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Method Development for Purification of γ-oryzanol from Hydrolyzed Rice Bran Acid Oil by Semi-preparative Chromatography

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Abstract: The objective of this study was to develop a method for isolation and purification of γ-oryzanol from hydrolyzed rice bran acid oil (RBAO) using semi-preparative chromatography by first applying silica coated-thin layer chromatography (TLC) to determine the suitable mobile phase. Subsequently, column chromatography was carried out to determine the effects of purification conditions such as the amount of and particle sizes of the sample silica gel, and elution modes, on the percentage of γ-oryzanol yield and recovery. The results from the TLC suggested that 75:25 (v/v) hexane to ethyl acetate mixture was a suitable mobile phase. The semi-chromatographic results indicated that the column containing 10 g of 25-40 μm silica gel with isocratic elution gave the highest yield (84%) of purified γ-oryzanol (> 95% purity). Further application of a step-gradient elution with 85:15 (v/v), followed by 75:25 (v/v) hexane to ethyl acetate mixture increased chromatographic resolution (Rs), resulting in enhanced separation efficiency, which in turn led to a higher yield of purified γ-oryzanol of 90%.

Key words: rice bran acid oil, γ-oryzanol, semi-preparative chromatography, purification

1 Introduction

γ-Oryzanol is a mixture of ferulic acids esters of phytosterols and triterpene alcohols and is an antioxidant[1, 2] and a hypo-cholesterolemic agent[3] found primarily in rice bran. It is also known for its ability to reduce allergic inflammation[4] and gastrointestinal inflammatory diseases[5]. Given the various therapeutic properties of γ-oryzanol, there has been growing global demand for this natural compound in cosmetic, nutraceutical and medical applications over the past decades[6].

In rice bran oil (RBO) processing, γ-oryzanol is generally extracted together with neutral oils, free fatty acids, and other impurities such as waxes and pigments. To improve the quality and to extend the shelf life of the final RBO product, FFAs are typically removed from the crude RBO in an early step, by saponification in the presence of aqueous solutions of NaOH or KOH. Through this process, FFAs are converted into rice bran oil soapstock (RBOS) which can be more easily separated from the neutral oil. Unfortunately, as high as 95% of the γ-oryzanol originally present in the crude oil is entrapped within the micellar structure of RBOS[7, 8], leading to a considerable γ-oryzanol loss in this by-product. For this reason, there has been a great deal of research and industrial interest in recovering γ-oryzanol from RBOS and its more stable derivative rice bran acid oil (RBAO), which is obtained by re-acidulating RBOS with sulfuric acid, followed by water removal, to extend the storage life of the by-product[9, 10].

RBAO is made up of comparable amounts of the two major components, FFAs and glycerides[11] and contains a relatively high amount of γ-oryzanol (up to 7 wt% on a dry basis). To recover γ-oryzanol from RBAO, previous studies suggested that FFAs can first be removed by distillation, either directly as FFAs, or after FFAs conversion into fatty

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acid methyl esters (FAMEs)[12]. The resulting dark distillation residue then undergoes alkali hydrolysis, in which the remaining glycerides and FFAs are converted into soap[12–15] before being extracted for γ-oryzanol with an organic solvent. With the removal of the FFAs, the hydrolyzed residue has a high concentration of γ-oryzanol. However, a large fraction (76.7 wt%) of γ-oryzanol is lost during the process[7]. Alternatively, without prior distillation, RBAO can be hydrolyzed and then directly extracted with an organic solvent such as ethyl acetate[16]. In either case, to obtain the purified product, additional steps are required to remove any remaining glycerides and other impurities such as phospholipids, waxes, sterols, glycolipids, tocopherol derivatives, resin and pigments[17] from the extracts. Although high purity γ-oryzanol (>98%) could be achieved by crystallization in a series of organic solvents[12], the percent recovery of γ-oryzanol is low (only 30-50%), potentially due to the limitation by phase equilibria of γ-oryzanol[18] and interference from the impurities within the sample.

