

# Pretreatment to increase yield and antioxidant activity of $\gamma$ -oryzanol in rice bran oil

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**ABSTRACT:** Six pretreatment processes to extract oil from Khao Dok Mali 105 rice bran were tested to assess the effect of the processes on the  $\gamma$ -oryzanol content and DPPH scavenging activity of the extract. The pretreatment processes consisted of the following: microwave heating (60–110 °C), hot air heating (70–180 °C), roasting (60–80 °C), parboiling (70 °C), autoclaving (121 °C), and enzyme (amylase) treatment (50 °C). The highest amount of oil was obtained by hot air heating (70 °C) of 0.27 g/g of dried rice bran, whereas the highest  $\gamma$ -oryzanol yield was obtained by parboiling 9.8 mg/g of dried rice bran. The highest  $\gamma$ -oryzanol concentration obtained with a roasting pretreatment (60 °C) was 46.9 mg/ml of rice bran oil.

**KEYWORDS:** Khao Dok Mali 105, extraction, ABTS, DPPH

## INTRODUCTION

Rice is a key industrial crop in Thailand and the main foodstuff in Asian countries, where many types of rice are grown. A famous type of rice grown in the central part of Thailand is Khao Dok Mali 105, which has a characteristic of long shape and white colour, as well as a jasmine-like aroma. Khao Dok Mali 105 is nutritionally rich in protein, vitamins, and ferrous and antioxidant compounds<sup>1</sup>.

The milling process divides rice into five parts: white rice, brown rice, broken-milled rice, rice husks and rice bran. The rice bran by-product accounts for about 10–12% of the rice grain and contains large amount of fibre, vitamins, minerals, and nutrients such as phenolic compounds and vitamin E and its components (tocols, tocopherol, tocotrienol, and  $\gamma$ -oryzanol)<sup>2,3</sup>. A group of ferulic acid esters of phytosterols, collectively called  $\gamma$ -oryzanol, is the main phytochemical in rice bran oil. The  $\gamma$ -oryzanol is used to treat a number of diseases, including high cholesterol, cancer and heart disease, and has antioxidant properties<sup>4-7</sup>.

Rice bran has previously been used as animal feed but the current recognition of the useful compounds contained in rice bran oil has prompted the extraction of antioxidant compounds<sup>8-11</sup>. The

oil extraction process has been extensively developed, starting with conventional extraction with organic solvents<sup>5,12</sup> and extending to ohmic heating<sup>3,13</sup>, hydrothermal treatment (subcritical water)<sup>13</sup>, and green technology using pressurised CO<sub>2</sub> (SC-CO<sub>2</sub>)<sup>3,14-16</sup>. Some extraction methods are still limited due to the high toxin content and cost; therefore, adaptation of the extraction methods is highly. Much research now indicates that the choice of heating pretreatment can enhance the yield of extracted oil and the fractions containing antioxidants, while also stabilizing the rice bran oil<sup>17-20</sup>. The grain also contains a large amount of carbohydrate, so an enzyme hydrolysis pretreatment has been used to obtain a high yield of oil containing antioxidant compounds<sup>21-23</sup>. Surprisingly, little research has focused on  $\gamma$ -oryzanol extraction or on increasing the extracted yield, although the pretreatment methods could conceivably enhance the yield of  $\gamma$ -oryzanol from rice bran.

The aim of the research presented here was to determine the effect of pretreatment methods on the yield of rice bran oil and  $\gamma$ -oryzanol and the resulting antioxidant activity. The results provide new information for improving the oil and  $\gamma$ -oryzanol extraction processes for rice bran.

## MATERIALS AND METHODS

### Rice bran

Khao Dok Mali 105 rice was cultivated in Suphan Buri Province, Thailand. The rice was milled using a typical milling process and the resulting rice bran was kept in a plastic bag and stored in a refrigerator at 4 °C.

