BRM which had the greatest lightness (L*) and highest number of starch granules originating from the endosperm. These techniques, particularly colorimetry, will allow the estimation of the DOM in industry by producing a calibration for each rice variety that is milled. The potentially high value albumin protein fraction is found mostly in the 1st and 2nd passes of BRM, suggesting that these materials are best suited for protein extraction.

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Highlights

- Colorimetry and particle size measurements can be used to estimate the degree of rice milling.
- The protein content and Osborne fraction composition changes after three abrasive milling passes.
- The protein fractions, albumin and glutelin, do not vary in molecular weight with degree of milling.
- BRM from the 1st and 2nd pass of milling are both suitable for protein extraction.

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Figure caption

Figure 1. The volume distribution of particle size in different BRM passes

Figure 2. Confocal microscopy of different BRM passes dyed blue with Calcofluor to highlight plant cell walls

Figure 3. SDS-PAGE gels of protein fractions in different BRM passes. Well number 1: 1st pass Albumin; 2: 2nd pass Albumin; 3: 3rd pass Albumin; 4: 1st pass Globulin; 5: 2nd pass Globulin; 6: 3rd pass Globulin; 7: 1st pass Glutelin; 8: 2nd pass Glutelin; 9:3rd pass Glutelin

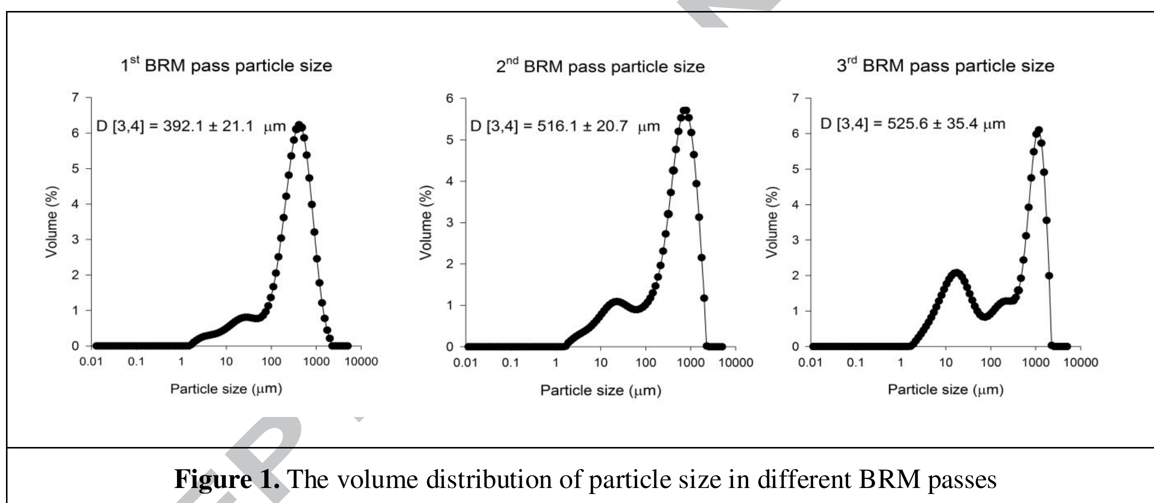

Figure 1. The volume distribution of particle size in different BRM passes

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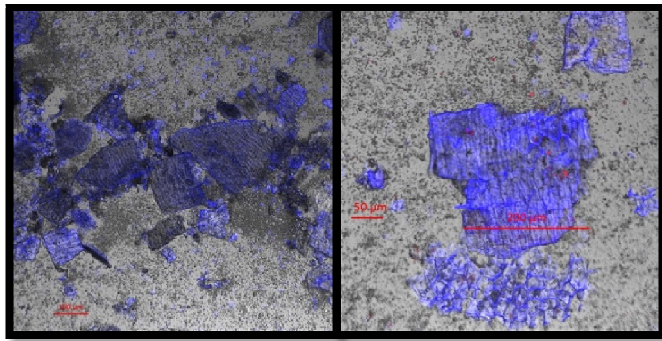
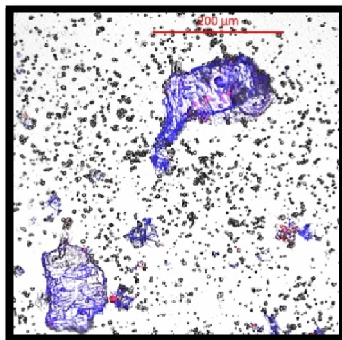
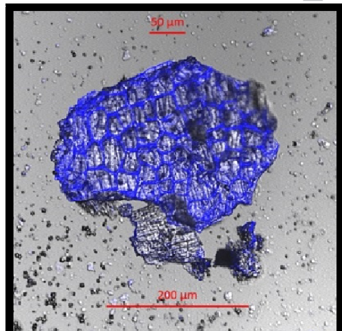


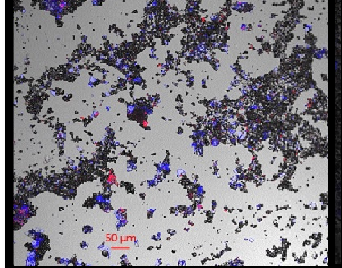
BRM from the 1st pass



BRM from the 2nd pass



BRM from the 3rd pass



Protein bodies (in red) from the 2nd and 3rd BRM passes

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Figure 2. Confocal microscopy of different BRM passes dyed blue with Calcofluor to highlight plant cell walls