Owing to the simple configuration required for semi-preparative/preparative-scale chromatography, and its scalability, high efficiency for product recovery and a high potential for industrial applications[19], this method has been extensively utilized for fractionation and purification of several compounds, including γ-oryzanol from crude RBO[20, 21]. Since the compositions of RBO by-products (RBOS and RBAO) differ from that of crude RBO, suitable chromatographic methods must be developed for specific systems under consideration. Even for the samples prepared from the same starting materials, the sample compositions will undoubtedly be different depending on the steps taken to prepare them, particularly whether distillation is employed in the recovery process or not. Previously, several studies have been conducted on chromatographic purification of γ-oryzanol isolated from RBOS[22, 23]. However, very few studies have reported the development of a method for chromatographic γ-oryzanol purification from RBAO. Das *et al.* (1998)[13] employed preparative normal-phase chromatography as a pre-purification step to recover γ-oryzanol from the hydrolyzed dark distilled residue of RBAO. Silica gel was used as a stationary phase and chloroform was used as an isocratic elution mobile phase. The purified product was obtained after the application of adsorption with activated charcoal, followed by crystallization[13, 14].

In our study, the ethyl acetate extract of hydrolyzed RBAO was used as a starting material for γ-oryzanol recovery without prior FFAs removal by distillation. Firstly, the contents of the key components, including γ-oryzanol, glycerides, and FFAs in the ethyl acetate extract, were analyzed. Secondly, a chromatographic method was developed to obtain purified γ-oryzanol from the sample using normal phase semi-preparative column chromatography packed with silica gel as a stationary phase. The suitable mobile phase was selected based on a preliminary study with thin layer chromatography. Finally, to further suggest a suitable chromatographic operation, the key operating conditions, including the silica gel particle sizes, the amounts of silica gel packing, as well as the elution modes, were evaluated for their effects on the column performance. In addition, the different components of the γ-oryzanol present in the purified fraction were analyzed with LC-MS.

2 Experimental Procedures

2.1 Materials

RBAO were obtained from the Thai Edible Oil Co. Ltd., Samutprakarn, Thailand. γ-oryzanol standard was purchased from Santa Cruz Biotechnology (Texas, USA). Oleic acid standard was purchased from Sigma-Aldrich (India). Chemicals such as ethyl acetate, methanol and isopropanol were purchased from Merck (USA). Hexane was purchased from Lab-Scan (Ireland) and Sodium hydroxide (NaOH) was purchased from Aps Chemicals (Sydney, Australia). Thin-layer chromatography and silica gel (LiChroprep® Si 60) were purchased from Merck (Germany).

2.2 Sample preparation

Since the presence of glycerides in the RBAO could potentially interfere with the subsequent purification of γ-oryzanol, as a primary step, alkali hydrolysis of RBAO was carried out to keep glyceride content at a minimum by converting it into soap. The method modified from Kittiruangthong (2005)[24] was used in which the reaction between RBAO and 2N NaOH solution at the ratio of 1:5 (v/v) took place at 80℃ for 10 min. The resulting hydrolyzed RBAO was then extracted with ethyl acetate at a 1:1 (v/v) ratio for 1 min and the mixture was then centrifuged at 4,000 rpm for 5 min to separate the extract from the RBAO sample. The extract was then analyzed for the amounts of FFAs, remaining glycerides, and γ-oryzanol using HPLC. The relative content of each component in the hydrolyzed RBAO was defined according to the following equation:

$$\text{Relative content of component} = \frac{\text{Amount of interested component in extract (mg)}}{\text{Total dry weight of RBAO (g)}} \quad (1)$$

2.3 Semi-preparative normal-phase chromatography

The semi-preparative chromatographic setup for this experiment consisted of a 14 mm-diameter 50 mL glass column (Pyrex®, Sigma-Aldrich, USA) packed with a specified amount of silica gel having a 60 Å pore diameter (Merck, Germany). The effects of the various semi-preparative chromatography conditions, including silica gel particle sizes (10-25 μm, 25-40 μm, and 40-63 μm), the amounts of silica gel packing (5, 10, and 15 g), and elution methods