### Chemicals

An authentic  $\gamma$ -oryzanol standard was obtained from Wako Pure Chemical Industries, Ltd, Japan. A CN5 HPLC column was bought from ACE. The solvents for HPLC, including methanol, isopropanol, ethyl acetate and hexane, were purchased from Carlo Erba. For extractions and solution preparation, hexane was purchased from Marcon Fine Chemical, USA; acetone and ethanol from Fisher Science, UK; and methanol from Avantor Performance Materials Inc., USA. The  $\alpha$ -amylase enzyme was purchased from Sigma-Aldrich Co., USA and 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma-Aldrich, Germany.

### Material preparation

The rice bran raw material was prepared by sieving through a 150–300  $\mu$ m standard sieve (Endecotts Ltd, England). The desired pretreatment was then applied, followed by continuous extraction of the rice bran oil by a standard extraction method. The moisture content of the rice bran was measured as approximately  $7.99 \pm 0.16\%$ .

### Pretreatment processes

The pretreatment methods were adapted from Thanonkaew et al<sup>19</sup> and Alrahmany et al<sup>22</sup>. The same amount of rice bran, weighting approximately 5 g, was used for all treatments. The six pretreatment methods are shown in Table 1. The first pretreatment method consisted of heating in a microwave at different temperatures (60, 90, and 110 °C) at a frequency of 2450 MHz (Samsung, GE87Q-S, Thailand) for 3 min. Pretreatment temperatures higher than 110 °C for durations longer than 3 min could burn the rice bran, which then turns dark brown; therefore, the limiting pretreatment condition was 110 °C for 3 min. The second pretreatment was heating with hot air at different temperatures (70, 100, and 180 °C) in an oven (Memmert, UN110, Germany) with an incubation time of 10 min. The third method was roasting in a 15 cm diameter domestic pan on a hot plate (Gerhardt, Germany) at 60 or 80 °C for 3 min. The

**Table 1** Pretreatment processing conditions.

Pretreatments	Conditions
Microwave heating	60, 90, and 110 °C for 3 min
Hot air heating	70, 100, and 180 °C for 10 min
Roasting	60 and 80 °C for 3 min in a domestic cooking pan, 15 cm diameter
Parboiling	75 °C for 60 min in a domestic cooking steamer, 30 cm diameter
Autoclave heating	121 °C for 15 min
Enzyme	50 °C for 120 min

fourth method consisted of parboiling in a 30 cm diameter domestic cooking steamer at 75 °C for 60 min. The fifth method consisted of heating in an autoclave at 121 °C and 103 kPa for 15 min—a condition typically used for sterilization and enzyme inhibition. The sixth method consisted of hydrolysis with  $\alpha$ -amylase (1375 unit/ml) under the optimum conditions for enzyme activity at a circulating rate of 180 rpm, and an incubation time of 120 min at 50 °C.

### Extraction method

The same extraction procedure was conducted after all pretreatment methods listed in the previous section. The maceration method described by Ruenngam et al<sup>24</sup> was followed, as this procedure extracted the highest amount of oil. The ratio of rice bran to solvent was 1:4 (g/ml), with hexane as the solvent, and the circulation rate was controlled at 200 rpm with a shaking time of 60 min at 30 °C. All extractions were conducted in 250 ml flasks covered with aluminium foil for light protection. Residues in the extracts were removed by filtration through Whatman No. 1 filter paper and the solvent was removed from the rice bran oil under vacuum in a rotary evaporator (Heidolph, Germany). The residues from the rice bran oil were weighed on a 4-digit balance.

### HPLC analysis

The amount of  $\gamma$ -oryzanol in the extracted oil was measured by HPLC. The stationary phase was a 250×4.6 mm CN5 HPLC column. The mobile phase was an isocratic mixture of hexane: ethyl acetate: acetic acid (97.3:1.8:0.9; v/v) run at a flow rate of 0.5 ml/min. A 20  $\mu$ l sample of each extraction was analysed at 330 nm. The data were reported using LC-solution software and the amount of  $\gamma$ -oryzanol was calculated by comparing the area under the

