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Integrated Subcritical Water and Methanol Extraction for Purification γ-Oryzanol from Rice Bran Using Silica Gel

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----------ABSTRACT----------
In this study was evaluate extraction of oryzanol from rice bran under subcritical condition and pressurized gas (N_2 and CO_2) to obtained rice bran oil (RBO) followed by purification oryzanol using silica gel. Temperature, reaction time and solvent ratio (methanol to water) were used at extraction method. The oryzanol content 2.16% ± 0,11 (2,75 mg oryzanol/g oil) were obtained using subcritical condition with initial free fatty acids (FFAs) content of 37.77%, under the following conditions: T= 200^{o}C, P= 4.0 bar (Used N_2 as a pressurized gas), methanol to water ratio 20/20 (v/v), ratio of rice bran/solvent of 1/8 (g/mL), and 7 h of reaction time. Using CO_2 as pressurized gas at the same operational condition, the yield and oryzanol content were obtained 13.82%±0.16; 2.03 %±0.19 or 2.84 mg oryzanol / g oil respectively. Employing silica gel as adsorbent on its weight at various solvent and temperature. The greatly effect of utilization of silica gel under methanol as a solvent at 32 ±1, ratio rice bran oil to silica gel (1/3) (w/w) were obtained 6.62% or 19.75 mg oryzanol/ g oil with low FFA content 4.38% which make it excellent adsorbent to oryzanol purification.
Keywords – *oryzanol, rice bran, silica gel, subcritical extraction*

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I. INTRODUCTION

An attractive alternative of biomass due to the great availability in nature. Rice production in Indonesia was increase 7 % in 2015 [1]. The main source of important component such as cellulose, hemicellulose, lignin and bioactive compounds. The main bioactive compounds contained in rice bran is γ-Oryzanol. Oryzanol content in rice bran is ten times higher than other bioactive compounds, such as α-tocopherol and α-tocotrienols [2]. Oryzanol content range is 1-3 % in rice bran depends on variety of rice and climate, reported 1,1 – 2,6% [3], 1,2 – 1,6 % [4] 1,5 - 2,9% [5] 1-2% [6] 1,8 – 3% [7]. Oryzanol is mainly composed of trans-ferulic acid esters with phytosterols (sterol and triterpene alcohol) wich extract from rice bran oil. Oryzanol is mixture of minimum ten phytosteryl ferulates (24 Methylene-cyclo-artenylferulates, Cycloartenylferulates, β-sitosterylferulate, Campesterylferulate, Sitostanil ferulates, Sitosteryl ferulates, Stigmasteryl ferulates, Δ-Stigmastenyl ferulates, Δ-Sitostanil ferulates, Δ-Campesterylferulate) [8].

Subcritical water technology has been recognized in past decade at various application (extraction and oxidation) [9]. Subcritical water is defined as hot water at temperatures ranging between 100 to 374 ^{0}C under high pressure to maintain water in the liquid state. Under subcritical conditions, the ion product of water increasing over ten thousands higher than that of water at room temperature [10]. In addition, the subcritical water dielectric constant decrease to those of polar organic solvent as increasing temperature [11] and it is greatly reduce with adding organic solvent such as methanol [12]. In order that, employing subcritical water and methanol extraction at various composition would obtain different yield of rice bran oil.

II. MATERIAL AND METHODS

2.1 Material

Rice bran (17.71% oil content) was obtained from Lamongan, East Java. Rice bran variant IR 64 was filtered to remove the impurities and kept in refrigerator to avoid increasing of FFA content. Oryzanol standard was obtained from Wako, Japan. Silica gel (70-230 mesh ASTM) was purchased from Merck, Germany. Any solvents and pressurized gases were purchased from commercial source.

2.2 Methods

2.2.1 Conventional Extraction

A conventional extraction technique (Soxhlet extraction) was used as reference for comparison with performance of subcritical water extraction. A 500 ml Soxhlet apparatus was employed in which 15 gr with n-hexane solvent for 6 hours [13].

