# Monitoring the Effect of Different Storage Conditions of Cold-Pressed Rice Bran Oil

Chutima Ponchurdchai and Jirada Singkhonrat

***Abstract*—This study investigated the effect of different storage conditions of cold-pressed rice bran oil from 2 different regions in Thailand (Lopburi province, LRBO and Yasothorn province, YRBO). All samples were stored at different temperature as opened and sealed bottle for 12 months. Fatty acid composition by nuclear magnetic resonance, NMR spectroscopy, chemical properties such as acid value, iodine value and peroxide value were reported. Also, the antioxidant content and its activity were evaluated, including FRAP assay (Ferric reducing antioxidant potential) and DPPH radical scavenging assay (1, 1-diphenyl-2-picrylhydrazyl radical reducing power methods). Cold-pressed rice bran oils sealed bottles for 12 months and freshly opened bottles have shown no affect to acid value, iodine value and peroxide value ($p<0.05$). Fatty acid composition of oil by $^1$H NMR technique obtained a good agreement with gas chromatography technique and observed mol% of unsaturated fatty acid was slightly decreased after year storage. After 12-month storage below 5 ℃ temperature, LRBO and YRBO showed good radical scavenging activities compared to quercetin with DPPH $IC_{50}$ of 0.647 ±0.02 and 0.663 ±0.02 mg/mL and with FRAP assay of 0.42106 ± 0.002 and 0.37471 ± 0.002 g quercetin equivalents/g, respectively.**

***Index Terms*—Rice bran oils, fatty acid, stability, $^1$H NMR.**

## I. Introduction

Oil obtained from the rice bran [1] has attracted much interest with many health benefits due to its rich source of natural antioxidant compounds (γ-oryzanol, tocopherols and tocotrienols). These compounds are considered to have an important role in preservation of the quality of oils due to their antioxidant activity. Rice bran is extracted or pressed to produce the rice bran oil, RBO which calls cold-pressed RBO if pressing and no further refinery process has been used. The major component of RBO is triacylglycerols, minor component consist of waxes, mono- and diglycerides, free fatty acids, and other compounds such as tocopherols, tocotrienols, and phytosterols [2]. Those unsaponifiable constituents (USC) of RBO have been reported and found in high levels, include phytosterols (1.5-2%), oryzanols (1.2-1.8%), tocopherols and tocotrienols (0.15-0.2%) which are bioactive components possessing powerful antioxidative activity [3]-[5].

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The composition of γ-oryzanol is mixture of phytosteryl ferulates including 4-hydroxy-3-methoxycinnamic acid (ferulate) esters of cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, β-sitosterol ferulate, and campesterol ferulate that can be separated and quantified by high performance liquid chromatography [6], [7].

In addition, the determination of the proportion of fatty acid in oils and fats can be analyzed by proton nuclear magnetic resonance spectroscopy, $^1$H NMR spectroscopy in a short period of time and also has a great deal of structural information, required small amount of sample. There are many reports of NMR application for olive oil, walnut oil and other vegetable oils by several authors [8]-[10].

The gas chromatography-mass spectrometry, GC-MS analysis and the integration of signals in the $^1$H NMR spectra were used for determining the fatty acid compositions and molecular degradation during a year storage. The antioxidant contents and its activity of RBO were also investigated. The aim of this study was to determine the impact of storage temperature on changes in molecular degradation for shelf life of RBOs and to evaluate the stability by monitoring acid, iodine and peroxide values from different storage conditions.

## II. Materials and Methods

### A. Chemical Properties of Oil

Cold-pressed RBO that obtained in 2013 from 2 major rice production regions in Thailand (Lopburi province LRBO and Yasothorn province YRBO) were used 3-6 bottles in this work. Only one bottle of each RBO samples was freshly opened and analysed at time 0 month, RBO-1 and at time 12 month, RBO-2. The rest of both RBO samples were kept as sealed bottle provided and stored in the darkness at room temperature, RBO-3 and in refrigerator below 5 ℃, RBO-4 throughout the period of 1 year (at time 12 months). Oil sample was evaluated the quality of oil by using classical method to determine acid value, AV, peroxide value, PV and iodine value, IV. All chemical analyses were carried out according to the association of official analytical chemists, AOAC method.