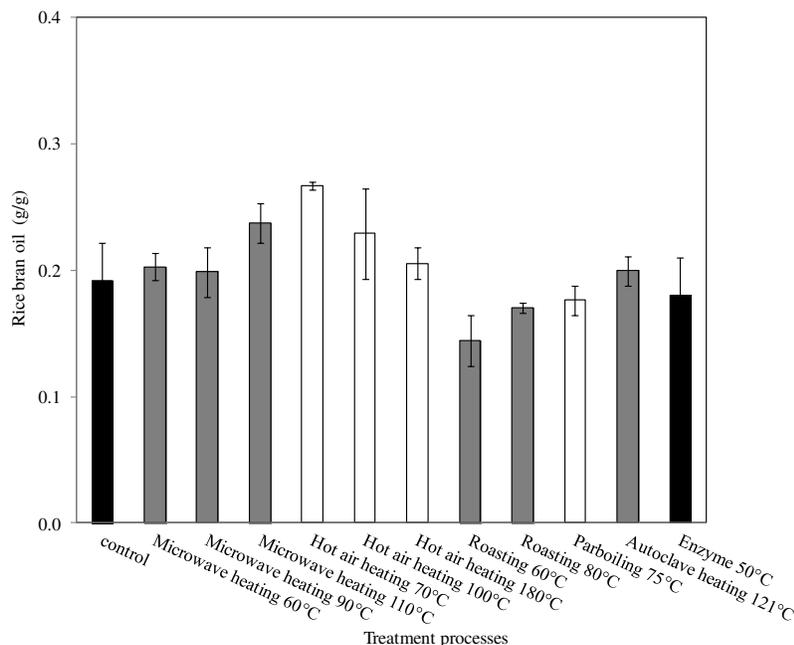


Fig. 1 Rice bran oil recovery from rice bran after different pretreatment processes.

peak appearing at around 27 min to that of a known concentration of the authentic  $\gamma$ -oryzanol standard, which eluted at that retention time.

#### DPPH radical scavenging activity

The free radical scavenging was evaluated using the assay described by Alrahmany et al<sup>22</sup>, with some modifications. Briefly, 100  $\mu$ l of each extract or a BHT standard was added to a 96-well plate containing 100  $\mu$ l 0.5 mg/ml DPPH prepared in ethanol. The mixtures were incubated at 30 °C in the dark for 30 min and then the absorbance was measured at 517 nm and compared to a control (as 100%) using a microplate reader (iEMS Reader MF, Finland). The radical scavenging or DPPH inhibition was calculated as  $1 - Ab_s/Ab_c$ , where  $Ab_c$  is the absorbance of the control sample and  $Ab_s$  is the absorbance of the test sample.

The scavenging activity was measured using a BHT standard prepared in the range of 6.25–100 g/l and then measuring the scavenging activity with the same method. The BHT concentration that gave the equivalent scavenging activity of the extracted oil was calculated.

#### ABTS assay

The ABTS assay was used to determine the antioxidant activity of the rice bran oil. The ABTS<sup>+</sup> was generated through a chemical oxidation reaction

with potassium persulphate<sup>6</sup>. The concentration of the blue-green solution of ABTS<sup>+</sup> radical solution was adjusted with ethanol to get the desired absorbance range (0.1–0.8). A 100  $\mu$ l volume of the extracted oil was then pipetted into a 96-well plate, mixed with 100  $\mu$ l ABTS<sup>+</sup> solution, incubated in the dark at 30 °C for 5 min, and then measured at 734 nm using ethanol as a blank. The ABTS activity was calculated using the same formula as above or expressed as  $\mu$ g/ml Trolox equivalents. The Trolox standard was prepared in ethanol and was linear in the range of 100–800  $\mu$ g/ml.