2.2.2 Subcritical Water and Methanol Extraction for Rice Bran Oil (RBO) Production

Rice bran (5g) was mixed with 40 ml solvent (deionized water + methanol) at subcritical reactor in a high pressure (40 bar) equipped with an external electrical heater. The reactor was made from stainless steel equipped with thread of screw for tightening with its caps in both bottom and top of reactor. The fittings purchased from Swagelok, Taiwan. Fig 1 is the subcritical reactor schematic diagram. Temperature in the reactor was measured by thermocouple and controlled at certain temperature. The extraction time was counted after the temperature reached at desired level.

After pre-determined time, the reactor was rapidly sequencing as a consequence, the pressure inside the reactor was decreased. The gas still remained in the reactor reduced by releasing the gas slowly. Reaction in the reactor stopped as soon as the pressure dropped. Liquid in the reactor was collected. Hexane (50 mL) was added in the mixture and stirred at 300 rpm for thirty minutes. The upper hexane phase which contained oil was withdrawn. This procedure was repeated three times. Hexane was removed and mass of the product were determined. Non-polar compound, FFA, and oryzanol content were analyzed.

2.2.3 Separation Oryzanol from Rice Bran Oil

Separation Oryzanol from rice bran oil with solid liquid extraction employing silica gel. Silica gels 70-230 mesh were heated to eliminating water content for 1 hour in 150 ^{0}C furnace temperature. A Soxhlet extraction were used to collecting non-polar component using n-hexane solvent from mixing silica gel and rice bran oil (1/3) (w/w) at 44 cycles. Thus, silica gel restrained the polar component (Oryzanol). The silica gel was extracted in Soxhlet apparatus with methanol as polar solvent at 27 cycles. The methanol phase was separated using rotary vacuum evaporator to obtained liquid phase (oryzanol rich) in various temperature 32±1, 51±1, 64±1 ^{0}C. The liquid phase were analyzed using Thin Layer Chromatography.

2.2.4 FFA Analysis

FFA content was analyzed with titrimatic method following with AOCS official method Ca-5a40 which has been modified by *Rukunuddin et al*, [14]. Sample was dissolved in ethyl alcohol at 60 ^{0}C and FFA content neutralized with natrium hydroxide with addition of penolphtalein solution. The weight of sample, volume of natrium hydroxide used for calculating the FFA content.

2.2.5 Thin Layer Chromatography analysis (TLC).

RBO has been purified with silica gel was analyzed with TLC for qualitative study of ester to ensure all non-polar compounds were removed from the solution. Sample was dissolved in hexane and spotted on TLC plate which was then developed in a solvent system of hexane/ethyl acetate/acetic acid (90:10:1). Spots were visualized under iodine vapor and identified by using authentic standards

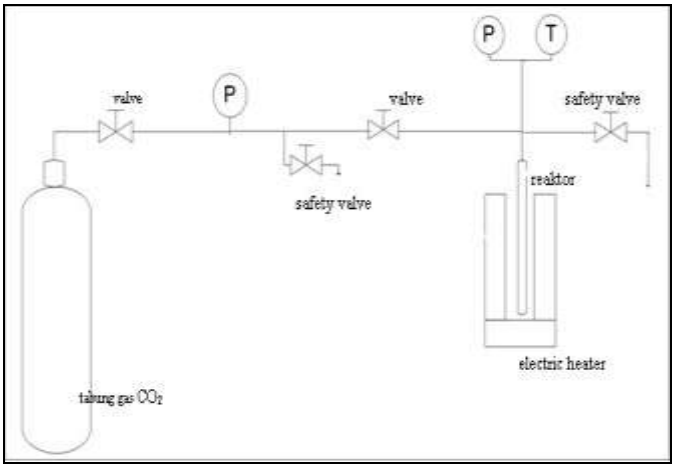

Figure 1. Methanol-water subcritical extraction schematic

2.2.6 Oryzanol Content Quantitative Analysis

Oryzanol content in the sample was determined by UV-vis Spectrophotometer. The sample was dissolved in hexane in a 1-cm quartz cell and absorption at multi wave length (200-400 nm) was measured. The analyses were performed under 100 nm/min, bandwith = 1 nm, and data pitch = 1 nm. The calibration curve was obtained with pure oryzanol in a concentration range 0-100 mg/100 mL. In this concentration range, the calibration curve obtained at λ_{316} is a straight line passing through the origin (R^2 = 0,9895).

