

## Extraction and Determination of Oryzanol in Rice Bran of Mixed Herbarium UKMB; AZ 6807: MR 185, AZ 6808: MR 211, AZ6809: MR 29

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**Abstract:** The level of total lipid and oryzanol content, an important antioxidant compound in locally produced bran was investigated. Total lipid in rice bran was extracted using 3:2 chloroform:methanol mixture yielding 16.4% fat. Oryzanol content was determined without saponification using a reverse-phase HPLC. Four fractions of oryzanol were successfully separated and quantitated. The 4 isomers were cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, campestryl ferulate and mixtures of  $\beta$ -sitosterol ferulate and cycloartenyl ferulate. The oryzanol content of local mixed varieties ranged from 23.7–43.0 mg g<sup>-1</sup>. The oryzanol concentration may depend on factors such as plant varieties, processing methods employed, extracting solvent used and ratio of extracting solvent to bran as well as extracting solvent temperatures. This study showed the potential of oryzanol extract from rice bran as a source of antioxidant.

**Keywords:** Vitamin, oryzanol, rice bran, food analysis

### INTRODUCTION

Rice bran, a by-product of the rice milling process constitutes about 10 wt % of rough rice grain. The bran layer contains 18 - 22% oil, making it the richest oil source from a grain by-product (Saunders, 1990). The hypocholesterolemic effect of rice bran has been attributed to various fractions of the bran such as the neutral detergent fiber, hemicellulose, rice bran oil and its unsaponifiable matter (Nicolosi *et al.*, 1991; Visser *et al.*, 2000). Compared to other vegetable oils, rice bran oil contains considerably high (4%) unsaponifiable matter which includes phytosterols, triterpene, alcohols, tocopherols and oryzanol (Nicolosi *et al.*,

1991; Raghuram and Rukmini, 1995). The oryzanol alone has been reported to constitute around 20 - 30% in the unsaponifiable matter of the bran and has been shown to have many pharmaceutical uses such as for growth acceleration, regulation of estrous cycle and an effective antioxidant compound (Seetharamaiah and Prabhakar, 1986).

Oryzanol or  $\gamma$ -oryzanol is a mixture of sterol esters of ferulic acid. This antioxidant compounds was first isolated in 1955 by Kaneko and Tsuchita. Norton (1995) reported that the complete oryzanol group is unique to rice bran oil and the exact composition of oryzanol depends on the rice cultivars. Gamma-oryzanol, a mixture of phytosterol ferulates comprises 3 major components;

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cycloartenyl ferulates, 24-methylene-cycloartenyl ferulate and campesteryl ferulate. Ten fractions of  $\gamma$ -oryzanol isomers from crude rice bran have been successfully identified and isolated using reverse-phase HPLC (Xu and Godber, 1999). There have been many reports on the physiological properties of  $\gamma$ -oryzanol such as having the superoxide dismutase-like antioxidant activity and hypocholesterolemic effects in animal models (Hundermer, 1991; Kahlon *et al.*, 1992, 1996; Rouanet *et al.*, 1993) and human subjects (Gerhardt and Gallo, 1998; Visser *et al.*, 2000). Besides the well-documented health benefits, oryzanol also has been reported as a potential additive in various food products, pharmaceuticals and cosmetics (Lloyd *et al.*, 2000).

Although numerous studies have been conducted (Seetharamaiah and Prabhakar, 1986; Shin and Godber, 1996; Hu *et al.*, 1996) on the various stabilization methods and solvent extraction systems to extract the maximum amount of this high-value antioxidant from rice bran, only a single study (Othman, 1987) has been done for the locally produced rice bran. Therefore the purpose of this study was to quantify and evaluate the amount of  $\gamma$ -oryzanol in mixed local Malaysian varieties.

In this study, the level of  $\gamma$ -oryzanol was determined in rice bran from local mixed varieties following two different stabilization methods. The stabilized rice bran samples were stored at room temperature (26°C) and analyzed at specific intervals for 48 weeks. In this study chloroform:methanol (3:2, v/v) was used in a ratio of 1 part of rice bran to 5 parts of extracting solvent.