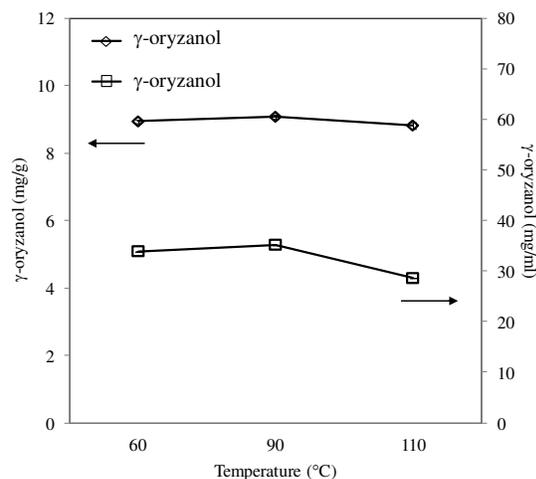
#### Statistic analysis

All experiments were done in triplicate and the data for the amounts of rice bran oil and  $\gamma$ -oryzanol and the antioxidant activities of ABTS and DPPH are presented as means and standard deviations. Statistical analysis was performed using SPSS 22.0. Duncan's multiple range test was used to determine significant differences with ANOVA 0.05 ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

#### Effect of pretreatment processes on amount of rice bran oil

The amount of rice bran oil obtained from each pretreatment process is shown in Fig. 1, where the x-axis is the pretreatment process and the y-axis shows the oil (in grams) extracted per gram of dried



**Fig. 2** Amount and concentration of  $\gamma$ -oryzanol in rice bran oil extracted after microwave pretreatment of rice bran at different temperatures.

rice bran. The amount of rice bran oil obtained with the microwave heating pretreatment was highest (0.24 g oil/g bran) for the 110 °C pretreatment, when compared to the 60 and 90 °C, as shown in Fig. 1 and Fig. 2. The statistical analysis shown in Table 2 indicates that the heating temperature had no significant effect on the amount of rice bran oil extracted (indicated by the same lowercase character for all heating temperatures). The microwave heating distorted and destroyed the original oil body membrane, resulting in the presence of pools of oil and small granular materials in the cells<sup>16</sup>; therefore, the oil was easily released into the bulk solvent. However, heat did not affect the amount of oil recovery even though the oil viscosity was reduced. The rice bran, when heated to 110 °C, turned from a pale yellow to a dark brown, with a rancid smell and bad oil quality (oxidative rancidity) because heat burned the bran. Residual lipase activity in the rice bran after heating at 110 °C released large quantities of free fatty acids and  $\gamma$ -oryzanol and added to the rancid smell<sup>25</sup>.

Hot air heating, which heated the material to the desired temperature by convection, decreased the amount of extracted rice bran oil, as shown in Fig. 1 and Fig. 3. This had the opposite effect to that seen with microwave heating, and was possibly due to the effect of too high a temperature (around 180 °C), which would accelerate the loss of moisture at the outside surface, resulting in collapse of the material. However, temperature increases between 70 and 180 °C had no significant effect on amount of

**Table 2** Amounts of extracted oil,  $\gamma$ -oryzanol content, and antioxidant activity for each pretreatment condition.

Treatments	RBO* (g/g)	$\gamma$ -oryz.† (mg/g)	ABTS (%)	Trolox (μg/ml)	DPPH (%)	BHT (mg/ml)
Microwave						
60 °C	0.20 <sup>a</sup>	8.94 <sup>ab</sup>	71 ± 7 <sup>b</sup>	7.54	51 ± 3	3.60
90 °C	0.20 <sup>aA</sup>	9.08 <sup>abBC</sup>	72 ± 5	7.58	51 ± 3 <sup>ab</sup>	3.61
110 °C	0.24 <sup>a</sup>	8.82 <sup>ba</sup>	63 ± 4	6.74	50 ± 2	3.58
Hot air						
70 °C	0.27 <sup>ba</sup>	9.26 <sup>bc</sup>	58 ± 6 <sup>a</sup>	6.24	50 ± 3 <sup>a</sup>	3.54
100 °C	0.23 <sup>b</sup>	8.93 <sup>a</sup>	58 ± 2	6.19	50 ± 1	3.54
180 °C	0.21 <sup>b</sup>	8.82 <sup>a</sup>	61 ± 5	6.53	50 ± 2	3.52
Roasting						
60 °C	0.14 <sup>c</sup>	8.81 <sup>a</sup>	69 ± 5 <sup>b</sup>	7.31	55 ± 4	3.87
80 °C	0.17 <sup>ca</sup>	9.10 <sup>aC</sup>	66 ± 1	7.00	54 ± 0 <sup>b</sup>	3.80
Parboiling						
70 °C	0.18 <sup>A</sup>	9.76 <sup>D</sup>	62 ± 2 <sup>ab</sup>	6.62	54 ± 1 <sup>b</sup>	3.83
Autoclave						
121 °C	0.20 <sup>A</sup>	8.86 <sup>B</sup>	68 ± 4 <sup>b</sup>	7.20	55 ± 2 <sup>ab</sup>	3.78
Enzyme						
50 °C	0.18 <sup>A</sup>	8.31 <sup>E</sup>	71 ± 1 <sup>b</sup>	7.53	53 ± 4 <sup>c</sup>	3.73