III. RESULT AND DISCUSSIONS

The content and composition of rice bran vary greatly depending on various factors such as climate, soil conditions, rainfall, and planted rice species [15]. Rice bran variant IR 64 has water content 12.74%. The content of FFA and oil content (RBO) in bran are 37,77 % and 17,71% ± 0,03 respectively. Previous researchers Zullaikah *et al* [16], with different rice varieties, also obtained oil content of 15-23%.

3.1 Effects of Reaction Time under Subcritical Condition

The yield increased with increasing reaction time, during 1 to 7 h of extraction time were obtained 11,2 % ± 0,19 to 13,82% ± 0,16 when using CO_2 as a pressurized gas. , but when reaction time rise to 8 h, the yield decrease to 9,55%±0,39. Employing N_2 as a pressurized gas had same trends with CO_2. The best condition at 7 h (12,75 % ± 0,071) reaction time then decreased slightly to 11.93% ± 0,27 at 8 h. When initial reaction time started, the rate extraction work slowly enough due to materials hasn't mixed completely. Long reaction time, the mixing of methanol, water and rice bran dispersed well so the rate extraction could run faster. Therefore, the yield increase significantly. However, *Ju et al* [17] reported that, a very long reaction time, the *fatty acid* could polymerized so the yield oils decrease badly. Water ionic produk were higher in subcritical condition (10^{-12}) than that of ambient (10^{-14}). The high content of H^+ and OH^- in subcritcal condition were indicated acid-base reaction. In addition, water density in subcritical was higher than ambient. In subcritical, had low enough compressibility although used high temperature [18]. High density and water ionic product pushed ionic reaction making yield of CO_2 got higher than that of N_2. The effect reaction time to yield oils can see at Fig. 2a. On the other hand, the FFA content (data not shown) has decrease continuously along with increasing reaction time. At initial reaction time, the FFA content decrease drastically doe to FFA have lower molecular weights than their respective triglycerides, which explained why in this study they are selectively extracted in the initial extraction process [19].

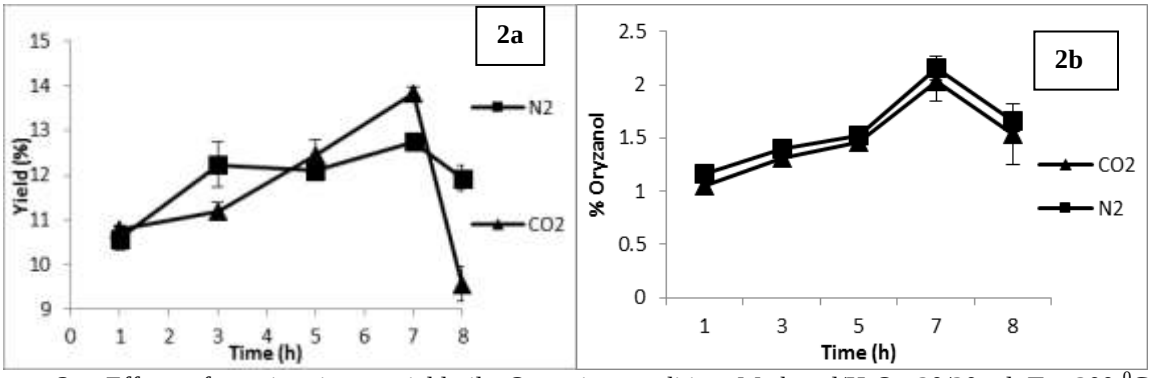

Figure 2a. Effects of reaction time to yield oils. Operation condition: Methanol/H_2O : 20/20 ml, T = 200 ^{0}C, P = 40 bar. **Figure. 2b** Effects of reaction time to oryzanol content. Operation condition: Methanol/H_2O : 20/20 ml, T = 200 ^{0}C, P = 40 bar.