## MATERIALS AND METHODS

### Materials

Rice bran sample was provided by a local milling company, Padiberas Nasional Berhad (BERNAS) at Sekinchan, Selangor, Malaysia. The rice bran used was from mixed local

varieties (Herbarium UKMB; AZ 6807: MR 185, AZ 6808: MR 211, AZ 6809: MR 29). Freshly milled rice bran was collected from mill break # 2, which was the first polisher immediately after the removal of hull. The sample was then transported immediately to Universiti Putra Malaysia (UPM) on dry ice in cold box containers. Upon arrival the sample was sieved using 600  $\mu$ m sieve to obtain uniform particle size before subjecting to the stabilization process.

Total lipid was extracted using a method described by Suzuki *et al.* (1996). Butylated Hydroxytoluene (BHT) (Sigma, UK), Chloroform (BDH, England) and Methanol (BDH, England) were used to extract lipid. All reagents were of analytical grade. Oryzanol content was analysed using HPLC and the method developed by Rogers *et al.* (1993). The solvent system consisted of HPLC-grade acetonitrile (Fisher Chemicals, UK), methanol (Merck, Germany) and isopropanol (Merck, Germany). Mobile phase was filtered and degassed under vacuum immediately prior to use. The oryzanol standard was purchased from Tokyo Kasei (Japan). Stabilization of rice bran was carried out using Autoclave (Tomy SS-325, US) and Microwave (National Microwave/Convection Oven IEC-750W, Japan) (Azzrina *et al.*, 2000).

### Standard and Sample Preparation

**Standard:** Stock solution of  $\gamma$ -oryzanol standard was prepared at a concentration of 50 mg ml<sup>-1</sup> of mobile phase. A series of daily working standards used were 2000 ppm, 1000 ppm, 500 ppm, 100 ppm and 50 ppm prepared from diluting stock solution with mobile phase.

**Sample:** A known amount of lipid was diluted in mobile phase at 20% concentration. The sample was vigorously vortexed (MS1 Minishaker, Malaysia) for 5 min. The slight emulsion formed was broken by centrifugation (Hettich, Germany) at 3000 rpm for 3 min. Aliquots were then filtered (Whatman, USA;

0.2  $\mu\text{m}$  pore size, PTFE membrane) prior to analysis.

### Total Lipid Extraction

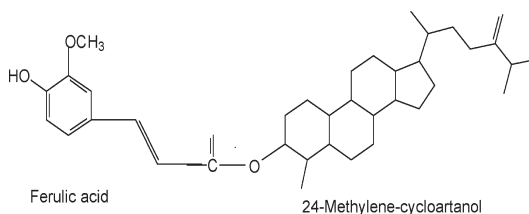
Twenty grams of stabilized rice bran sample stored for a specific period of time was homogenized with 100 ml chloroform:methanol (3:2, v/v) mixture containing 0.05% butylatedhydroxytoluene (BHT) on an orbital shaker (Protech, Malaysia) for 1 hour. The homogenate was centrifuged, and re-homogenized twice with another 25 ml mixture of chloroform:methanol. The extracts were combined and concentrated on a rotary evaporator (Butchi, Switzerland). The lipid extract was kept in 20 ml chloroform:methanol solution (3:2, v/v) at temperature below  $-20^{\circ}\text{C}$  for further analysis.

### Determination Oryzanol Content

The oryzanol components of rice bran lipid were separated by reverse-phase HPLC. The HPLC system consisted of a Hewlett-Packard (Germany) Model G1311A High Performance Liquid Chromatography connected to ALS Autoinjector Series 1100 (Hewlett Packard, Germany). Oryzanol components were detected at 325 nm with a Hewlett-Packard Model 1100 Series Photodiode Array Detector (PDA). Oryzanols were separated on a Hewlett-Packard 250 x 4 mm packed with 5-mm ODS (C18) Hypersil silica. The mobile phase consisted of acetonitrile/methanol/isopropanol (50:45:5 by volume).