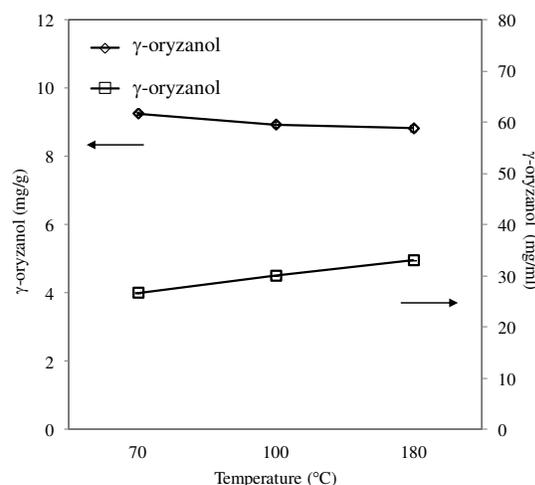
\* RBO: rice bran oil (extracted amount).

†  $\gamma$ -oryz.:  $\gamma$ -oryzanol content.

Values are means ± SD of triplicate.

The same superscript lower-case letter in a column indicates no significant difference in each pretreatment process at  $p < 0.05$ .

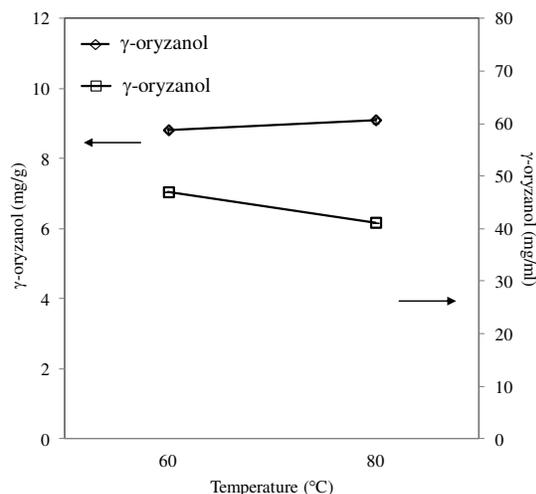
The same superscript capital letter in a column indicates that there is no significant difference between different pretreatments at  $p < 0.05$ .



**Fig. 3** Amounts and concentrations of  $\gamma$ -oryzanol in rice bran oil extracted after hot air pretreatment of rice bran at different temperatures.

extracted oil, as shown by the statistical analysis in Table 2. This might indicate that the collapse of the material prevented the penetration of the solvent inside the material.

The effects of pretreatment of the rice bran by roasting at different temperatures are shown in



**Fig. 4** Amounts and concentrations of  $\gamma$ -oryzanol in rice bran oil extracted after pretreatment of rice bran by roasting at different temperatures.