The highest oryzanol content (2.16% ± 0,11 %) used N_2 as a presurized gas were obtained also at 7 h reaction time Fig. 2b. At initial reaction time, only 1% oryzanol was more difficult to extract from rice bran because the slower extraction of oryzanol of lower solubility. Another explanation, at initial time, the extraction rate almost equal with degradation rate and at optimum time the the extraction rate was greater than degradation rate, whereas at the longest reaction time, the degradation rate was greater than extraction rate. Therefore, the oryzanol content at 8 h reaction time was decrease.

Employing N_2 as a pressurized gas in subcritaical extraction got high oryzanol than that of CO_2 due to oryzanol easier degradate at acid medium such us CO_2 [20] while N_2 is inert gas. Therefor,oryzanol content in rice bran oil tend to increase [21].

3.2 Effects of temperature reaction under subcritical condition

Temperature is one of parameter that affecting subcritical extraction. The increasing temperature effected rising diffusion rate and decreasing viscosity, surface tension. Therefore, diffusion of subcritical water into matrix ran quickly [22]. The yield oils increase constantly as increase temperature. Under high solubility condition (>170 ^{0}C), it appears that oil was extracted more quickly and diffusion-controlled extraction is reached, whereas

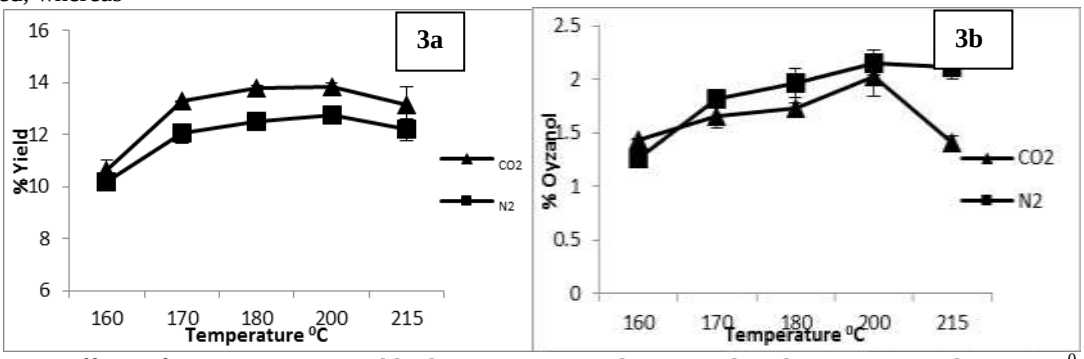

Figure. 3a Effects of reaction time to yield oils. Operation condition: Methanol/H_2O : 20/20 ml, T = 200 ^{0}C, P = 40 bar. **Figure. 3b** Effects of temperature to oryzanol content. Operation condition: Methanol/H_2O : 20/20 ml, reaction time= 7 h, P = 40 bar.

Raising temperature greatly affected oryzanol extraction. The yield and oryzanol content using N_2 as pressurized gas 12.75%±0.07 ; 2,15%±0.11 or 2.75 mg oryzanol/g oil at 200 ^{0}C whereas employing CO_2 as a pressurized gas, yield and oryzanol content 13.82%±0.16 ; 2.03 %±0.19 or 2.84 mg oryzanol / g oil. The present work, both using N_2 and CO_2 as pressurized gas were higher than *Shen* , 1996 which is obtained only 1,5% with supercritical carbondioxide. Accelerating temperature made positive affecting to oryzanol conten doe to in high temperature, the outer layer of rice bran easier to brake so more released oryzanol easily [2]. The presence of CO_2 made the acid medium, therefore oryzanol easier degrade [20] become phytosterol [25].

