Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.12922



# Separation of γ-oryzanol from immature rice seeds by nanofiltration membrane

Dan LI[1], Chao ZHANG[1], Hui-min ZHANG[1], Dong-jie ZHANG[1*], Feng ZUO[1,2*]

**Abstract**

γ-Oryzanol, which is rich in rice seeds, is natural phytonutrition with many functions such as antioxidant. However, γ-oryzanol has a large loss in the traditional processing, resulting in a huge waste of available resources. In this study, the rice seeds highest content of γ-oryzanol during the development of rice seeds was selected and separated processes by nanofiltration. Several nanofiltration membranes were dramatically applied to condensing phytonutrition under 0.5 Mpa pressure and the flux ranged from 5.8 to 7.7 $kg•m^{-2}•h^{-1}$. The first processing supplied the separation improving ethanol-extraction system enrichment in γ-oryzanol. In the second, refining ethanol-extraction dramatically reduced the content of free fatty acids, while γ-oryzanol in the retention solution was further significantly increased. Moreover, the isolated γ-oryzanol added in the soybean oil, notably improved antioxidant ability of oil. Our founding can be widely used in the functional food manufacturing and also provides as an available method.

**Keywords:** γ-oryzanol; nanofiltration membranes; rice seeds development; antioxidant.

**Practical Application:** The content of bioactive substances in immature rice seeds was higher than that of mature rice seeds, and the content of γ-oryzanol was suitable for harvesting 20 ~ 30 days after flowering (DAF), which is natural phytonutrition with many functions such as antioxidant. And the rice seeds with highest content of γ-oryzanol during the development were selected and separated processes by nanofiltration. Separating γ-oryzanol from food by nanofiltration technology can explore feasible application and it could obtain high-value economic. In addition, this separating method will also provide potential application value for extracting γ-oryzanol directly from rice and promoting the industrial utilization of immature rice.

## 1 Introduction

Rice (*Oryza sativa L.*) is an important staple resource for global mankind. Over the past five decades, the increase in grain yield has been mainly contributed to the tripling of world total rice production, according to the Food and Agriculture Organization of the United Nations (2015) statistical databases. Thus, it can meet the needs of most of the world's population.

Many nutrients, such as polyphenolic compounds (ferulic acid and ferulic esters), anthocyanins and proanthocyanins are wasted because mature rice seeds are usually dehulled and then milled and polished to obtain white rice. Therefore, whole rice grains will preserve more beneficial compounds and provide health for consumers. But these parts are usually used as feeds and have no food value (Worasuwannarak et al., 2007). In developing countries, such as China, India and Indonesia, researchers have become interested in the widespread availability of large quantities of rice husks which are produced annually by milling and the parts have other valuable applications to avoid waste, such as for electricity and heating (Lim et al., 2012). The conversion of rice husks to energy using different technologies, such as direct combustion or pyrolysis, has shown potential in meeting the energy needs, especially in large populations (Lim et al., 2012; Pode, 2013). Butsat et al. (2009) reported the content of phenolic acid and their antioxidant activities were decreased in rice husk during rice seeds development. In addition to researching rice husk samples at different stages of rice seed development, it has recently been found that immature rice seeds contain more bioactive components than mature seeds, such as protein, reducing sugar, vitamin B2, B3, B6 and vitamin E (tocopherol + tocotrienol) (Ji et al., 2013). Moreover, the total phenolic/flavonoids content and antioxidant activity of immature rice were higher than those of mature rice (Lin & Lai, 2011; Shao et al., 2014). γ-Oryzanol is a mixture of ferulic acid and several phytosterols (cycloartenol, 24-methylene cycloartanol, β-sitosterol, campesterol and so on), which is rich in rice seeds than other grains. It was reported that γ-Oryzanol synthesized rapidly in the early stage of grain development and reached the maximum on about 20 ~ 30 days after flowering (Chen & Bergman, 2016). γ-Oryzanol not only has natural antioxidant ability but also has a significant role in reducing cholesterol (Islam et al., 2011; Kozuka et al., 2012).

The main separation methods of γ-oryzanol include soaping, liquid phase preparation and supercritical fluid extraction (carbon dioxide and liquefied hydrocarbons) (Lai et al., 2005; Narayan et al., 2006; Yoon et al., 2014). However, these methods have the disadvantages of large solvent consumption and high expenditure cost, which affect the industrial interests. Compared with the traditional biological treatment technology, membrane technology has many advantages in the field of bioprocessing. In recent decades, there were many studies on degumming, dewaxing, deacidifying, and decolorizing of edible

*Received 30 Jan., 2022*
*Accepted 05 Mar., 2022*
[1] *HeilongJiang BaYi Agricultural University, College of Food Science, Daqing, People's Republic of China*
[2] *Ministry of Education, Engineering Research Center of Processing and Utilization of Grain By-Products, Daqing, People's Republic of China*
*Corresponding authors: zuofeng-518@126.com; byndzdj@126.com

oil by membrane technology, but few of them were used for the separation of γ-oryzanol (Manjula & Subramanian, 2009; Ladhe & Kumar, 2010; Roy et al., 2014). It is well known that many solvents, such as hexane, benzene or petroleum ether, are commonly used in the commercial processing of extraction oil from rice bran. Hexane has no significantly difference in the yield of oil and the concentration of γ-oryzanol in the extract oil comparing with other solvents (i-Propanol, Acetone, Ethyl acetate) (Sereewatthanawut et al., 2011), but hexane and benzene are harmful to the environment and low quantity for healthy compared to ethanol or methanol. Koike et al. (2002) reported a method for extracting edible oil using ethanol, which has been widely studied by researchers. However, there are few reports on the extraction of γ-oryzanol with ethanol and methanol.

Through the previous research, we found that the content of bioactive substances in immature rice seeds was higher than that of mature rice seeds, and the content of γ-oryzanol was suitable for harvesting 20 ~ 30 days after flowering (DAF). The materials in our study were also immature rice seeds. The purpose of this paper was to explore feasible application of separating γ-oryzanol from food by nanofiltration technology and improving its nutritional value, which could obtain high-value economic. In addition, this separating method will also provide potential application value for extracting γ-oryzanol directly from rice and promoting the industrial utilization of immature rice.

## 2 Material and methods

### *2.1 Material and chemicals*

The rice seeds were planted in an experimental field of BQRF (Bao-quanling Reclamation Farm, northeast of China, 47°37′N, 131°01′E) situated in Heilongjiang province. Immature rice seeds (from DAF-14th to DAF-35th, grains collected at 7-day intervals) were drying at less than 50 °C. After they were ground in a cyclone sample mill (Foss Analytical Co., Ltd, Suzhou, China) installed with a 0.5 mm or 1 mm sieve. All samples were stored at -20 °C until analysis.