(isocratic and gradient) on the chromatographic performance, were investigated. For each chromatographic run, 1 mL of ethyl acetate extract of hydrolyzed RBAO was loaded into the column. The sample was then eluted with a mobile phase comprising hexane and ethyl acetate at an appropriate volume ratio, as suggested by the preliminary TLC experiment (Supplementary material, Fig. S1). In all experiments, the mobile phase was set to flow gravimetrically downward through the column. The eluted samples were collected in fractions at 5 min intervals and were analyzed with HPLC for the contents of glycerides, γ-oryzanol, and FFAs. In addition, the effect of a step-gradient elution was determined by sequentially eluting the column with two mobile phases of different hexane / ethyl acetate compositions. The chromatographic performance was evaluated in terms of γ-oryzanol recovery (%) and yield (%), defined according to Eq. 2 and Eq. 3, as follows:

$$\gamma\text{-oryzanol recovery}(\%) = \frac{\text{Total } \gamma\text{-oryzanol weight from all collected fractions (mg)}}{\text{Weight of } \gamma\text{-oryzanol in loaded extract (mg)}} \times 100 \quad (2)$$

$$\gamma\text{-oryzanol yield}(\%) = \frac{\text{Total } \gamma\text{-oryzanol weight from all fractions having } \gamma\text{-oryzanol purity} > 95\% \text{ (mg)}}{\text{Weight of } \gamma\text{-oryzanol in loaded extract (mg)}} \times 100 \quad (3)$$

where the purity of γ-oryzanol in each collected fraction was determined using Eq. 4 as follows:

$$\gamma\text{-oryzanol purity} = \frac{\gamma\text{-oryzanol peak area}}{\text{Total area under all peaks}} \times 100 \quad (4)$$

In addition, the chromatographic resolution Rs, a value indicative of how well two eluted components (A and B) can be separated, is defined as the difference in retention times (t_R) between the two adjacent peaks: $t_{R,B} - t_{R,A}$ (where, by convention, A elutes first), divided by the average of the peak widths, w_A and w_B, according to the following equation (Eq. 5).

$$R_S = \frac{2(t_{R,B} - t_{R,A})}{w_B + w_A} \quad (5)$$

The retention time ($t_{R,i}$) was determined by Eq.6 and the peak width (w_i) was determined by Eq.7, for each component i:

$$t_{R,i} = \frac{\sum_{i=1}^{n_p} t_i C_i}{\sum_{i=1}^{n_p} C_i} \quad (6)$$

$$w_i = 4\frac{\sum_{i=1}^{n_p} (t_i - t_{R,i})^2 C_i}{\sum_{i=1}^{n_p} C_i} \quad (7)$$

in which t_i is the time at which the fraction i was collected, C_i is the concentration of the component of interest in fraction i, and n_p is the total number of collected fractions.

2.4 Component analysis by HPLC

The amounts of γ-oryzanol, glycerides and FFAs in the extract of the original RBAO and the hydrolyzed RBAO, as well as in all collected chromatographic fractions, were quantified using HPLC equipped with a pump (Alltech model 626, USA) and an ELSD detector (Alltech ELSD 2000ES, USA). For each analysis, 5 μL of the sample was injected through the 3.9 mm × 300 mm μBondapak C18 column with 10 μm particle size and 125 Å pore size at room temperature. The detector condition was set at 60℃ with a nitrogen flow at the rate of 1.7 L/min. A mixture of methanol and isopropanol at the ratio of 60:40 v/v was used as the mobile phase, with a flow rate of 0.9 mL/min. The amounts of γ-oryzanol, glycerides, and FFAs in the extract were calculated based on the corresponding calibration curves generated using standard solutions of γ-oryzanol, glycerides, and FFAs. The composition of the γ-oryzanol in the fractions was also verified by LC-MS (Table S1).

3 Results and Discussion

3.1 Relative contents of components in ethyl acetate extract of hydrolysed RBAO

The ethyl acetate extract of the starting RBAO sample and that of the hydrolysed RBAO to be loaded onto the semi-preparative column were analysed to determine the relative contents of γ-oryzanol, glycerides, and FFAs. As shown in Table 1, after alkali hydrolysis of the RBAO, the relative contents of the components in the ethyl acetate extract changed considerably. Specifically, the relative content of glycerides was reduced from 105.3 to 8.65 mg/g dry weight of RBAO, while that of FFAs was reduced from 516.0 to 135.0 mg/g dry weight of RBAO. The decrease in glycerides and FFAs contents observed was a result of their conversion into soap. In addition, an approximately

Table 1 Relative contents of γ-oryzanol, glycerides and FFAs in extract of RBAO and hydrolyzed RBAO.