Under low solubility (<170 ^{0}C) not all oil is extracted even after 7 h. The highest yield oils 13,82% ± 0,16 at 200 ^{0}C with CO_2 as a pressurized gas were described at Fig.3a. However, yield oils decrease to 13,14% ± 0,67 at 215 ^{0}C. The same trend was occurred at N_2 as pressurized gas. The increasing extraction temperature above the certain value gives rise to the degradation of the essential oil components. The possibilities explanation, as increasing temperature, the extraction rate come faster due to an increase in diffusion rate of subcritical water decrease viscosity and surface tension [23].

Using N_2 also has the same trend but given lower oil recovery than CO_2. The presence of CO_2 made acidifying medium. Therefore, accelerate the rate extraction oil due to lipid easier dissolved in acid medium [24].

At high temperature oryzanol degraded much slower than α-tocopherol (another antioxidant in rice bran), indicating that oryzanol is a promising antioxidant for high temperature application [26].The oryzanol content were obtained in this study, have longer time degradation that is 8 h at 215 ^{0}C Fig. 3b compared with earlier study *Khuwijitjaru* [26] degraded at 120 ^{0}C for 10 h and [27] began degrade at 180 ^{0}C for 6 h.

3.3 Effects of ratio solvent (methanol – water) under subcritical reaction.

The amount of ratio solvent have correlation with polarity. The polarity water (10,2) and methanol (5,6) is totally different. However, under subcritical condition, the dielectric constant of water decrease to 27, same with dielectric constant of methanol at ambient temperature. Therefore, water can act as solvent to non-polar compounds [28]. The adding of organic solvent namely methanol could increase yield of rice bran oil.

Yield oils increase as increasing ratio methanol to water. The highest yield (17,43% ± 0,04) oils was obtained at ratio 30/10 methanol to water used CO_2 as a pressurized gas. The N_2 also has the same trend but the yield oils lower than CO_2 Fig.4a.When the amount of methanol increase to ratio 35/5, the yield oils decrease slightly. It's proved that the excess of methanol, disposed to extract more polar compounds [29]. The greater amount of solvent, the bigger extraction efficience. However, the abundant solvent doesn't extract more,the certain amount of solvent can extract optimally [29]. The FFA content decrease continuesly as increasing ratio methanol to water. The presence methanol, give possibilities esterification occcured and convert FFA to non polar compounds. Therefore, the FFA content decrease drastically [21]. The ratio methanol-water great influence capabiity to extract oryzanol. The characteristic oryzanol tends to solved in more polar solvent like methanol, isopropanol, ethyl acetate etc [2]. The highest oryzanol content could be extracted 2.15% ±0.11 or 2.75 mg oryzanol /g oil with yield 12.75% ±0.07 at ratio solvent was (20/20 ml) used N_2 as pressurized gas. Thi may due to, methanol had lower polarity and viscosity and preferentially extracted lipids than water. In contrast, the