**Fig. 1** and **Fig. 4**. Increasing the roasting temperature from 60–80 °C increased the amount of rice bran oil recovered; however, this result was not significantly different for either temperature (**Table 2**). The highest amount of rice bran oil (0.27 g oil/g bran) was obtained after hot air heating at 70 °C, while the lowest amount (0.14 g/g) was obtained by roasting at 60 °C. This difference might reflect the possibility that heat from the roasting process directly affects the surface of the material, so that moisture moves quickly from the surface, causing collapse of the material. In contrast, heat from air passes through the material and disrupts the original oil body membrane, causing an oil pool to build up<sup>16</sup>. The hot air might also cause swelling of the material, which would allow the solvent to penetrate more easily into the material and aid in oil removal. The microwave heating process produced a lower amount of extracted oil than hot air heating at the same range of pretreatment temperatures (90 and 100 °C) because microwave heating strongly lowered the moisture content, which then made the material more brittle and caused it to collapse<sup>19</sup>.

Heating processes involving steaming, such as parboiling and autoclave heating, extracted a lower amount of oil compared to dry heating processes, such as microwave heating and hot air heating. On the other hand, the roasting process released even less oil than parboiling and autoclave heating processes, in contrast to previous research<sup>19</sup> conducted at a higher temperature of 150 °C. The effect of pretreatment temperature on the removal

of moisture might be the critical factor determine the final amount of extracted oil. Even through steam could penetrate through the material, it did not increase the extracted oil yield. Steam under high pressure could have caused the material to collapse, thereby blocking oil release. Steam and pressure would also break the bulk oil into small droplets, which could then be trapped in water inside the material, creating difficulty in extraction with a non-polar solvent. The amount of oil released following the enzyme hydrolysis pretreatment was in the same range as that obtained with the roasting and parboiling processes because amylase hydrolyses carbohydrates, thereby facilitating oil extraction.

The mean values of the greatest amounts of oil extracted by each pretreatment process were analysed for significant differences. As shown in **Table 2**, the highest amounts of rice bran oil extracted from each pretreatment did not differ significantly at the  $p < 0.05$  level.

#### Effect of pretreatment processes on amount of $\gamma$ -oryzanol

The effects of the pretreatment processes on the amounts of  $\gamma$ -oryzanol are presented in **Fig. 5**, where the  $x$ -axis shows the pretreatment processes and the primary  $y$ -axis shows the amount of  $\gamma$ -oryzanol (mg/g dried rice bran) (bars) and the secondary  $y$ -axis shows the  $\gamma$ -oryzanol concentration (mg/g rice bran oil) (dots). The highest amount of  $\gamma$ -oryzanol was obtained from the parboiling process at 75 °C (9.76 mg/g dried rice bran), whereas the highest  $\gamma$ -oryzanol concentration was achieved with the roasting process at 60 °C (64.93 mg/ml). The roasting process accelerates removal of moisture from the material surface, causing collapse of the surface and trapping the oil inside the material; therefore, the highest concentration of  $\gamma$ -oryzanol was obtained.

The means of the highest amounts of oil obtained from each pretreatment process were also evaluated for significant differences, as demonstrated in **Table 2**. The amount of  $\gamma$ -oryzanol obtained from microwave heating (90 °C), hot air heating (70 °C), and roasting (80 °C) showed no significant differences at  $p < 0.05$ .

The effect of microwave heating temperature on the amount of  $\gamma$ -oryzanol is presented in **Fig. 5**, where the  $x$ -axis shows the pretreatment temperature from 60–110 °C produced by the microwave heating process. The primary  $y$ -axis shows the amounts of  $\gamma$ -oryzanol (mg/g dried rice bran) and secondary  $y$ -axis shows the  $\gamma$ -oryzanol concentra-