### B. $^1$H NMR Analysis

Oil sample (approximately 70 mg per sample) was added to 5mm NMR tubes and dissolved in 600 μL of deuterated chloroform and a small proportion (a drop) of dimethyl sulfoxide-*d6*. The $^1$H NMR spectroscopy was performed on a Bruker Avance 400-MHz Spectrometer. The chemical shift was expressed in δ scale (ppm). All NMR data were analyzed by NMR software "Mnova" from Mestrelab Research and

solvents were used as internal references.

### C. Fatty acid Composition

The fatty acid composition was determined by converting oil to methyl esters through esterification reaction by heating oil (30 g) to 80 ℃. Then, added 3%wt potassium hydroxide in methanol into reaction solution and stirred this solution for 30 min. After refluxing, the solution was cooled to room temperature and collected the upper layer and then separated glycerol out. The solution was neutralized by warm water and dried with sodium sulfate anhydrous. The methanol was evaporated after filtrate the sodium sulfate anhydrous.

The fatty acid methyl esters were analyzed by using a gas chromatography (Shimadzu GC-2010 plus) equipped with a split/splitless capillary injector and mass spectrometer detector. Analytical separation was achieved on HP-INNOWAX column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was He, which was supplied at a flow rate of 1.41 mL/min. The amount injected was 0.1 μL and the split ratio was 1:60. Temperature settings were as follows: injector 230 ℃; The oven temperature was held at 160 ℃ for 5 min. and then programmed to 210 ℃ at 4 ℃/min, and held for 6 min at 210 ℃.

### D. Antioxidant Activity Determination

*2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay* [11]: the stock solution 0.24 mg/mL of DPPH in methanol was prepared and the working solution was obtained by mixing 10 mL of the stock solution with 45 mL methanol to obtain a solution having a concentration of about 43 μg/mL.

Samples stock solution (10 mg/mL in methanol) was further diluted with methanol to obtain several concentrations test sample in a range of 0.1-2.0 mg/mL. Ascorbic acid was used as positive standards. An aliquot (30 μL) of these solutions (samples and positive standards) were allowed to react with 170 μL of the DPPH working solution for 30 min in the dark. The absorbance was then measured at 515 nm. Sample concentrations providing 50 %inhibition, $IC_{50}$ was calculated from the graph plotted between %inhibitions against sample concentrations. The tests are carried out in triplicate for each sample. The percentage of inhibition was calculated according to (1)

$$\%\text{Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

where

$A_{control}$ = absorbance of DPPH solution

$A_{sample}$ = absorbance of DPPH reacts with sample

*Ferric reducing antioxidant power, FRAP Assay* [12]: Assay of reducing power was carried out by potassium ferric cyanide method. Sample solutions were prepared in methanol to have concentrations in a range of 5 mg/mL. Sample (200 μL) was mixed with 500 μL of phosphate buffer (0.2 M, pH 6.6) and 500 μL of 1% w/v potassium ferricyanide. The mixture was then incubated at 50 ℃ for 30 min. The 500 μL of 10% w/v trichloroacetic acid was added into the mixture, which was then centrifuged at 3000 rpm for 30 min. Finally, 600 μL of the supernatant solution was collected and mixed with 600 μL of distilled water and 120 μL 0.1% w/v ferric chloride and absorbance was measured at 700 nm. Quercetin (0.01 - 10 μg/mL) was used as a standard and phosphate buffer was used as a blank solution. Result were compared and expressed in quercetin equivalents (mg/g sample).

### E. γ-Oryzanol Analysis

UV-Vis spectroscopy was applied to determine the oryzanol contents directly from oil sample without any extraction by weighed 10 mg of RBO and dissolved in hexane to make the volume up to 10 mL. The OD was measured in a 1.0 cm cell at 314 nm in a Shimadzu UV-240 double beam recording spectrophotometer. The oryzanol content in the oil was calculated by using (2) [7]

$$\text{oryzanol, g\%} = \frac{\text{OD of hexane solution}}{\text{weihgt (g) of oil}} \times \frac{100}{358.9} \quad (2)$$

where OD is optical density and 358.9 is the specific extinction coefficient of oryzanol.

### F. Statistical Analysis

The determinations of conventional analysis and NMR analysis were carried out in triplicate and reported as mean values ± SD. Analysis of covariance (ANACOVA) was carried out on the experimental data by Window and revealed that these significant differences ($p$<0.05) were between conditions (time and treatment).