## RESULTS AND DISCUSSION

Soxhlet extraction method was the most common technique used in the extraction of lipid from plant and animal tissues. The extractability of rice bran lipid using this method has been reported in the range of 18 - 20% (Saunders, 1986). In the present study, approximately 3.3 g of crude oil was obtained from 20 g of rice bran (16.4% yields). This result was supported by Xu and Godber (1999), who reported that 14% of lipid was extracted from 25 g rice bran. However using



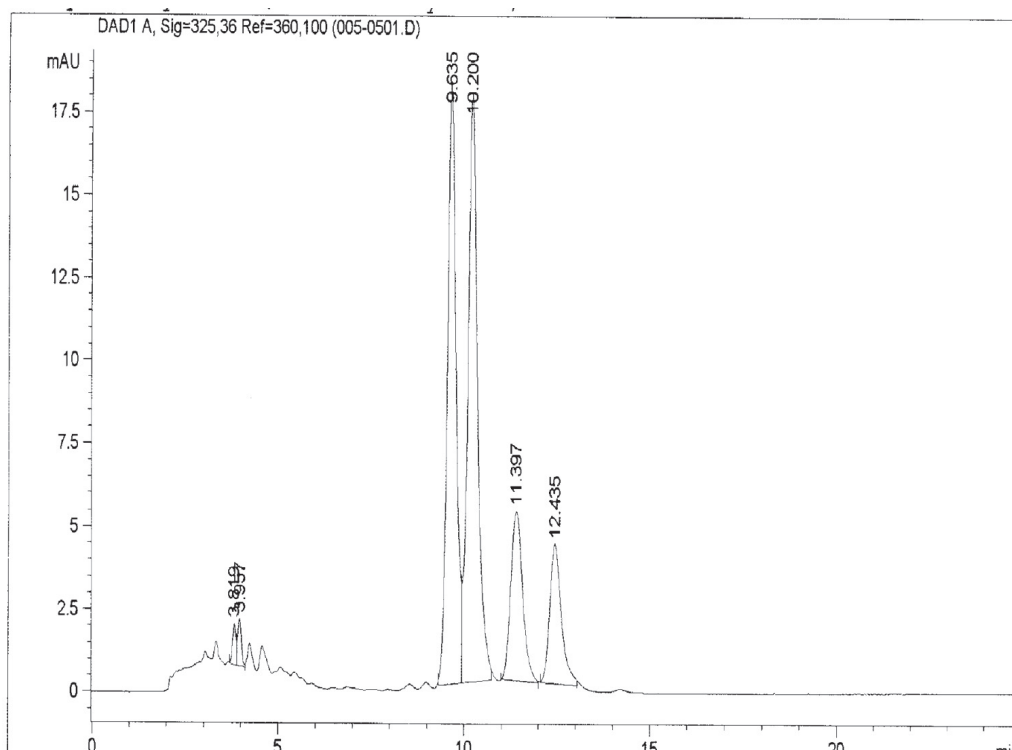
**Figure 1:** Molecular structure of ferulic acid esterified with 24-methylene-cycloartanol (Source: Lloyd *et al.*, 2000)

preheated solvent extraction, a higher percentage of crude oil can be extracted ( $24.9 \pm 0.9\%$ ) (Hu *et al.*, 1996).

A typical HPLC chromatogram obtained from crude rice bran oil extracted is shown in Figure 2. There were 4 major isomers detected namely; cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, campesteryl ferulate and mixtures of  $\beta$ -sitosteryl ferulate and cycloartenyl ferulate. The total  $\gamma$ -oryzanol concentration ranged from 23.7 - 43.0  $\text{mg g}^{-1}$  in the crude oil of the stabilized samples without saponification. Generally saponification has been employed in most lipid-extracted samples to remove interfering triglycerides and other hydrolysable materials and to aid the release of lipids from a sample matrix (Diack and Saska, 1994). However, in the case of oryzanol, the saponification process may hydrolyze the ester bond between triterpenoids and ferulic acids.

The level of  $\gamma$ -oryzanol detected in crude oil in this study was higher compared to the earlier investigations such as 9.8  $\text{mg g}^{-1}$  (Xu and Godber, 1999), 12.8 - 13.9  $\text{mg g}^{-1}$  (Hu *et al.*, 1996), 14  $\text{mg g}^{-1}$  (Zhao *et al.*, 1987), 12.2  $\text{mg g}^{-1}$  (Nicolosi *et al.*, 1994) and only 2.4 - 3.1  $\text{mg g}^{-1}$  (Shin and Godber, 1996) using different extracting solvents and mixture of solvents.