γ-Oryzanol standards powder were bought from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) (≥ 98% purity, cycloartenol ferulate 23%, cyclobranol ferulate 51%, campesterol ferulate 15% and β-sitosterol ferulate 10%). 1,1 Diphenyl-2-picrylhydrazine (DPPH) was purchased from Sigma-Aldrich (St. Louis, USA). All chemicals used were HPLC grade and obtained from Shanghai Ambrosia Pharmaceutical Co. Ltd. (Shanghai, China). Other chemicals and solvents used in study were of analytical grade. Membranes used in this study were made of a thin aromatic (or semi-aromatic) polyamide active layer and a thicker more porous supporting layer. NF 90 (MWCO: 100) and NF 245 (MWCO: 200) purchased from The Dow Chemical Company (Michigan, American), NF SS-NF7-2540 (MWCO: 800) provided by Risingsun Membranes Co. Ltd. (Beijing, China).

### *2.2 γ-oryzanol saturation*

The solubility of γ-oryzanol was measured under different conditions of acid, alkaline and temperature. Under different conditions, 10 mL of γ-oryzanol standard powder was added to each solvent, and it was saturated at room temperature for 12 h. The appropriately diluted solution is then analyzed by HPLC as described below, and the solubility concentration was determined using a calibration curve. The values presented were an average of three measurements.

### *2.3 Solvent extraction*

In this study, we first investigated the effect of particle size (< 0.5 mm and < 1 mm) on content of extracts. The content of γ-oryzanol in rice flour was determined by Soxhlet extraction with ethanol/methanol. Then the experiments were carried out under the condition of ultrasonic (120 W, 50 Hz). The effects of ultrasonic extraction time and the ratio of solvent to powder on the content of γ-oryzanol were investigated.

The extraction yield and content of γ-oryzanol in the extractable substances were calculated as follows (Equations 1-2):

$$\text{yield} = \frac{\text{Extract substances}}{\text{Mass of rice seed powder}} \times 100\ \% \quad (1)$$

$$\text{yield} = \frac{\gamma - \text{oryzanol}}{\text{Mass of extract substances}} \times 100\ \% \quad (2)$$

The solvent extraction yields presented in the extraction solution was an average of the three measurements with a standard deviation of less than 3 wt.%. The content of γ-oryzanol in the extraction solution presented was an average of the three measurements and the standard deviation was less than 1 wt.%.

### *2.4 Pretreatment of extraction solution*

The separation and extraction process includes: (1) removing coarse particles with filter paper under vacuum condition; (2) ultrafiltration membrane (0.45 μm) to remove particles under vacuum; (3) followed by a solution for membrane processing.

### *2.5 Membrane component and parameters*

Amicon stirred cell (Model: UFSC40001, Milipore) was used in experiment. In a nitrogen atmosphere, an electrolytic cell with an O-ring membrane (7.6 cm diameter with 41.8 $cm^2$ effective area) was placed on a magnetic stirrer and forced with a stirrer bar (rotation speed 400 rpm) to reduce the polarization effect on the concentration. Tests were carried out at room temperature (25 ~ 30 °C) with different dilutions (100%, 70%, 50%, 30%) of the extracts at 0.5 MPa. Solution of feed, retentate section and permeate were collected every 30 min for analysis of γ-oryzanol, FFA (free fat acids), and chlorophyll. In our study, the permeation flux (F) and the solution rejection (R) were considered as the parameters, to characterize the membrane.

Permeate flux ($kg/m^2/h$; LMH) was measured by determining the volume of the solution permeating through the membrane per unit area on fixed time, using the following equation (Equation 3):

$$F = \frac{V_m}{St} \quad (3)$$

In which $V_m$ is the weight permeated through the membrane (kg) and $S$ is the membrane area ($m^2$) and $t$ is the time taken for the volume to permeate (h).

To obtain cells with varying feed concentrations in the process, the rejection rate is defined as the ability of the membrane to separate. The more one is kept by the membrane, the more the one's rejection is, by using following equation (Equation 4):

$$R = \left(1 - \frac{C_p}{C_R}\right) \times 100\% \quad (4)$$

In which $C_p$ is the concentration of one in the permeate solution and $C_R$ is the concentration of one in the retention solution.

### *2.6 Methods of determination*

*γ-oryzanol*

The content of γ-oryzanol was determined by high performance liquid chromatography (HPLC) system (Agilent 1260; USA) with UV-vis diode array detector, gradient pump system, and automatic sampling injection system. The chromatogram was an Agilent C18 column (250 mm × 4.6 mm, 5 μm, USA). In brief, 10 μL of prepared sample was injected with the mobile phase, isocratic mode, containing mixture of AcOH, MeCN, and MeOH (2 : 44 : 54, by volume), the flow rate was 1.4 mL/min and the detection wavelength was 325 nm. The retention time and quantification of the γ-Oryzanol peaks in samples were dependent on the standards in the library constructed in this study.

*FFA content*

The AOCS Official method Ca 5a-40 (American Oil Chemists' Society, 1997) was used to titrate the content of FFA with KOH in this study.

*Spectroscopic determination of chlorophyll a and b*

The contents of chlorophyll a and b in the process were determined by spectrophotometry at the selected wavelength of 663 and 645 nm. The wavelength of 663 nm corresponds to the absorption maximum of chlorophyll a and the wavelength of 645 nm corresponds to the absorption maximum of chlorophyll b, using the following equation of Arnon (1949) (Equations 5-7):

$$OD_{663} = 82.04C_a + 9.27C_b \quad (5)$$

$$OD_{645} = 16.75C_a + 45.6C_b \quad (6)$$

$$C_T = 8.02OD_{663} + 20.21OD_{645} \quad (7)$$

In which $C_a$ and $C_b$ are milligram per liter of chlorophyll a and b respectively, and OD the density values at the respective wavelengths as obtained on the spectrophotometer. $C_T$ is sum of $C_a$ and $C_b$.

### *2.7 DPPH radical scavenging activity*

1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) radical scavenging activityof model oil (soybean oil with 5% of γ-oryzanol separated by the above nanofiltration membrane) and soybean oil was determined. DPPH was configured as a 0.25 mM methanol solution, and model oils and soybean oil were diluted to different concentration. 100 μL of the sample oil was thoroughly mixed with 3 mL of DPPH solution during the test for 1 h in the dark. Then the absorbance of the reaction solution was measured at 517 nm, and 100 μL of hexane was used in the control group instead of the oil sample. The free radical scavenging ability was calculated by the following expression (Equation 8):

$$\text{Scavenging capacity}(\%) = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \quad (8)$$

### *2.8 Statistical analysis*

All statistical analyses were performed in triplicate and presented as means ± standard deviations (SDs). Data processing was conducted using SPSS (version 20 for Windows 2010, SPSS Inc., Armonk, NY, USA). Comparison of each group during each stage of grain development was determined by one- and two-way ANOVA. Duncan's tests were used to calculate the statistically imperative different among each groups. Statistical significance was defined as $P \leq 0.05$.