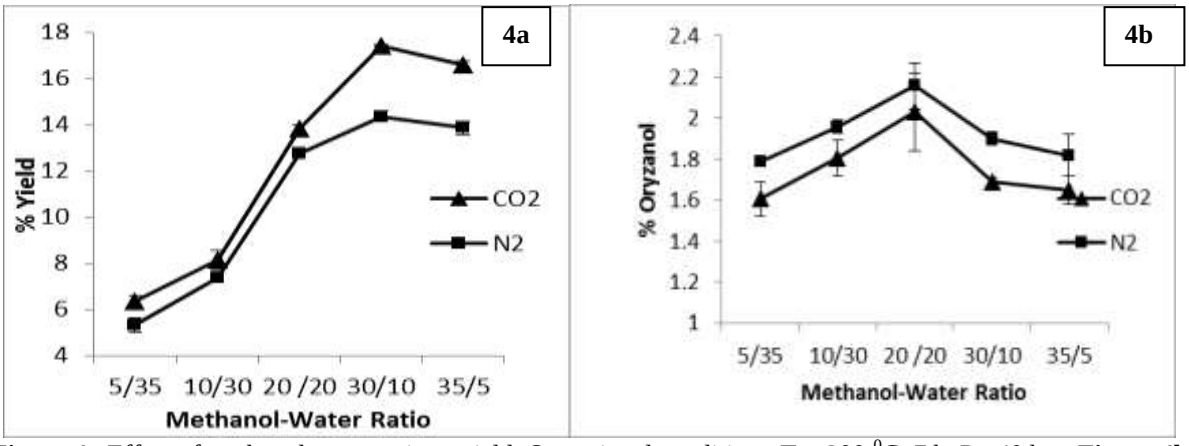

Figure 4a Effect of methanol water ratio to yield. Operational condition : T = 200 ^{0}C, 7 h, P= 40 bar **Figure 4b** Effect of methanol water ratio to yield and oryzanol content. Operational condition : T = 200 ^{0}C, 7 h, P= 40 bar.

extraction using solvent containing a high percentage of methanol caused lower extracbility oryzanol due to an excess methanol prefer extrac more polar compound like carbohydrate [30].Fig.4b showed a trend toward a possitive relationship between ratio solvent to oryzanol extracted.

3.4 Effect of Temperature to Purification Oryzanol Using Silica Gel

Purification oryzanol using silica gel using silica gel as adsorbent. Parameters affect adsorbtion such as temperature, presssure, mixing rate [31], partilce size, pore size and surface area [32]. Physical properties of silica gel are amorph and high porisity. In addition, silica gel has more properties like hirdofilic, large surface area and high posrosity also. High porosity of silica gel making polar compund (oryzanol, TAG, FFA, tocopherol, tocotrienols) adsorbed easier came into silica gel pore. In contras, dominant non-polar compounds like FAME stucked only at outer layer of silica gel. After silca gel surface area coated by rice bran oil, extracted at various temperature by solvent hexane in 44 cycle to collect all non polar compunds followed by methanol to collect all polar compounds in 27 cylcle then. There were two phase, hexane phase and methanol phase described at Tabel 1.

Table 1 . Effect of Temperature to Oryzanol Content. Operating Condition : ratio rice bran oil / silica gel = 1/3 (w/w)

	TEMPERATUR (^{0}C)	HEXANE PHASE			METHANOL PHASE		
		Oryzanol (%)	FFA (%)	Others (%)	Oryzanol (%)	FFA (%)	Others (%)
	32±1	6.29	11.77	81.93	92.03	4.07	3.9
Content		(0.19)			(6.62)		
	51±1	7.86	7.86	84.27	85 .98	2.34	18.7
Content		(0.22)			(10.10)		
	64±1	10.58	6.28	83.13	77.08	2.47	20.44
		(0.25)			(9.03)		

Table 1 showed that the oryzanol content in hexane phase only 0.19 -0.25% The logical reason is adsorbtion occur exothermicly. The increasing temperature affect small part polar compounds adsorb into silica gel. The optimum purification between polar and non-polar compounds at low temperature. Hence, difference polarity oryzanol with hexane making unsoluble between them. On the other hand, solubilty oryzanol into methanol was high. It tend to extracted in methanol phase due to oryzanol is lipid polar. Consequently, almost all oryzanol run into methanol phase at low temperature. The optimum purification oryzanol was obtained 6.62% or 19.75 mg oryzanol/g oil at 32±1 ^{0}C. Whereas at 51±1 ^{0}C and 64±1^{0}C were obtained 10.10% or 18.46 mg oryzanol/g oil ; 9.03% or 16.55 mg oryzanol/g oil respectively. This study also corresponding to [33] adsorb vitamin E (tocol and tocotrienols) using silica gel with maximum collected at low temperature. Adsorption at high temperature (>45 ^{0}C) could reduce the adsorption rate, made decreasing of saturated silica gel capacity [34] reported . In addition, oryzanol would degraded at high temperature. In this study, oryzanol content (19.75 mg oryzanol/ g oil) were collected higher than [35] with supercritical Carbondioxide ($SCCO_2$) at 350 bar , 40 ^{0}C were obtained 15.2 mg oryzanol/ g oil.