The higher extractability of  $\gamma$ -oryzanol in the present study could be due to the use of 3:2 chloroform:methanol mixture as the extracting solvent, where oryzanol in both non-polar (Xu and Godber, 1999) and polar (Qureshi *et al.*, 2000) lipid fractions were extracted. Earlier, Seetharamaiah and



**Figure 2:** Ultraviolet detection of  $\gamma$ -oryzanol components.

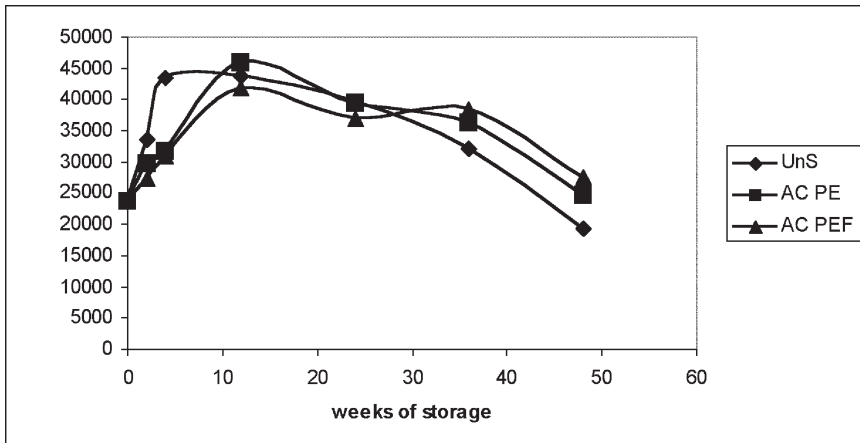
1= cycloartenyl ferulate; 2= 24-methylene cycloartenyl ferulate; 3= campesteryl ferulate; 4= $\beta$ -sitosteryl ferulate and cycloartanyl ferulate.

Prabhakar (1986) have shown that a higher concentration of oryzanol was extracted using chloroform:methanol mixture (2:1, v/v). Besides the solvent type, other factors such as solvent to bran ratio and extraction temperatures may also influence the extractability of rice lipid and its minor components (Hu *et al.*, 1996). Diack and Saska (1994) found that when separating antioxidants of rice bran such as vitamin E and oryzanol compounds, their concentrations also varied substantially according to the origin of the rice bran.

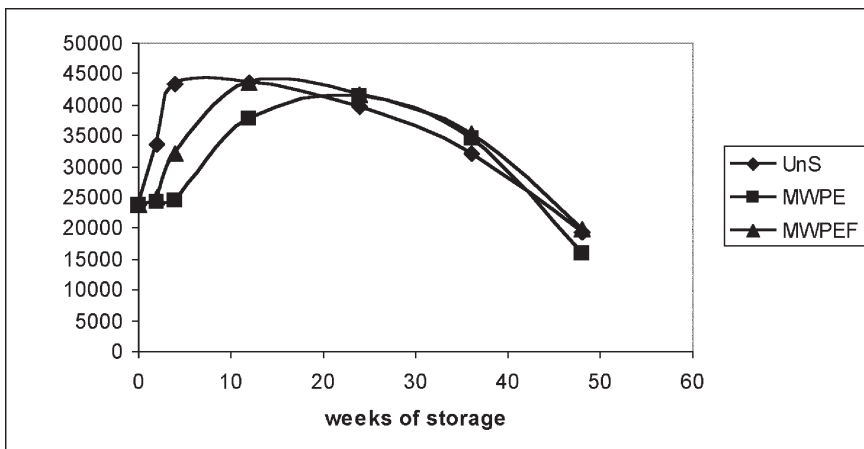
Figures 3 and 4 show the changes in oryzanol content over 48 weeks of storage following unstabilized (raw) and two different stabilization methods (microwave and autoclave). Overall, the oryzanol levels in raw and stabilized samples reduced with storage time. The highest  $\gamma$ -oryzanol concentration

detected in raw samples was in week 2, while in stabilized samples the highest concentration was during week 12. There was steady increment detected in the content of oryzanol from 0 - 5 and 0 - 12 weeks in raw and stabilized samples, respectively. The slow release of oryzanol in rice bran lipid observed could be due to the fact that fat-soluble antioxidants such as tocotrienol and tocotrienol-like compounds (including oryzanol) are bound to insoluble cellular components of the plant tissues (Qureshi *et al.*, 2000). In this study, the effect of autoclave and microwave methods to stabilize rice bran was comparable as shown by the observed similar trend.