## 3 Results and discussion

### *3.1 Changes of γ-oryzanol content in different layers*

The main components of γ-oryzanol in all samples were evaluated and it could be proved that the peak presented in all samples were consistent with the standard (Figure 1).

As shown in Table 1, the content of γ-oryzanol was the high in the rice bran layer, followed by brown rice. It was also found that there was a small amount of γ-oryzanol in milled rice and rice husk, and the content of γ-oryzanol in rice husk was higher than that in milled rice. During grain developing, the content of γ-oryzanol in polished rice and rice husk has not been changed much. In DAF-14th, the content of γ-oryzanol in the bran layer was the lowest (115.26 mg/100 g dry matters), in DAF-21st, the content of γ-oryzanol was the highest (190.35 mg/100 g dry matters), and then decreased by 8.31 mg/100 g dry matter. The γ-oryzanol content inbrown rice was the highest at DAF-21st (22.80 mg/100 g dry matters), and then decreased slightly. The immature rice seeds can be obtained after peeling and polishing,as shown in Figure 2.

Previous studies showed that the content distribution of γ-oryzanol in rice was in the order of bran (3,174.2-3,176.4 mg/kg) > whole grain (413.3-473.3 mg/kg) > rice husk (102.4-323.2 mg/kg) > endosperm (49.1-231.8 mg/kg), and Huang & Ng (2011) also obtained the same trend when studying the distribution of γ-oryzanol in rice. However, there is no report about the distribution of γ-oryzanol during rice seeds maturation. The results of this experiment showed that the content of γ-oryzanol in the bran layer of mature rice was not only rich, but also higher than

**Table 1**. Changes of γ-oryzanol content in different layers of development rice seeds.

| | γ-oryzanol (mg/100 g) | | | |
|---|---|---|---|---|
| | DAF-14th | DAF-21th | DAF-28th | DAF-35th |
| Brown rice | 20.43 ± 1.37[b] | 22.80 ± 1.13[b] | 19.47 ± 1.23[b] | 21.45 ± 1.37[b] |
| Bran | 115.26 ± 25.52[a] | 190.35 ± 31.54[a] | 182.04 ± 34.33[a] | 181.83 ± 34.81[a] |
| Polished rice | 0.39 ± 0.09[c] | 0.16 ± 0.06[c] | 0.46 ± 0.07[c] | 0.71 ± 0.11[c] |
| Husk | 2.79 ± 0.55[c] | 1.29 ± 0.31[c] | 2.77 ± 0.40[c] | 1.60 ± 0.31[c] |

Three measurements ± SD. DAF = day after flowering. The same letters are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple tests.

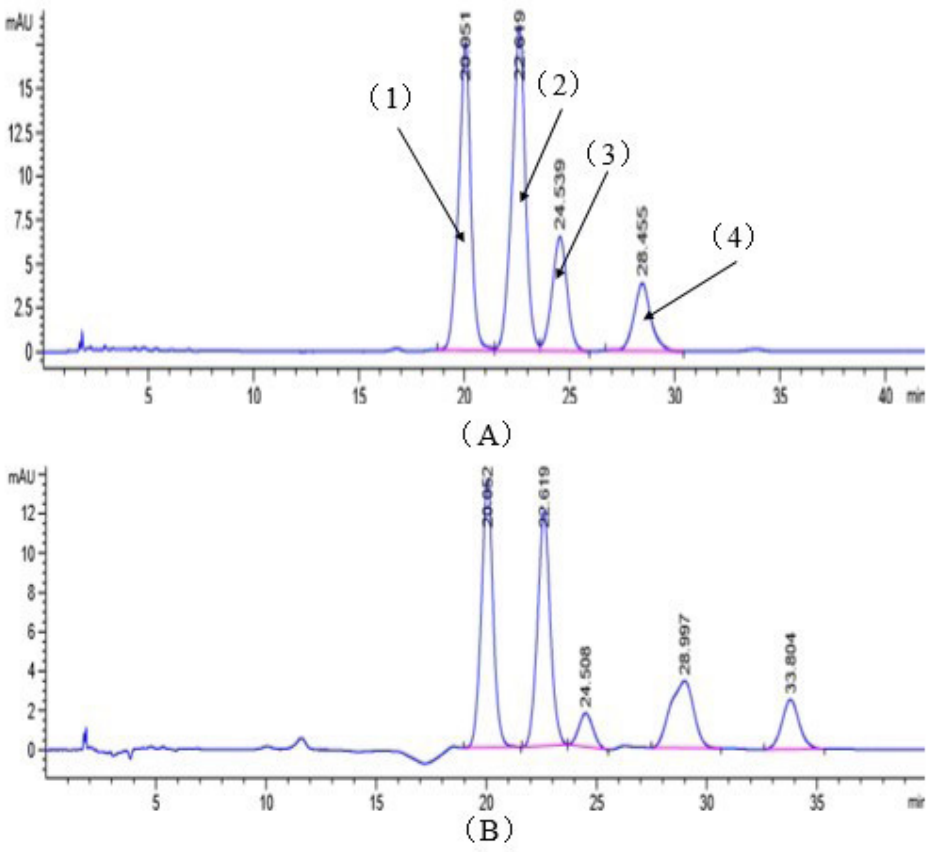


**Figure 1**. (A) Chromatogram of γ-oryzanol standard (0.2 mg/mL); peak (1): cycloartenolferulate; peak (2): cyclobranolferulate; peak (3): campesterolferulate; peak (4): β-sitosterolferulate; (B) chromatogram of sample.

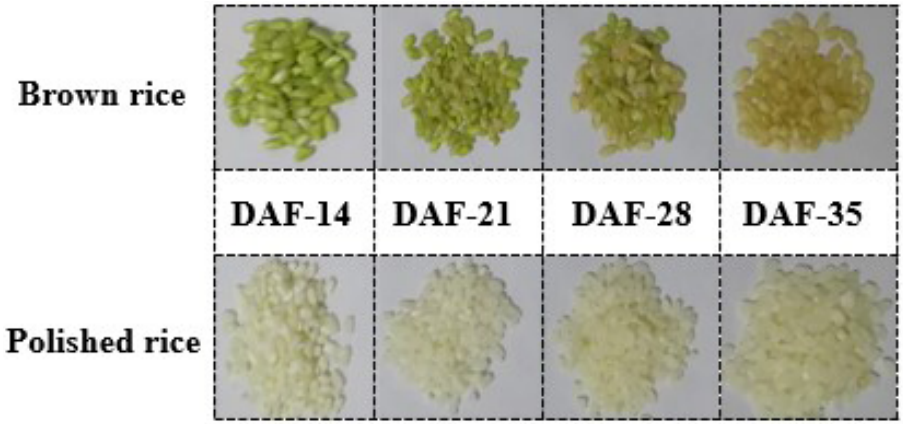


**Figure 2**. The immature rice seeds dehulled and polished.

that in other parts of rice during grain development. γ-Oryzanol has good antioxidant activity, so it is found in the bran layer to protect other plant tissues from oxidative damage (Munné-Bosch & Alegre, 2002). In the view of the fact that the content of γ-oryzanol in the rice seeds of DAF-21st stage was higher than that in the seeds of other stages, γ-oryzanol was extracted from the seeds of DAF-21st stage.