Components (mg/g dry weight of sample)	In extract of	
	RBAO	Hydrolyzed RBAO
γ-oryzanol	135.5	53.84
Glycerides	105.3	8.65
FFAs	516.0	135.0

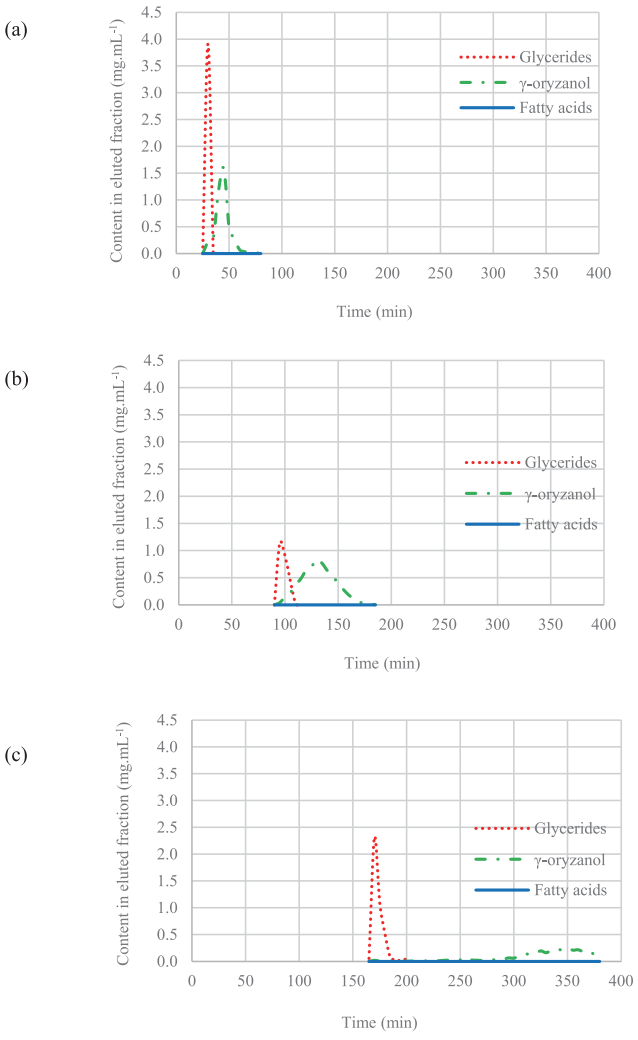

Fig. 1 Component concentrations of collected fractions of hydrolyzed RBAO extract by isocratic elution with 75:25 (v/v) hexane:ethyl acetate from semi-preparative normal-phase chromatography packed with 10 g of silica gel, of particle size a) 40-63 μm, b) 25-40 μm, and c) 15-25 μm as stationary phase.

60% decrease in the relative content of γ-oryzanol was seen in the extract of the hydrolyzed RBAO (from 135.5 to 53.84 mg/g dry weight of RBAO). Such inevitable γ-oryzanol loss of up to 60% depends on the reaction conditions employed, has also been reported in previous literatures[15, 25, 26]. It should be noted here that further purification steps are needed to separate γ-oryzanol from the remaining glycerides and FFAs, albeit the high reductions of these compounds by alkali hydrolysis.

3.2 Semi-preparative normal-phase chromatography

Semi-preparative normal-phase chromatography was performed to further purify γ-oryzanol from the extract of hydrolyzed RBAO. The effects of particle sizes, amounts of silica gel packing, and elution modes on the chromatographic performance were evaluated by comparing the chromatograms, constructed by plotting the contents of γ-oryzanol, and the glycerides and FFAs in the eluted fractions. The chromatograms obtained from the columns by varying the particle sizes are shown Fig. 1 and the amounts of silica gel are shown in Fig. 2. For all chromatographic runs, it was evident that glycerides were eluted first since low polarity components (such as glycerides) are unfavorably adsorbed on silica gel, which possesses highly polar surface hydroxyl groups[27]. Next to glycerides, γ-oryzanol,