The reduced concentrations of oryzanol after the 5<sup>th</sup> week and 12<sup>th</sup> week for raw and stabilized samples, respectively, indicated that the involvement of this compound in combating non-enzymatic lipid oxidation



**Figure 3:** Total  $\gamma$ -oryzanol in unstabilized and autoclaved rice bran samples stored for up to 48 weeks. AC- Autoclaved, UnS- Unstabilized samples, PE- Polyethylene bags, PEF- Polyethylene bags covered with foil



**Figure 4:** Total  $\gamma$ -oryzanol in unstabilized and microwave-heated rice bran samples stored for up to 48 weeks. MW- Microwave-heated, UnS- Unstabilized samples, PE- Polyethylene bags, PEF- Polyethylene bags covered with foil

(Sowbhagya and Bhattacharya, 1976). The oxidation that occurred could have been catalyzed by the presence of the naturally present metal ions in the rice bran or introduced by contamination from the shelling equipment during polishing (Champagne, 1994).

The quantity of plant-sterol in the lipid fraction of rice bran is influenced by plant genetics, growing and harvesting conditions, the state of maturity at harvest and processing techniques employed (Houghton and Raman, 1998). The unsaponifiable fraction of crude rice bran oil contains a unique complex of

naturally occurring antioxidant compounds such as oryzanol and vitamin E. However in this study, the vitamin E content was not determined. Simultaneous assessment (identification and quantification) of oryzanol and vitamin E in rice bran oil has been developed by Rogers *et al.* (1993) using reverse-phase HPLC. Through the HPLC methods, it has been clearly established that  $\gamma$ -oryzanol is a mixture of several components (Diack and Saska, 1994; Norton, 1995). However, depending on the chromatographic approach, different numbers of individual components have been identified. In this study, only 4 major components of oryzanol were successfully identified following the method and experimental conditions of Rogers *et al.* (1993).

Oryzanol, is a unique compound that imparts various physiological functions such as decreasing plasma cholesterol level in animals (Nicolosi *et al.*, 1991; Seetharamaiah and Chandrasekhara, 1989; Sunitha *et al.*, 1997), decreased platelet aggregation (Seetharamaiah *et al.*, 1990) and showed antioxidant functionality (Duve and White, 1991). In plant tissues,  $\gamma$ -oryzanol is present at a very high concentration such as 43 000 ppm in these mixed local varieties (Herbarium UKMB; AZ 6807: MR 185, AZ 6808: MR 211, AZ6809: MR 29). The high concentration of oryzanol is important as part of the plant defense system.

During saponification, caustic refining of rice bran oil removes the oryzanol from the oil to the soap stock. Recovery of this compound may be carried out by ether extraction at pH 9.5 (Seetharamaiah and Prabhakar, 1986). Purification and crystallization of oryzanol can be carried out using methanol-acetone solvent, producing a compound with antioxidant properties similar to tocopherols (Hui, 1996). The normal refining methods also affect the oryzanol content in the refined oil. Nicolosi *et al.* (1994) reported that more than 90% of the oryzanol and tocotrienols were lost during oil refining.

In a study that compares the concentration of oryzanol in 5 different brands of commercial oils, Roger *et al.* (1993) showed that the range of oryzanol concentration was 0.115-0.787 mg g<sup>-1</sup> of oil. Besides the effect of the specific oil processing step, rice varieties used also may influence its' final concentration of oryzanol in commercial cooking oil.

## CONCLUSION

In conclusion, factors such as rice varieties, procedures used using brown rice and extraction parameters, influenced the level of oryzanol in rice lipid. The level of oryzanol in mixed Herbarium UKMB was higher than some earlier reports and contained four major isomers (cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, campesteryl ferulate and  $\beta$ -sitosteroyl ferulate). This finding is important as oryzanol is a potent and high value antioxidant compound and locally produced bran could be the source of this compound.

## ACKNOWLEDGEMENTS

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