## III. Results and Discussion

### A. Chemical Properties of Oil

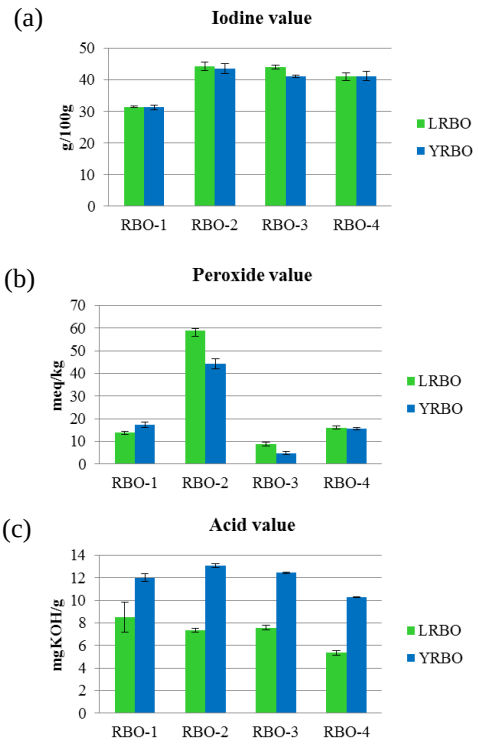


Fig. 1. (a) Iodine values, (b) acid values and (c) peroxide values of L-RBO and Y-RBO in different storage conditions: freshly opened **RBO-1**; opened over 12 months **RBO-2**; sealed at room temperature over 12 months **RBO-3**; sealed below 5 ℃ over 12 months **RBO-4**.

According to different storage conditions, Fig. 1a showed

that the IV of both RBOs after one-year storage strangely insignificantly different, which may be subjected by complexity of crude cold-pressed RBO matrixes. LRBO-2, LRBO-3 and L-RBO-4 observed the IV of 44.19±1.32, 43.98±0.56 and 41.06±1.19 g/100g, respectively. YRBO-2, YRBO-3 and YRBO-4 observed the IV of 43.56±1.55, 41.13±0.29 and 41.16±1.42 g/100g, respectively. When the different storage conditions were investigated through the year, all samples were appeared with sediment and colloid of micro particles and suggested to free the unsaturated fatty chains and unbound double bonds. That was suggested as a reason that both freshly opened bottles obtained lower IV in LRBO-1 and YRBO-1 which observed the IV of 31.45±0.18 and 31.24±0.73 g/100g, respectively.

Freshly opened bottle of LRBO-1 and YRBO-1 observed the PV of 14.07±0.23 and 17.32±1.14 meq/kg, respectively. From Fig. 1b, after year storage, both RBO obtained significantly difference of peroxide value except YRBO obtained below 5 ℃ is not significant difference from fresh oil. LRBO-2, LRBO-3 and LRBO-4 observed the PV of 58.76±0.83, 8.85±0.63 and 15.84±0.81 meq/kg, respectively. YRBO-2, YRBO-3 and YRBO-4 observed the PV of 44.21±2.35, 4.82±0.78 and 15.52±0.50 meq/kg, respectively. The oil exposed to the air obtained higher peroxide value due to higher oxidation rate with oxygen molecules in the direct air exposure, but without air exposure oil storage below 5 ℃ has lower peroxide value than storage at room temperature. It can be indicate that after one year storage of oil as sealed bottle is not affected to peroxide value significantly ($p$<0.05). In addition, AV in Fig. 1c of both oils in all conditions is not significant difference ($p$<0.05). LRBO and YRBO observed the AV range of 5.34-8.52 and 12.00-13.29 mgKOH/g, respectively. It can be suggested that after one year storage of oil is not affected to hydrolysis according to relatively stable acid value in every condition.