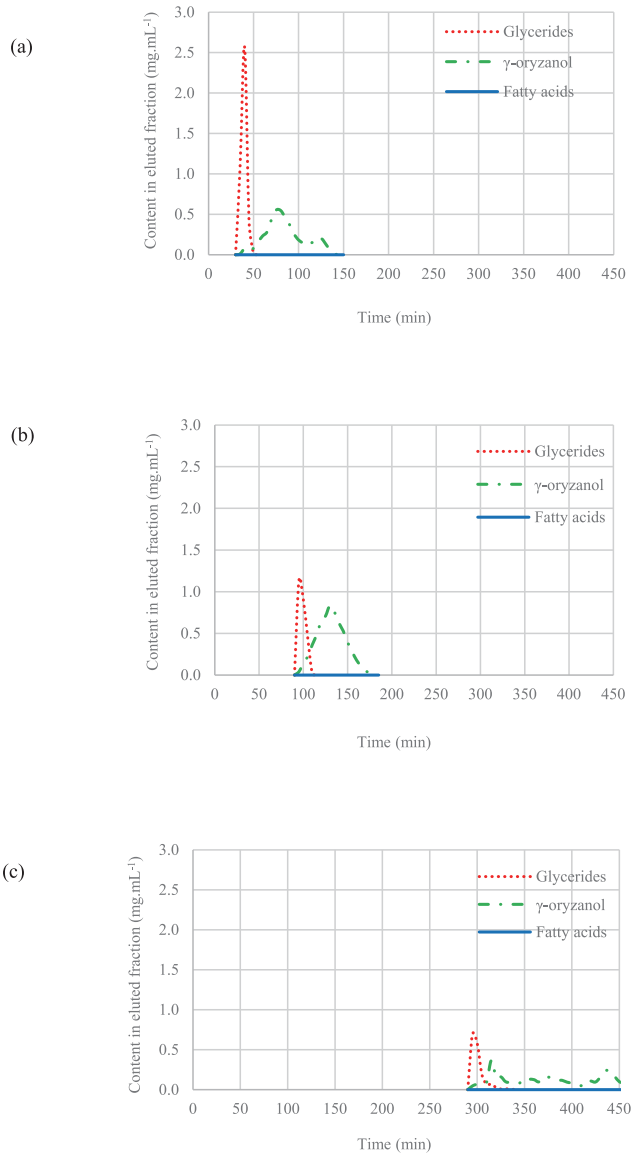

Fig. 2 Component concentrations of collected fractions of hydrolyzed RBAO extract by isocratic elution with 75:25 (v/v) hexane:ethyl acetate from semi-preparative normal-phase chromatography packed with a) 5 g, b) 10g, and c) 15 g of silica gel of particle size 25-40 μm.

whose molecular structure consists of a hydroxyl group and a carboxylic ester group, was eluted, while on the other hand, FFAs, being the most polar component containing carboxylic acid functional groups, were strongly bound to the silica gel column, and were not eluted by the mobile phase applied in our study. The TLC results in Fig. S1 also showed the same order of the components migrating up the silica gel plate, being first the glycerides, followed by the γ-oryzanol and then the FFAs.

3.2.1 Effect of silica gel particle size

The chromatograms of components isocratically eluted with 75:25 (v/v) hexane to ethyl acetate mixture from various columns packed with 10 g of silica gel of different particle sizes: 40-63 μm, 25-40 μm, and 15-25 μm are shown in Figs. 1a, 1b, and 1c. The corresponding γ-oryzanol recovery, yield, and Rs values obtained from these columns are summarized in Table 2. With the large size particles (40-63 μm), the column void fraction, and thus the mobile phase velocity, was high (10.94 mm/min), causing the glycerides and γ-oryzanol components to elute from the column quickly as narrow peaks. However, at such high velocity, the components did not have sufficient time to reach equilibrium partitioning between the mobile phase and the silica gel phase, resulting in low separation efficiency between them. As summarized in Table 2, the corresponding Rs value determined based on Eq. 5. was relatively low (Rs = 0.82), and an overlapping area between the γ-oryzanol peak and the glycerides peak was observed, while clear separation between the two peaks would be expected if the Rs value is greater than 1.5[28, 29]. In addition, at high mobile phase velocity, a part of the γ-oryzanol could travel through the column quickly, leaving behind a part of the loaded γ-oryzanol that was adsorbed on the inner regions of the silica gel particles but was not desorbed quickly enough, causing low recovery of 63% and a low yield of 60%. As the particle size becomes smaller, the equilibration times become shorter as components can reach the adsorption site more quickly. Furthermore, in

Table 2 γ-Oryzanol recovery, γ-oryzanol yield, chromatographic resolution (Rs), and mobile phase velocity (u_t) at various chromatographic conditions.