### *3.2 γ-oryzanol saturation*

The γ-oryzanol can dissolve in methanol/ethanol. The solubility of γ-oryzanol in methanol/ethanol was affected by pH. The results of γ-oryzanol saturation are shown in Table 2 and Table 3. The yield and purity of final γ-oryzanol product depend on its solubility in solvents. From Table 2 and Table 3, it can be obviously seen that γ-oryzanol solubility in acid solvent is lower. The solubilities in ethanol and methanol were 98.3 mg/100 mL and 103.3 mg/100 mL respectively under neutral conditions, while the solubilities in solvents were the highest under alkaline conditions, such as 0.30% base (1,874.0 mg/100 mL and 1,918.7 mg/100 mL respectively). This was to be expected, considering that changes in pH can affect the solubility of γ-oryzanol in methanol/ethanol. γ-Oryzanol is an amphiphilic compoundwith a phenolic hydroxyl group (-OH) - itself having some weak acidity and a long hydrophobic tail. Under alkaline conditions, γ-oryzanol is converted to γ-oryzanol salt, which has significant solubility in methanol/ethanol, while under acidic conditions, γ-oryzanol is precipitated by adjusting the pH of the solution.

The temperature also affects the solubility of γ-oryzanol in methanol/ethanol. However, compared with acid and temperature changes, alkaline was the main factor affecting on the solubility of γ-oryzanol. As shown in Table 4, we found that at 25 °C and in neutral solvent condition, methanol/ethanol could fully dissolve. γ-oryzanol in that rice seeds powder, so we chose to extract γ-oryzanol at room temperature and neutral solvent condition.

### *3.3 γ-oryzanol extraction*

The percentage of the extract determined substances by Soxhlet extraction with ethanol and methanol are shown in Figure 3, and the percentage of γ-oryzanol in extractable was also determined. When the particle size was less than 0.5 mm, the extraction rate and γ-oryzanol content were higher in the extraction solution of methanol and ethanol, and there was no significant difference between the extraction rate and γ-oryzanol content of methanol and ethanol. The results showed that the decrease of particle size could promote the dissolution of soluble substances in rice seed, which was due to the increase of the surface area of rice seeds in contact with solvent. In previous studies, it has been reported that the finer the grape pomace and berries are ground, the higher the extraction efficiency (Meyer, 2002; Cacace & Mazza, 2003). In fact, for smaller particle sizes, the diffusion of the solvent in the solid can be improved due to shorter diffusion distances improving the extraction rate, thus allowing the solution to be successfully leached in a shorter time. Figure 3 shows that there was no different in the content of γ-oryzanol between the methanol-extractables and ethanol-extractables. Studies by FDA agencies consistently show that ethanol is preferable to traditionalsolvents such as chloroform, hexane, acetonitrile because it is considered less toxic and more environmentally friendly (Food and Drug Administration, 1997). Above all, ethanol was used as extraction solvent to extract γ-oryzanol from rice seeds, and rice seed powder with particle size less than 0.5 mm was selected.

Because ethanol is not traditionally used in the processing of rice byproducts, the effects of extraction time and the ratio of solvent to power on the extraction rate of γ-oryzanol from rice grain powder were studied. The results of each key factor researched are shown in Table 5 and Table 6. Table 5 presents that under ultrasonic conditions, the extraction time within 30 min has a remarkably effect on the extraction of γ-oryzanol, and the extraction rate of γ-oryzanol is slightly increased even if the extraction time is 60 min. The shorter the extraction time,

**Table 2**. The effect on the different acid to the solubility of γ-oryzanol in methanol/ethanol at room temperature.

| HCl (%) | 0.05 | 0.10 | 0.15 | 0.30 | 0.40 | 0.50 |
|---|---|---|---|---|---|---|
| Methanol-Solubility (mg/100 mL) | 87.7 ± 6.5 | 82.3 ± 4.5 | 75.0 ± 4.6 | 45.7 ± 5.0 | 22.0 ± 3.0 | 13.7 ± 1.5 |
| Ethanol-Solubility (mg/100 mL) | 89.3 ± 5.5 | 82.3 ± 7.1 | 78.7 ± 3.5 | 48.3 ± 6.0 | 28.3 ± 3.5 | 16.0 ± 2.0 |

**Table 3**. The effect on the different alkaline to the solubility of γ-oryzanol in methanol/ethanol at room temperature.

| NaOH (%) | 0 | 0.05 | 0.10 | 0.15 | 0.30 | 0.40 | 0.50 |
|---|---|---|---|---|---|---|---|
| Methanol-Solubility (mg/100 mL) | 98.3 ± 6.5 | 602.3 ± 19.0 | 973.7 ± 84.8 | 1,646.0 ± 109.5 | 1,874.0 ± 96.1 | 1,851.3 ± 98.1 | 1,137.3 ± 73.2 |
| Ethanol-Solubility (mg/100 mL) | 103.3 ± 5.9 | 616.0 ± 27.2 | 970.3 ± 72.5 | 1,652.3 ± 97.2 | 1,918.7 ± 104.1 | 1,881.0 ± 66.6 | 1,150.7 ± 33.9 |

**Table 4**. The effect on the different temperature to the solubility of γ-oryzanol in methanol/ethanol.

| Temperature (°C) | 25 | 30 | 35 | 40 | 45 | 50 |
|---|---|---|---|---|---|---|
| Methanol-Solubility (mg/100 mL) | 76.7 ± 5.7 | 95.0 ± 6.0 | 106.3 ± 8.5 | 118.7 ± 7.0 | 112.7 ± 9.0 | 136.7 ± 11.1 |
| Ethanol-Solubility (mg/100 mL) | 79.7 ± 6.7 | 98.7 ± 6.5 | 112.3 ± 6.5 | 121.0 ± 7.5 | 124.7 ± 7.6 | 114.0 ± 9.0 |