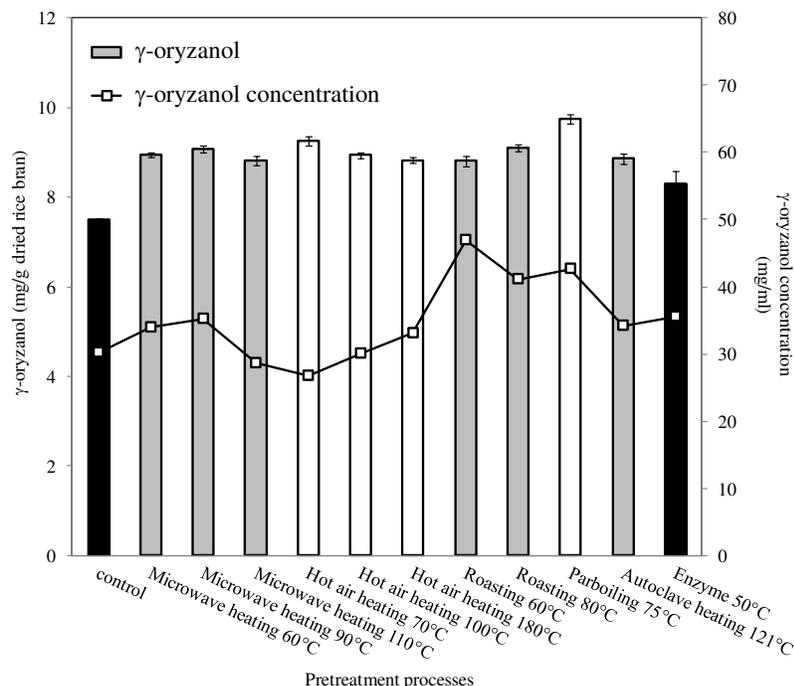


Fig. 5 Amount of  $\gamma$ -oryzanol in rice bran oil extracted from rice bran after different pretreatment processes.

tion (mg/ml rice bran oil). Increasing the heating temperature from 60 °C to 110 °C resulted in a slight increase in the  $\gamma$ -oryzanol concentration in the oil; this concentration then dropped for increases in the range of 90–110 °C. However, the highest value of 35.2 mg/ml was achieved at 90 °C. The highest amount of oil was extracted at 110 °C, which resulted in the lowest value for the  $\gamma$ -oryzanol concentration for material pretreated at that temperature. The amount of  $\gamma$ -oryzanol remained quite stable as the heating temperature was increased from 60 °C to 110 °C, as confirmed elsewhere<sup>20, 25–27</sup>. However, heating at a temperature more than 120 °C enhanced degradation of  $\gamma$ -oryzanol, as reported elsewhere<sup>20, 27</sup>.

The statistical analysis shown in Table 2 confirmed that all experiments showed significant differences. Heating promotes fatty acid degradation by cleavage of oils to free fatty acids and low molecular weight compounds such as  $\gamma$ -oryzanol<sup>25, 28</sup>. Even though microwaves are considered the most energy-efficient type of heating and a rapid method for bran stabilization, microwave pretreatment still has limitations that require preliminary tests of feasibility for any application<sup>26</sup>.

The effects of a hot air heating pretreatment on the amounts of  $\gamma$ -oryzanol are presented in Fig. 5. The amount of  $\gamma$ -oryzanol decreased when the pre-

treatment temperature increased from 70–180 °C and reached the highest value (9.3 mg/g dried rice bran) at 70 °C. Lipase is one type of enzyme present in rice bran and it promotes rancidity by cleaving fats to low molecular weight compounds, such as free fatty acids. The optimum temperature for lipase activity is around 60–70 °C, so that the increased amount of low molecular weight compounds might be  $\gamma$ -oryzanol<sup>26, 29</sup>. However, at the higher temperature of 100 °C, the loss of moisture inside the material resulted in no activity of enzyme, so the  $\gamma$ -oryzanol level was low<sup>25, 27</sup>. Moreover, a pretreatment temperature higher than 110 °C promotes degradation of  $\gamma$ -oryzanol, which resulted in the lowest value, as shown for the 180 °C pretreatment in Fig. 3<sup>25</sup>. The  $\gamma$ -oryzanol concentration was high following the 180 °C pretreatment because the amount of extracted oil was the lowest obtained. The statistical analysis shown in Table 2 indicates that the pretreatment temperature of 70 °C gave a significantly higher amount of  $\gamma$ -oryzanol than that obtained with any other pretreatment temperatures.