### *B. Fatty Acid Composition*

TABLE I: THE EQUATIONS FOR DETERMINING A FATTY ACID COMPOSITION BY $^1$H NMR TECHNIQUE

| | Method A | Method B |
|---|---|---|
| S (%) | 100 [B/ (A+B)] | 100 [2B/3D] |
| O (%) | 100 [(E/D)-2[B/(A+B)]] | 100 [(3E-4B)/3D] |
| L (%) | 100 [(C/2D) - (E/D) + [B/(A+B)]] | 100 [Ln+L+S]* |
| Ln (%) | 100 [1-(C/2D)] | 100 [(4A+4B-3C)/6D] |

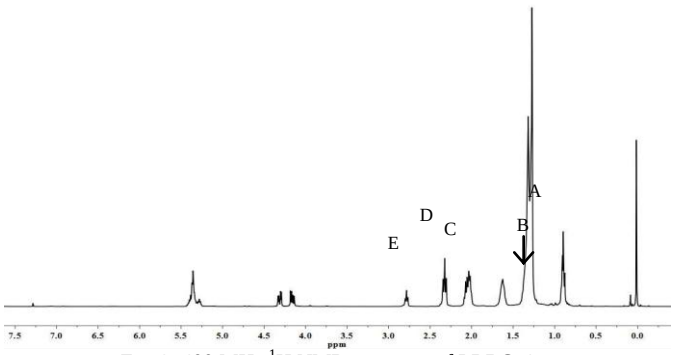


Fig. 2. 400 MHz $^1$H NMR spectrum of LRBO-4.

The determination of the proportions of oleic, O, linoleic, L, linolenic, Ln and saturated, S acyl groups in RBO carried out by using the equations in Table I. The parameter A-E is the integration of some signals of the spectra as show in Fig. 2. Method A [13] based on proportional methyl group in sample and method B [14] based on the methylenic hydrogen atom in α position. From Table II, it can be observed that $^1$H NMR technique was analyzed and calculated the intensity for the unsaturated fatty acid compositions of RBO samples and GC-MS technique was provided all the saturated fatty acid compositions of the RBO samples. When compared with GC-MS technique, $^1$H NMR can be made in a few minutes and without any chemical manipulation of sample. Therefore, equation in method A, which obtained with reasonable standard deviations (<0.7), was used for comparing the stability of RBOs and the results were analyzed from their intensity according to assigned $^1$H NMR spectra, shown in Fig. 3.

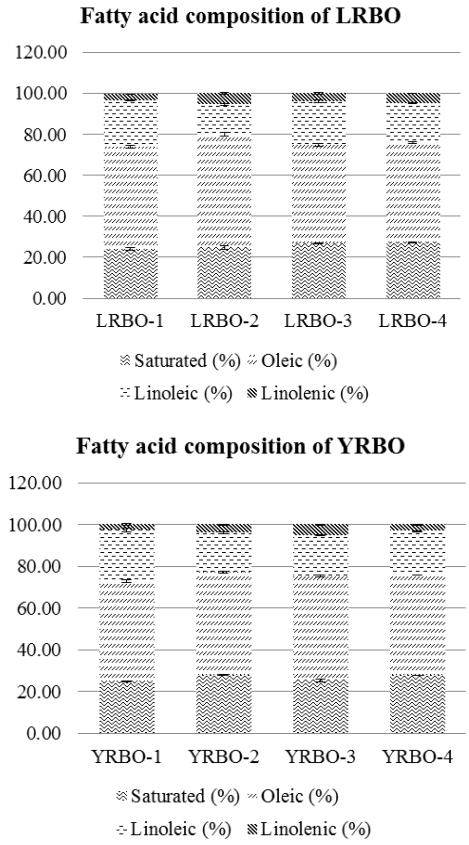


Fig. 3. The fatty acid composition of RBO in different storage condition derived from $^1$H NMR spectra and calculated by method A.

LRBO samples revealed no impact of the storage condition by keeping the fatty acid profiling consistently, whereas YRBO sample, observed that sealed bottles at room temperature in the dark, showed the best condition on steadying the level of unsaturated fatty acid composition significantly over 12-month storage.

### *C. Antioxidant Activity*

RBOs, kept as sealed bottle below 5 ℃, were screened to determine its free radical scavenging activities. Two different methods were used to test the antioxidant activity of the extract, including FRAP assay and DPPH radical scavenging assay. From Table III, LRBO-4 and YRBO-4 showed good DPPH radical scavenging activities with $IC_{50}$ of 0.647±0.02 and 0.663±0.02 mg/mL and FRAP of 0.42106± 0.002 and 0.37471± 0.002 quercetin equivalents g/g, respectively.