Conditions		Total γ-oryzanol recovery (%)	Total γ-oryzanol yield (%)	Rs	u_t * (mm/min)
Varied Parameters	Fixed Parameters				
Effect of Particle size					
40-63 μm	*Silica gel amount*:10 g *Elution method*: Isocratic	63.67	60.37	0.82	10.94
25-40 μm		89.42	83.64	0.82	2.99
15-25 μm		50.36	49.77	1.80	1.70
Effect of Silica gel amount					
5 g	*Particle size*: 25-40 μm *Elution method*: Isocratic	72.41	69.25	0.90	5.56
10 g		89.42	83.64	0.82	2.99
15 g		63.25	28.39	0.70	2.22
Effect of Elution method					
Isocratic	*Silica gel amount*: 10 g *Particle size*: 25-40 μm	89.42	83.64	0.82	2.99
Step-gradient		90.49	90.15	1.77	2.99

* The mobile phase velocity u_t (mm/min) was determined for each experiment using the following equation for the evaluation of the effects of the chromatography conditions.

$$u_t = \frac{u_s}{\varepsilon}$$

$$u_s = \frac{Q}{A}$$

in which u_s is the average fluid superficial linear velocity (mm/min), Q is the volumetric flow rate of the mobile phase (mL/min), A is the cross-sectional area of the column (m^2), ε is the interparticle or external porosity which can be calculated from the following equation:

$$\varepsilon = 1 - \frac{(\text{Amount of silica gel used} / \text{Bulk density of silica gel})}{\text{Volume of column}}$$

in which the volume of the column (m^3) is calculated from the cross-sectional area of the column (A, m^2) multiplied by the length of the column (L, m) which was measured for each experiment. The average bulk density of 500 kg/m were calculated based on the range of these values as described by the manufacture (Merck, Germany) and were herein used for the estimation of the porosity.

this column, the components are retained longer, allowing them to reach equilibrium partitioning between the two phases. The component separation was therefore expected to be improved. For the column packed with silica gel particles of intermediate size (25-40 μm), the recovery was increased to 89%. Nevertheless, the resolution remained at Rs = 0.82 due to band-broadening, resulted by increased pressure drop and increased retention time. Considering the fractions with >95% γ-oryzanol purity, the yield was therefore lower than the recovery (84 vs. 89%). Despite the advantages of shorter path length and more uniform packing, the smallest particle size (15-25 μm) resulted in high-pressure drop across the column which caused the blockage of the mobile phase flow down the column. This effect could lead to an extremely long retention time and high longitudinal diffusion, and thus severe band-broadening of higher retention of the γ-oryzanol component. Although the resolution was found to be high (Rs = 1.8), slow mobile phase velocity (1.70 mm/min) caused by high pressure drop could not supply enough convective flow that pushed the adsorbed γ-oryzanol down the column. As a result, only approximately 50% of the γ-oryzanol was recovered.

Based on these results, the appropriate particle size was found to be 25-40 μm. This size was therefore employed in the subsequent study to determine the effects of the amount of silica gel packing on the chromatographic performance.

3.2.2 Effects of silica gel amount

Having chosen the suitable silica gel particle size of 25-40 μm, the effect of the various amounts of silica gel on γ-oryzanol isolation can be seen in the chromatograms of the sample components, eluted from the columns packed with 5, 10, and 15 g as shown in Figs. 2a, 2b, and 2c. As shown in these figures, the retention time of the components eluted from the column packed with 5 g was the shortest, since the resistance to mobile phase flow was low in such a short column. This quick elution could not allow the retained solute (γ-oryzanol) to properly reach the stationary-mobile phase equilibrium partitioning, leading to poor peak separation. In this case, the γ-oryzanol recovery was 72%, the yield was 69%, and Rs value was 0.9.