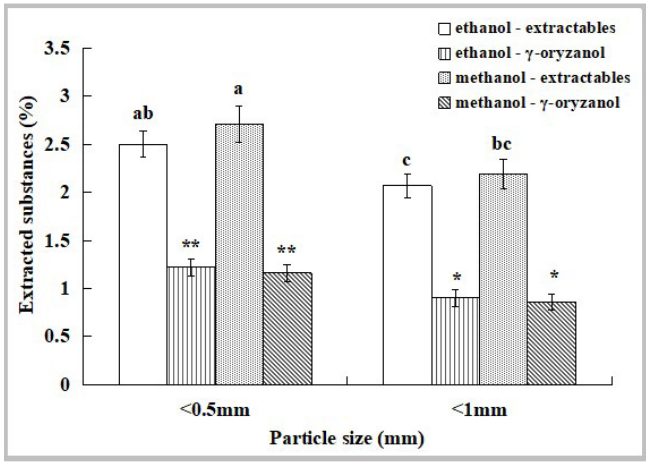


**Figure 3**. Percentage of extractable substances and γ-oryzanol obtained by ethanol/methanol extraction from rice seeds in Soxhlet: results for different particle size ranges. (Note: Within the range of < 0.5mm, ab and a represent the difference in particle size of extractable substances extracted from ethanol/methanol, and ** represents no significant difference. In the range of <1 mm, c and bc represent different particle sizes of extractable substances extracted from ethanol/methanol, and * represents no significant difference.)

**Table 5**. Extraction yield of γ-oryzanol using different solvent to powder ratio for a 30 min at room temperature.

| Solvent | Solvent: powder (V/W, %) | Yield of extraction (%) | γ-oryzanol in extraction (%) |
|---|---|---|---|
| Ethanol | 2 : 1 | 1.60 | 1.14 |
| | 3 : 1 | 1.65 | 1.16 |
| | 4 : 1 | 1.72 | 1.10 |
| | 5 : 1 | 1.82 | 1.15 |
| | 6 : 1 | 2.02 | 1.12 |

**Table 6**. Extraction yield of γ-oryzanol at different extraction times at room temperature (2 : 1 the ratio of solvent and powder).

| Solvent | Extraction time (min) | Yield of extraction (%) | γ-oryzanol in extraction (%) |
|---|---|---|---|
| Ethanol | 10 | 1.58 | 0.65 |
| | 20 | 1.54 | 0.73 |
| | 30 | 1.63 | 1.22 |
| | 40 | 1.69 | 1.08 |
| | 50 | 1.60 | 1.34 |
| | 60 | 1.65 | 1.29 |

the higher the industrial availability. Therefore, half an hour is appropriate as a parameter of the extraction time. The results showed that after saponification in the solvent for half an hour, the extraction rate did not increase significantly, and remained stable. It is believed that there is no mass transfer after saponification for half an hour, but the extraction efficiency decreases significantly. The time it takes for the leachate to reach the maximum value indicates how tightly the substance is bound in the structure. Xiao et al. (2011) reported that extraction of brown pigment from Rosa laevigata reached the peak at 2 h, though there seems to be no apparent obstruction to the extraction process; other studies have reported the extraction time influence antioxidant nature on extractable materials.

Moreover, the content of γ-oryzanol increased with the increase of the amount of solvent. In fact, it's a slight improvement over the 1 : 10 ratio. Therefore, the ratio of powder and solvent is 2:1 in the extraction process, and the technology is applied in industry to reduce the consumption of solvent.

From the extraction step (Tables 5-6), the extractable substances of rice seeds presenting in extraction solution system includes approximately 1% total of γ-oryzanol, in which the majority consitituents are cycloartenol ferulate, cyclobranol ferulate, campesterol ferulate, β-sitosterol ferulate (MW: cycloartenol ferulate ~ 601 Da, cyclobranol ferulate ~ 615 Da, campesterol ferulate ~ 575 Da, β-sitosterol ferulate ~ 585 Da). Moreover, there are chlorophyll (MW: chlorophyll a ~ 895 Da, chlorophyll b ~ 907 Da), tri-glycerides (MW ~ 900 Da) and FFA (MW ~ 280 Da). There is divergence of molecular weight, hence, each compound above all was first separated from the ethanol-extracted solution using specified nanofiltration membranes with different nominal molecular weight cut-off (MWCO) characteristics.

### *3.4 Selectivity of nanofiltration membrane for γ-oryzanol in ethanol extraction*

Three kinds of nanofiltration membranes, NF-90, NF-245 and NF-270, were used to investigate the ethanol solvent tolerance of nanofiltration membranes by standing on a shaker at 120 r/min for 1.5 h. And the results were listed in Table 7. The repellency of NF-90, NF-245, SS-NF7-2540 membrane to MgSO4 and NaCl was lower than that of control group, but the difference was not significant, which indicated that NF-90, NF-245, SS-NF7-2540 membrane had good tolerance. Polyamide composition membranes (NF-90, NF-245, SS-NF7-2540) showed some separation ability in preliminary experiments with undiluted ethanol extraction solutions (Table 8). The polyamide composite membrane was a hydrophilic membrane, hence the flux obtained in this week polar solvent-ethanol extraction solution was not low [NF-90 ~ 6.51 kg/(m²·h), NF-245 ~ 5.58 kg/(m²·h), SS-NF7-2540 ~ 7.77 kg/(m²·h)]. γ-Oryzanol (cycloartenol ferulate, cyclobranol ferulate, campesterol ferulate, β-sitosterol ferulate) and FFA, chlorophyll a, chlorophyll b had difference in rejection among three membranes, and we found the rejection (%) of each individual component were in the order of chlorophyll a > γ-oryzanol > chlorophyll b > FFA > ethanol. As shown in Table 7, NF-245 demonstrated intermediate rejection effect on γ-oryzanol and FFA, chlorophyll a, chlorophyll b comparing with NF-90 (highest rejection for these compounds) and NF-270 (lowest rejection for these compounds). The molecular weight has an effect on the rejection of the membrane. (Figure 4), and we can get a trend that the bigger MW, the more rejection it gets. In theory, chlorophyll b (MW 907) should get a better rejection than FFA and γ-oryzanol as they are low molecular weight. However the chlorophyll b was too low rejection to provide a viable separation, which was probably owing to the

**Table 7**. Determining membrane changes of permeate flux and solute rejection after immersing in ethanol (100%) for 1.5 hour.

| Type | Management | Permeate flux (kg/(m²·h) | Saline solution | Solute rejection |
|---|---|---|---|---|
| NF 90 | Untreated | 7.04 ± 0.40 | $MgSO_4$ | 19.89% |
| | | | NaCl | 5.53% |
| | Treated | 9.92 ± 1.27 | $MgSO_4$ | 14.29% |
| | | | NaCl | 3.46% |
| NF 245 | Untreated | 9.93 ± 0.57 | $MgSO_4$ | 16.85% |
| | | | NaCl | 3.98% |
| | Treated | 11.95 ± 0.92 | $MgSO_4$ | 12.56% |
| | | | NaCl | 3.01% |
| SS-NF7-2540 | Untreated | 16.01 ± 0.68 | $MgSO_4$ | 15.23% |
| | | | NaCl | 3.10% |
| | Treated | 20.02 ± 1.32 | $MgSO_4$ | 13.56% |
| | | | NaCl | 2.8% |

Untreated: not immersing in ethanol (100%); treated: immersing in ethanol (100%) for 1.5 hour.