TABLE II: FATTY ACID COMPOSITION OF LRBO-4 AND YRBO-4

| | LRBO-4 | | | YRBO-4 | | |
|---|---|---|---|---|---|---|
| | GC-MS | $^1$H NMR | | GC-MS | $^1$H NMR | |
| | | Method A | Method B | | Method A | Method B |
| **Saturated fatty acid** | | | | | | |
| C14:0 | 0.75±0.02 | + | + | 0.65±0.10 | + | + |
| C16:0 | 20.87±0.14 | + | + | 20.57±0.45 | + | + |
| C18:0 | 4.6±0.17 | + | + | 4.47±0.44 | + | + |
| Total | 26.22±0.33 | 27.28±0.2 | 32.48±0.27 | 25.69±0.99 | 27.72±0.03 | 42.99±0.06 |
| **Unsaturated fatty acid** | | | | | | |
| C18:1 | 42.22±0.33 | 48.70±0.38 | 43.75±0.44 | 44.15±1.56 | 48.21±0.18 | 33.39±0.27 |
| C18:2 | 29.31±0.54 | 19.17±0.21 | 18.67±0.21 | 28.02±0.87 | 21.02±0.29 | 20.09 ±0.31 |
| C18:3 | 2.25±0.03 | 4.85±0.02 | 5.10±0.02 | 2.13±0.14 | 3.06±0.09 | 3.53±0.11 |

- (+) cannot be calculated from the equations.
- C14:0-myristic acid, C16:0-palmitic acid, C18:0-stearic acid, C18:1-oleic acid, C18:2-linoleic acid, C18:3-linolenic acid

These result showed that LRBO-4 had slightly better antioxidation activities than YRBO-4 with higher value (2.47%) in $IC_{50}$ of DPPH and lower quercetin equivalent (12.4%) in FRAP.

TABLE III: ANTIOXIDANT ACTIVITY OF RBO

| Sample | DPPH [1] $IC_{50}$ (mg/ml) | FRAP[2] Quercetin Equivalent (g/g) |
|---|---|---|
| **LRBO-4** | 0.647±0.02 | 0.42106±0.002 |
| **YRBO-4** | 0.663±0.02 | 0.37471±0.002 |

[1] $IC_{50}$ = concentration that can reduce Free radical from 100% to 50%
[2] Quercetin Equivalent (g/g) is amount of quercetin that gives antioxidant property equivalent to the antioxidant property of sample.

The differences in the oxidation activity of the oils (LRBO and YRBO) may be attributed to the different processing and refining conditions applied. It was noticed that rice bran (DPPH activity in $EC_{50}$ of 0.56 mg/mL [15]) possessed a greater activity than one-year old cold-pressed rice bran oils. This may be due to the fact that natural polyphenols and other antioxidants in oil can vary with the conditions of extraction and processing and can be decreased to a greater extent while following the pressing methods for obtaining commercial products and period of storage.

### *D. γ-Oryzanol Contents*

The samples of RBO were observed the level of antioxidant contents. According to UV-Vis spectroscopy technique, both oil samples (LRBO and YRBO) obtained a high oryzanol contents. The different storage condition indicated the effect of oryzanol contents as shown in Table IV. RBO which is kept as sealed bottle below 5℃ revealed higher level of oryzanol than other condition.

TABLE IV: ORYZANOL CONTENTS OF RBO

| | %Oryzanol | |
|---|---|---|
| | **Lopburi, L** | **Yasothorn, Y** |
| **RBO-2** | 1.39±0.02 | 1.69±0.05 |
| **RBO-3** | 1.50±0.21 | 1.72±0.03 |
| **RBO-4** | 1.57±0.05 | 1.89±0.10 |

## IV. CONCLUSION

The different storage conditions affected to the stability of cold-pressed rice bran oil over a year. On the other hand, the comparison of freshly opened and one-year old sealed bottle samples had shown indifferent in acid value peroxide value and iodine value. In molecular level, the fatty acid composition of oil by $^1$H NMR technique can be alternative method to GC-MS technique also showed slightly decreasing on unsaturated fatty acid composition in all condition over a year storage. RBO in sealed bottle and storage below 5℃ over a year showed a good level of antioxidant activity and oryzanol contents. A year shelf-life of cold-pressed RBO is suggested and appreciated for consuming as an alternative source of antioxidants.

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