The amount of  $\gamma$ -oryzanol obtained is shown in Fig. 5, where the x-axis is the temperature ranging between 60 and 80 °C for the roasting pretreatment. The amount of  $\gamma$ -oryzanol was the highest at 80 °C (9.1 mg/g dried rice bran) because of the enzyme activity. The statistical analysis shown in Table 2

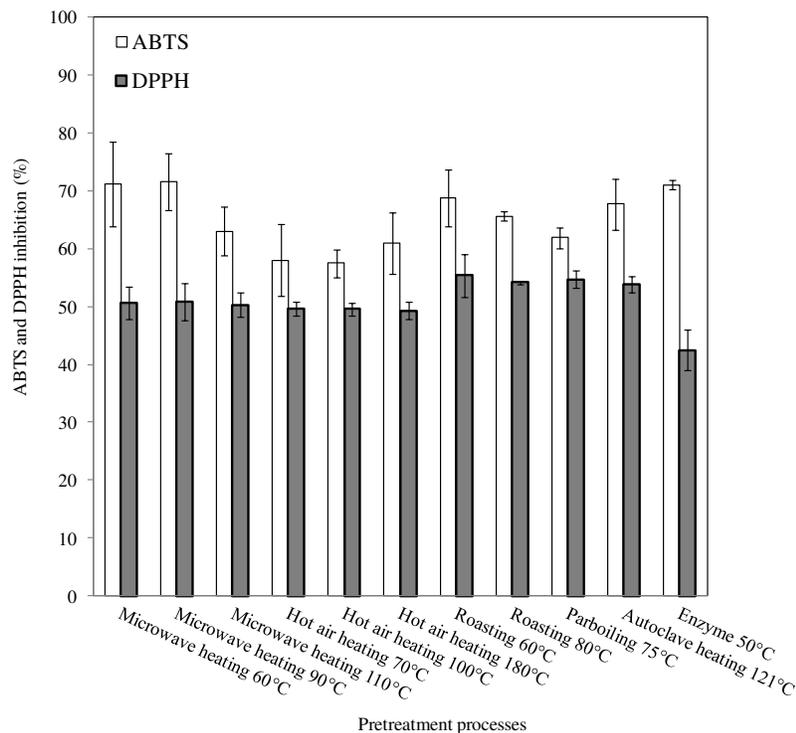


Fig. 6 Antioxidant activity of compounds in rice bran oil extracted from rice bran after different pretreatment processes.

indicates no significant differences for the two pretreatment temperatures.

#### Effect of pretreatment processes on antioxidant activity

The results for both antioxidant activities are displayed on the y-axis in Fig. 6; the x-axis lists the pretreatment processes. The activity of DPPH scavenging (grey bars) shows a higher percentage scavenging for the oil extracted after roasting (60 °C) and parboiling processes, when compared to the other pretreatment processes. The overall trend for the antioxidant activity parallels that seen for the  $\gamma$ -oryzanol concentration in Fig. 2.

The amount of BHT equivalents for DPPH scavenging is indicated in Table 2. The percentages for the ABTS assay are also shown in Fig. 3 as white bars. The trend of ABTS inhibition differed from the trend of  $\gamma$ -oryzanol concentration, which might indicate effects on the polyphenol and vitamin E content of the rice bran oil<sup>19,25</sup>. The highest amounts of oil from each pretreatment process were tested for significant differences between means (Table 2).

#### CONCLUSIONS

This study was conducted assuming that pretreatment proceeds prior to extraction can affect the raw

material resulting in increased amount of extracted compound (rice bran oil and  $\gamma$ -oryzanol). Heat and lipase activity had physical effects on the material and  $\gamma$ -oryzanol stability. The amount of oil achieved the highest value from pretreatment by hot air heating at 70 °C and the amount of  $\gamma$ -oryzanol obtained was the highest by parboiling pretreatment process whereas  $\gamma$ -oryzanol concentration reached the highest value by roasting pretreatment process at 60 °C. This study also demonstrated that rice bran oil acts as free radical scavengers to inhibit DPPH activity and bleach ABTS.

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