**Table 8**. Permeate flux and rejection in the ethanol system.

| Type | Rejection A (%) | Rejection B (%) | Rejection C (%) | Rejection D (%) | Rejection E (%) | Rejection F (%) | Rejection G (%) | Permeate flux kg/(m²·h) |
|---|---|---|---|---|---|---|---|---|
| NF 90 | 54.22 | 53.73 | 61.03 | 58.44 | 33.84 | 69.67 | 26.73 | 6.51 |
| NF 245 | 42.93 | 38.21 | 42.11 | 40.71 | 25.23 | 69.12 | 25.36 | 5.58 |
| SS-NF7-2540 | 26.39 | 27.31 | 34.86 | 28.09 | 20.56 | 52.54 | 24.52 | 7.77 |

A = cycloartenol ferulate; B = cyclobranol ferulate; C = campesterol ferulate; D = β-sitosterol ferulate; E = FFA (free fat acid); F = chlorophyll a, G = chlorophyll b.

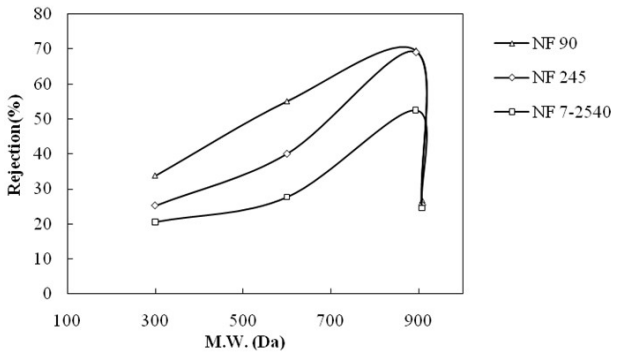


**Figure 4**. Comparative rejection of solutes of various M.W. (molecular weight) in the methanol systems.

concentration of chlorophyll b lower than chlorophyll a. When the membrane negatively repels the FFA, the FFA positively permeates all the membranes. The retention rates of γ-oryzanol and chlorophyll-a of NF-90 and NF-245 membranes were higher than that of SS-NF7-2540 membrane.

Although the three membranes used in this study were obtained from the same membrane materials (polyamide composite membrane), the different rejection values discovered were obvious, possibly due to their differences in the thickness of their surface layer (Azaïs et al., 2016). Comparison of flux values of NF-90, NF-245 and SS-NF7-2540 membranes (Table 8) also suggested differences in their surface layer. Considering SS-NF7-2540 membrane had a higher selectivity for chlorophyll, but a low rejection of γ-oryzanol, and NF-90 membrane had a high rejection for γ-oryzanol. Therefore, in order to effectively separate, SS-NF7-2540 and NF-90 membranes were used in further experiments (Figure 5).

### *3.5 Analysis of membrane SS-NF7-2540 separated solution by UV-vis absorption spectrum*

Spectrophotometry is the selective absorption of specific wavelengths of light by the molecules or ions of a substance, and the qualitative, quantitative, and structural analysis of the substance is performed. Determine or measure the content of the substance based on the level of absorbance at certain characteristic wavelengths in the absorption spectrum. This method is widely used for its simple operation, high efficiency, good selectivity, sensitivity, precision, and accuracy.

UV-vis absorption spectra of feed solution (immature rice grain ethanol extract), permeate solution and γ-oryzanol standard solution (1 mg/mL, 0.8 mg/mL) measured are shown in Figure 6, respectively. The spectrum of feed solution (Figure 6, black line) shown an absorption peak at 248 nm, 312 nm, a shoulder peak at 413 nm, moreover it also had an absorption peak at 665.5 nm. The absorption peaks at 248 nm and 312 nm are attributed to γ-oryzanol [peak absorption, λ = 248 nm and 312 nm (Figure 6, red line)], which consists of ferulic acid esters with phytosterols. Ferulic acid is an aromatic compound with three characteristic absorption peaks, Ethylenic bands [E1 (at 184 nm), E2 (at 204 nm)], Benzenoid bands [B (254 nm)], in addition the methoxy (-OCH3) and hydroxyl (-OH) substituent