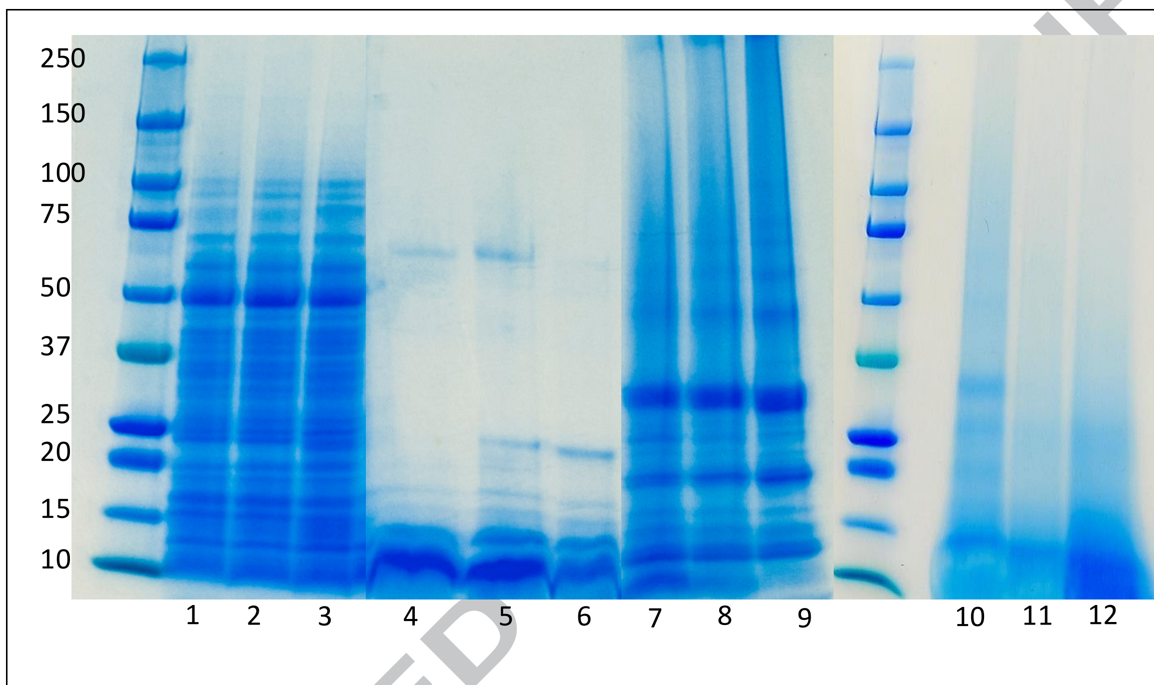

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Table 1. The colour properties of different BRM different passes

Hunter	BRM 1st pass	BRM 2nd pass	BRM 3rd pass
L*(lightness)	43.44^b ± 1.73	48.22^b ± 0.15	60.55^a ± 1.5
a* (red to green)	1.97^a ± 0.1	0.48^b ± 0.03	-0.5^c ± 0.01
b* (yellow to blue)	22.6^a ± 0.13	19.25^b ± 0.28	9.41^c ± 0.18

Different letters show significant differences (P<0.05) between samples in the same row

Table 2. The chemical composition of different BRM passes (different DOM)

Composition (% wb)	1st pass BRM	2nd pass BRM	3rd pass BRM
Protein	12.70^b ± 0.12	13.47^a ± 0.06	8.88^c ± 0.02
Lipid	21.83^a ± 0.23	22.00^a ± 0.0	8.50^b ± 0.71
Free fatty acid as oleic	12.43^a ± 0.28	10.31^b ± 0.08	4.68^c ± 0.03
Starch	11.65^c ± 0.06	19.78^b ± 0.04	55.51^a ± 1.20
Water	10.55^b ± 0.06	10.70^b ± 0.02	11.75^a ± 0.06
Ash	9.18^a ± 0.01	8.05^b ± 0.05	3.38^c ± 0.01
Phytic acid	5.19^a ± 0.10	4.65^a ± 0.11	2.90^b ± 0.27
Non-starch carbohydrate	28.96^a ± 0.21	21.37^b ± 0.06	9.08^c ± 2.10

Different letters show significant differences (P<0.05) between samples in the same row

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Table 3. Relative concentration of protein fractions in different BRM passes

Protein fraction	BRM 1st pass (%)	BRM 2nd pass (%)	BRM 3rd pass (%)
Albumin	$78.25^{a} \pm 3.80$	$75.94^{a} \pm 2.11$	$27.30^{b} \pm 0.07$
Globulin	$9.47^{a} \pm 2.65$	$7.32^{a} \pm 1.01$	$13.46^{a} \pm 1.70$
Glutelin/Oryzenin	$7.73^{c} \pm 1.13$	$13.63^{b} \pm 0.98$	$53.19^{a} \pm 1.16$
Prolamin	$3.20^{b} \pm 0.37$	$3.10^{b} \pm 0.12$	$6.12^{a} \pm 0.73$
Total extractability % (based on N content)	$74.78^{a} \pm 3.95$	$77.25^{a} \pm 3.55$	$68.94^{a} \pm 1.60$

Different letters show significant differences ($P<0.05$) between samples in the same row

Table caption

Table 1. The colour properties of different BRM different passes

Table 2. The chemical composition of different BRM passes (different DOM)

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