3.5 Qualitative Analyses Using Thin Layer Chromatography (TLC)

The oryzanol content also measured in qualitative analyses using Thin Layer Chromatography. Table 1 showed that high oryzanol concentration at 51±1 ^{0}C indicated by dot appear clearly. However the maximum recovery oryzanol were obtained at 32±1 ^{0}C by using UV-Vis Spectrophotometri as seen at tabel.

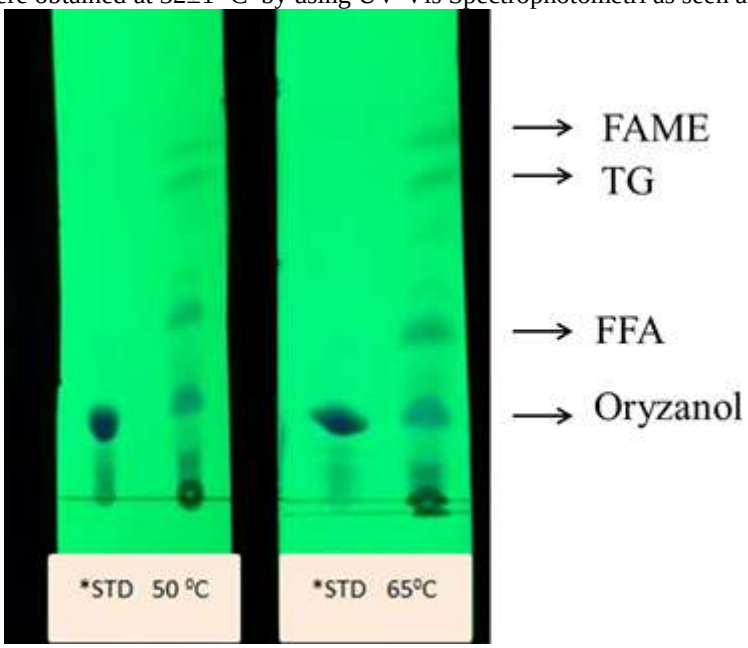

Figure 5. TLC Analyses. Effect of Temperature to Oryzanol Content. Mobile phase (heksan/etyl acetate/ acetic acid) (90:10:1, v/v/v). * STD = Oryzanol Standard

FFA content was obtained 4.07% at 32±1 ^{0}C, the increasing temperature at 64±1^{0}C , the FFA content decrease significantly at 2.47%. Free Fatty Acid (FFA) as another polar compounds in rice bran oil. FFA would be impurities for the next purification and isolation of oryzanol. FFA tend to extracted in methanol phase, due to the same polarity, but at high temperature FFA could extract in hexane phase. Consequently, FFA remain in silica gel didn't appeared well (see Fig. 5).

IV. CONCLUSIONS

The subcritical water and methanol extraction of oryzanol gave the better of yield oil, oryzanol content, time efficiency compared to soxhlet extraction, indicating that oryzanol as a promising antioxidant for high temperature application. Using CO_2 as a pressurized gas, temperature 200 ^{0}C, ratio methanol 20/20 for 7 h, the yield and oryzanol content were obtained 13.82%±0.16 ; 2.03 %±0.19 or 2.84 mg oryzanol / g oil respectively. Employing N_2 as pressurized gas at the same condition, the yield and oryzanol content were obtained 12.75%±0.07 ; 2,15%±0.11 or 2.75 mg oryzanol/g.Whereas the optimum purification oryzanol from rice bran oil using oil to silica gel (1/3) were obtained 6.62% or 19.75 mg oryzanol/ g oil with low FFA content 4.38% at 32±1 ^{0}C maked it easier to the next step for isolation and cristallization.

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