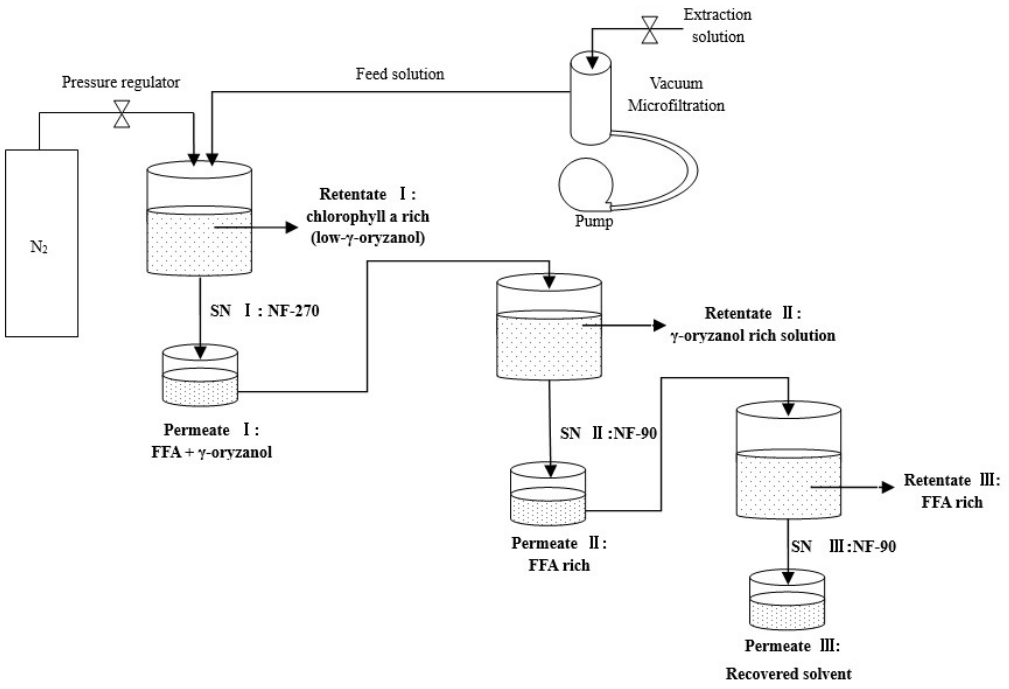


**Figure 5**. Schematic of membrane filtration system.

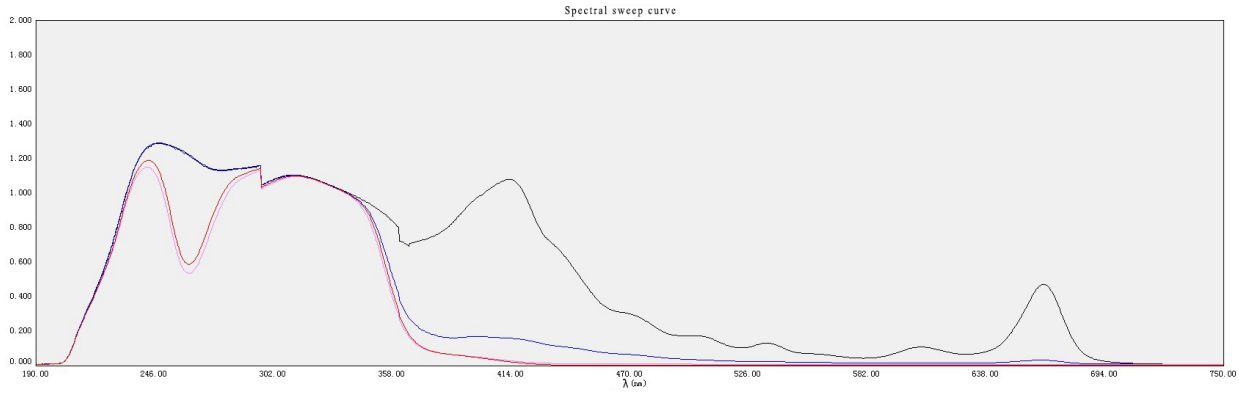


**Figure 6**. Effect of SS-NF7-2540 filtering on Absorbance by UV (Ultraviolet Light) scanning in the methanol system.

groups on the benzene ring have a red shift effect on spectrum, and that is to say, γ-oryzanol has absorption in the 200-300 nm region in theory (Kaewboonnum et al., 2010). Several carotenoids in rice seeds had a broad absorption at 413 nm, while chlorophyll a and chlorophyll B had a broad absorption at 660 nm (Manjula & Subramanian, 2009; Roy et al., 2014).

The spectrum of permeate solution (Figure 6, black line) presents that the concentration of chlorophyll and carotenoids compounds observed in the solution was obviously decreased, and the absoption spectra of the permeate (blue line) and the standard solution of γ-oryzanol (red/pink line) were observed to be similar at 200-350 nm. In other words, the SS-NF7-2540 membrane provides a good separation for these ethanol extracts.

### *3.6 Effect of ratio (powder/ethanol) on rejection and flux*

Compared with ultrafiltration and reverse osmosis, nanofiltration membranes have different rejection mechanisms due to different pore sizes and charges. Unlike reverse osmosis membranes, nanofiltration membranes are porous membranes that perform separation based on a mechanism known as convection-diffusion, which is generally accepted (Wijmans & Baker, 1995). In general, it is worth noting that the main properties affecting membrane separation depend on the solubility and diffusion of the compound in the membrane material. In addition, the coupling effect of compound-compound/solvent and the solubility also have influence on the separation of the membrane (Bhanushali et al., 2001; Bhosle et al., 2005; Wei et al., 2010; Qiu et al., 2016).

The performance of the membrane (SS-NF7-2540, NF 90) with ratio of powder/ethanol is shown in Figure 7. Under SS-NF7-2540 and NF90 membranes, the permeation flux decreased with the increase of the extract concentration from 30% to 100% (V/V).This system can be viewed as a mixed solution containing ethanol, γ-oryzanol, chlorophyll a, chlorophyll B, and FFA. Viscosity has an adverse effect on convective flow in the extraction solution system and affects the variation of flux with extraction concentration, which may be the key factor to explain the phenomenon that flux decreases with increasing extraction concentration. Meanwhile, the polarization in process of membrane separation, which is unavoidable in fact, has a negative impact on permeate flux and the stirring in membrane cell could inhibit effect of polarization on this process, thereby reducing viscosity and increasing osmotic pressure promoting permeate flux when the feed solution is in higher concentrations.

The rejection rate of SS-NF7-2540 membrane to FFA was low, and the rejection rate was between 20% and 26% at all concentrations. Palmitic acid, oleic acid and linoleic acid are the main fatty acids present in rice seeds (Kim et al., 2015). The retention of γ-oryzanol was lower than that of chlorophyll a, ranging from 30% to 40%.

It was found that the retention rates of each component (chlorophyll a, chlorophyll b, γ-oryzanol) increased gradually with increasing concentrations, except for the retention rate of FFA, which remained stable in Figure 7A. The NF-90 membrane exhibited a similar trend in rejection of individual components as the SS-NF7-2540 membrane over a solute concentration range of 30(%) to 70(%), whereas the rejection of these component decreased as the solute concentration changed from 70(%) to 100(%), as shown in Figure 7B. The SS-NF7-2540 is considered as a "loose NF" membrane, whereas NF-90 is a "tight NF" membrane. Consequently, the rejection of individual constituents measured by NF-90 performed preferentially compared to that measured by SS-NF7-2540. The effect of diffusion is more positive than effect of viscosity in low concentration, but effect of viscosity is prominent in high concentration, which may explain those findings.

Although rejection of γ-oryzanol have been reported in various studies (Manjula & Subramanian, 2008; Sereewatthanawut et al., 2011), there have been few published studies of rejection of γ-oryzanol monomers (cycloartenol ferulate, cyclobranol ferulate, campesterol ferulate, β-sitosterol ferulate). Therefore, this study was conducted to assess NF-90 selecting γ-oryzanol monomers. The results of rejection (cycloartenol ferulate, cyclobranol ferulate, campesterol ferulate, β-sitosterol ferulate) are shown in Figure 7C. There were significantly reduce in rejection of each γ-oryzanol monomers from 70% to 100% (W/V). These results of this study demonstrated that each γ-oryzanol monomers exhibited differential permeability in liquid mixtures, although their molecular weights were similar, Cycloartenol ferulate permeated preferentially over other γ-oryzanol monomers.