As the silica gel amounts increased, the time of the components spent in the column increased, allowing better equilibration of the components between the two phases. As seen in Table 2, the column with 10 g showed more favorable γ-oryzanol recovery (89%) and yield (84%). Nevertheless, although the separation between glycerides and γ-oryzanol should be increased when the larger amount of packing material is available for the components to separate as they move through the column, the additional column length influenced the fluid dynamics within the column, which contributed to the increased axial dispersion of the components, leading to band broadening and dilution of the target components[30]. As a result, the separation efficiency decreased, which in turn lowered the resolution of the components as can be seen from the lower resolution of the column with 10 g silica gel compared with that of the 5 g silica gel column (0.82 vs. 0.9). This effect can be seen even more clearly in Fig. 2c which shows the chromatogram of components eluted from the column packed with 15 g of silica gel. In addition to the effect of the extra column length on axial diffusion, pressure drop across the column increased considerably in the column packed with larger amount of silica gel, thus lowering the velocity of the gravimetric flow of mobile phase down the column. This, not only led to an unfavorably long retention time, and thus low productivity, it also resulted in low γ-oryzanol recovery (63%) due to insufficient convective transport of the γ-oryzanol down the column. In addition, at low mobile phase velocity, axial dispersion and band-broadening of the eluted components were worsen. As a result, the resolution was found to be the lowest among all the conditions studied (Rs = 0.7), and an extremely low γ-oryzanol yield of only 28% was obtained.

3.2.3 Effects of elution mode

In previous investigations, the particle size and the amount of silica gel packing were shown to be key factors influencing chromatographic performance, and the highest glycerides and γ-oryzanol recovery and yield were obtained with the column packed with 10 g of silica gel particles with a diameter range between 25-40 μm, and under isocratic elution with 75:25 hexane/ethyl acetate mixture. However, there appeared to be a limit on the improvement in the chromatographic resolution that could be achieved by adjusting just these two parameters. When the thermodynamic approach was taken, in our study, the mobile phase compositions were adjusted stepwise during the chromatographic run. Specifically, the column was eluted initially with the 85:15 hexane to ethyl acetate mixture for 1 hr before switching to the 75:25 hexane to ethyl acetate solvent mixture, while the other chromatographic conditions remained the same. The resulting chromatograms, shown in Fig. 3, can be compared to those obtained with isocratic elution with 75:25 (v/v) hexane/ethyl acetate mixture.

As illustrated in Fig. 3, the step gradient elution resulted in a clear baseline separation between the γ-oryzanol and the glycerides, which corresponded to the increased Rs value from 0.82 to 1.77, as can be seen in Table 2. The selection of the mobile phase compositions used was strongly suggested by the TLC results (Fig. S1), which showed that the solvent mixtures with relatively high hexane to ethyl acetate ratios, i.e., 85:15, appeared to provide superior separation between the glycerides and the γ-oryzanol, at the expense of a longer retention time compared with the 75:25 hexane to ethyl acetate mixture. Therefore, the mixture at 85:15 v/v ratio was initially used to allow suffi-

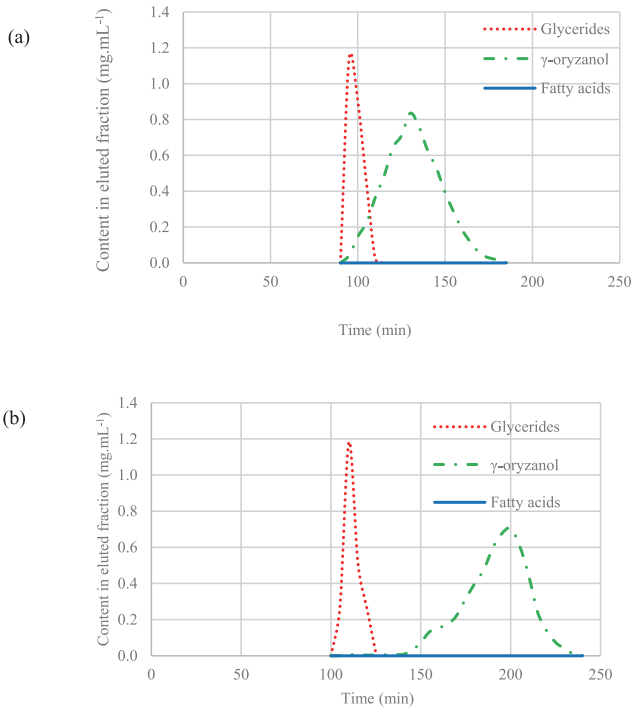

Fig. 3 Effects of elution mode on γ-oryzanol isolation using 10 g of the 25-40 μm silica gel as stationary phase a) isocratic elution mode with 75:25 hexane to ethyl acetate mixture b) step gradient elution mode with 85:15 hexane to ethyl acetate mixture for 1 hour, followed by elution with 75:25 hexane to ethyl acetate mixture.

cient separation between the two components, followed by the higher polarity solvent mixture (larger ratios of ethyl acetate) that could more rapidly elute the more polar (and more retained) γ-oryzanol from the column. Due to the increased resolution, the γ-oryzanol yield and recovery were improved (90%, Table 2). By the analysis with LC-MS (Table S1), the purified fraction was confirmed as being comprised of four main γ-oryzanol components, namely cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate and β-sitostery ferulate.

To sum up, the semi-preparative chromatographic method proposed in this study is a simple and promising method for the purification of γ-oryzanol from hydrolyzed RBAO. Even though the method was developed particularly for the γ-oryzanol purification from hydrolyzed RBAO, a comparison of the results with the data reported in the literature, albeit for different starting materials as summarized in Table 3, provides some insights into the possibility of applying the process at a larger scale. Specifically, the comparison with the process reported by Lai *et al.*[21] for γ-oryzanol recovery from RBO indicates that a similar level of γ-oryzanol recovery and purity could be obtained when using a simple open-column two-step gradient system (versus three-step gradient elution with medium pressure column chromatography). This was possible because the hydrolyzed RBAO was less complex and contained lower amounts of impurities than the crude RBO. When compared with the results reported by Kasim *et al.*[31] for the chromatographic purification of γ-oryzanol from biodiesel residue, the percentage of γ-oryzanol recovery and purity obtained were higher in our study. This could be because

Table 3 Comparison results with literatures.

Sample	Chromatographic method and conditions	%Recovery and Purity	Reference
RBO	Injection volume: 2 mL Column: stainless-steel 1 cm i.d. 25 cm length Packing material: silica gel 12 μm Mobile phase: Three-step gradient elution with a solvent composition of hexane/ethyl acetate from 90:10 to 50:50 to pure ethyl acetate Mobile phase flowrate: 8.0 mL/min	Purity: 98.9% Recovery: 94.1%	[21]
Biodiesel residue	Injection volume: 10 mL Column: 2.4 cm i.d. and 35 cm length Packing material: silica gel (74–250 μm) Mobile phase: isocratic hexane:ethyl acetate (97:3, v/v) Mobile phase flowrate: 1.0 mL/min	Purity: 79.37% Recovery: 80.73%	[31]
Hydrolyzed RBAO	Injection volume: 1 mL Column: stainless-steel 1.4 cm i.d. 23 cm length Packing material: silica gel 25-40 μm Mobile phase: Two-step gradient elution with a solvent composition of hexane/ethyl acetate from 85:15 to 75:25 Mobile phase flowrate: 0.2 mL/min	Purity: >95% Recovery: 90.49%	This work

silica gel of larger particle sizes (74–250 μm versus 25-40 μm) than ours were employed in their study, and the chromatographic system had not been optimized.

4 Conclusions

This study demonstrated that high-purity γ-oryzanol (> 95%) could be isolated from RBAO through a simple semi-preparative normal-phase chromatographic system with isocratic elution operated at a suitable condition, providing up to 84% γ-oryzanol yield. The particle size and the amount of the adsorbent used in the column are crucial factors directly influencing mass transport and fluid dynamics within the column, which in turn determined the overall performance of chromatographic separation. Furthermore, the chromatographic resolution could be improved via a thermodynamic approach by applying a step-gradient elution mode, resulting in the increased γ-oryzanol recovery and yield. The findings in this study form the basis for further development of large-scale preparative chromatography for the production of purified γ-oryzanol from hydrolyzed RBAO.

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Conflict of Interest

The authors have declared no conflict of interest.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess22217

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