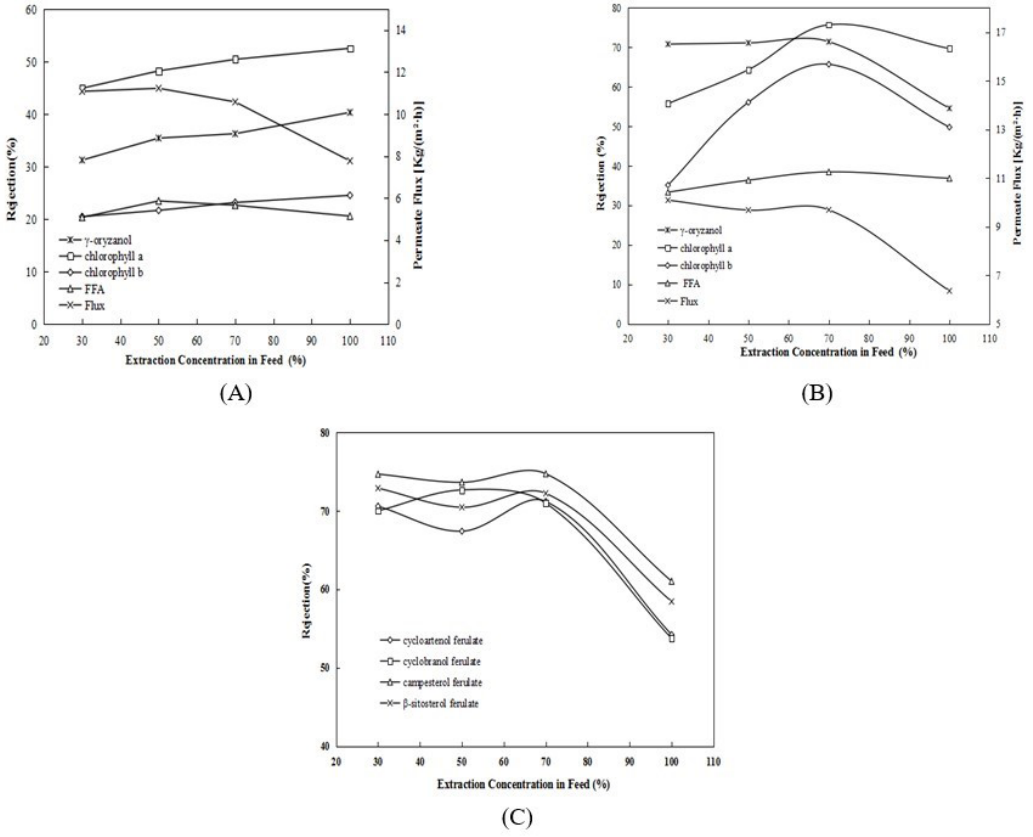


**Figure 7**. Effect of extraction concentration on permeate flux and rejection values in the methanol system, (A) NF SS-NF7-2540; (B) NF 90. (C) Effect of extraction concentration on γ-oryzanol (cycloartenol ferulate, cyclobranol ferulate, campesterol ferulate, β-sitosterol ferulate) rejection values in the ethanol system (NF 90).

### *3.7 The antioxidant of γ-oryzanol added in soybean oil*

The antioxidant activity was evaluated by the method of anti-free radical activity. In this study, DPPH free radical scavenging capacity was used as an index to determine the antioxidant capacity of oil. The mechanism of DPPH free radical scavenging method was that DPPH· scavenged by devoting hydrogen of antioxidant constituents was transformed to 2,2-diphenylpicryl-hydrazyl (DPPH). After DPPH combines with the electron or hydrogen radical provided by the antioxidant, the color of the reaction solution changes from purple to yellow and the absorbance at 517 nm decreases rapidly.

The antioxidant capacity results of model oils (soybean oil with addition 5% separated γ-oryzanol by nanofiltration membrane) and soybean oil are shown in Figure 8. It can be clearly seen that the effect of DPPH free radical scavenging activity was also increased significantly with the increasing oil concentration because different γ-oryzanol concentrations presented in mode oil. It was found that the slope rate of the fitted line of the model oil free radicals scavenging activity was significantly greater than that of the soybean oil. It is known that phenolic hydroxyl groups are active units in free radical scavenging reactions, each all four major constituents γ-oryzanol including 7′-hydroxyl group on substitution of ferulic acid were demonstrated DPPH· free radical scavenging activities by Akiyama et al. (2005), and the study has been reported that the antioxidant activity was mainly affected by cyclobranol ferulate of γ-oryzanol monomers (Massarolo et al., 2017). The fitting equation is y = 67.171x + 0.5932 calculated by model oil scavenging DPPH free radical, and the fitting equation for DPPH free radical scavenging of soybean oil is y = 48x - 0.3333.

The researchers exploit natural sources to maintain beneficial compounds with antioxidant activity. These materials can be used to prevent food deterioration by oxidation and to replace synthetic preservatives. γ-oryzanol was stable and resistant to high temperature in bread baking (Laokuldilok et al., 2011), which is suitable as a valuable source of natural antioxidants.

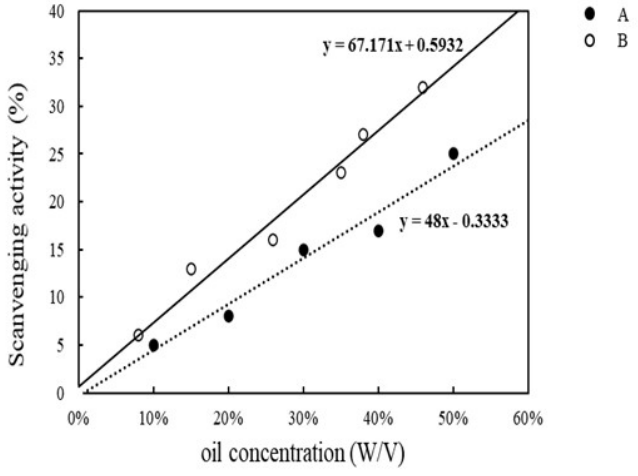


**Figure 8**. Antioxidant activity of soybean oil and model oil at different concentrations. Note: A is the DPPH scavenging rate of soybean oil, and B is the DPPH scavenging rate of model oil.

## 4 Conclusion

The results showed that the content of γ-oryzanol was the highest in the immature rice seeds of DAF-21st, which could be used as an extraction resource. Our study presented that γ-oryzanol separated by nanofiltration membranes was an effective antioxidant in DPPH free radical assay. As discuss above, it can be used in food supplements to promote human health, as suggested by that addition of soybean oil in this study.

## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

Feng Zuo and Dong-jie Zhang contributed to the conception of the study; Dan Li and Chao Zhang contributed significantly to analysis and manuscript preparation; Huimin Zhang and Chao Zhang performed the data analyses and wrote the manuscript.

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