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COMPARISON OF HYDROLYSIS METHODS FOR REMOVAL OF GLYCERIDES IN RICE BRAN ACID OIL

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A Thesis Submitted in Partial Fulfillment of the Requirements
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อมรรัตน์ มีดำ : การเปรียบเทียบวิธีไฮโดรไลซิสเพื่อกำจัดกลีเซอไรด์ในน้ำมันกรดรำข้าว (COMPARISON OF HYDROLYSIS METHODS FOR REMOVAL OF GLYCERIDES IN RICE BRAN ACID OIL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อาทิวรรณ โชติพฤกษ์, 86 หน้า.

วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาวิธีการไฮโดรไลซิสและหาวิธีไฮโดรไลซิสที่เหมาะสมสำหรับกำจัดกลีเซอไรด์ในน้ำมันกรดรำข้าว โดยพิจารณาจากร้อยละการเปลี่ยนแปลงกลีเซอไรด์ ปริมาณสารแกมมาออริซานอลที่เหลืออยู่ และความสามารถในการต้านอนุมูลอิสระ ซึ่งการทดลองถูกแบ่งออกเป็นสองส่วนคือ ส่วนแรก เป็นการเปรียบเทียบวิธีการไฮโดรไลซิสที่เหมาะสมสำหรับการกำจัดกลีเซอไรด์ทั้งหมด 3 วิธี ได้แก่ ไฮโดรไลซิสด้วยน้ำสภาวะกึ่งวิกฤติ ไฮโดรไลซิสโดยใช้ตัวเร่งปฏิกิริยาชนิดกรดและไฮโดรไลซิสโดยใช้ตัวเร่งปฏิกิริยาชนิดเบส วิธีที่ถูกเลือกจะถูกนำไปศึกษาต่อเพื่อหาสภาวะที่เหมาะสมในการไฮโดรไลซิสในส่วนที่สอง จากการพิจารณาพบว่าการไฮโดรไลซิสด้วยน้ำสภาวะกึ่งวิกฤติและไฮโดรไลซิสโดยใช้ตัวเร่งปฏิกิริยาชนิดเบส สามารถกำจัดกลีเซอไรด์ได้มากกว่าการไฮโดรไลซิสด้วยตัวเร่งปฏิกิริยาชนิดกรด ขณะเดียวกันฤทธิ์การต้านอนุมูลอิสระของผลิตภัณฑ์ทั้งสามไม่แตกต่างกันอย่างมีนัยสำคัญ ดังนั้นทั้งสองวิธีนี้จึงถูกศึกษาสภาวะที่เหมาะสม จากการพิจารณาผลร้อยละการเปลี่ยนแปลงกลีเซอไรด์และปริมาณคงเหลืออยู่ของสารแกมมาออริซานอล พบว่า อุณหภูมิและเวลาที่เหมาะสมที่สุดสำหรับการไฮโดรไลซิสด้วยน้ำกึ่งวิกฤติคือ 200 องศาเซลเซียส เป็นเวลา 30 นาที โดยสภาวะนี้มีการเปลี่ยนแปลงปริมาณกลีเซอไรด์ที่ลดลง และมีสารแกมมาออริซานอลคงเหลืออยู่จากเริ่มต้น เท่ากับ 86.49±1.18 และ80.34±1.80 เปอร์เซ็นต์ ตามลำดับ สำหรับการไฮโดรไลซิสโดยใช้ตัวเร่งปฏิกิริยาชนิดเบส สภาวะที่เหมาะสมที่สุดคือการใช้ความเข้มข้นของสารละลายโซเดียมไฮดรอกไซด์ที่ 2.5 นอมอล์ อุณหภูมิ 90 องศาเซลเซียส ระยะเวลาระหว่าง 5 ถึง10 นาที ที่สภาวะเหล่านี้สามารถกำจัดกลีเซอไรด์ได้อย่างสมบูรณ์ และมีสารแกมมาออริซานอลเหลือในผลิตภัณฑ์เท่ากับ 56.48±1.54 % และ 65.88±0.25 เปอร์เซ็นต์ เมื่อใช้เวลาที่ 5 และ 10 นาที ตามลำดับ นอกจากนี้ยังพบว่าฤทธิ์การต้านอนุมูลอิสระของผลิตภัณฑ์ที่ได้จากไฮโดรไลซิสด้วยตัวเร่งปฏิกิริยาเบสสูงกว่าผลิตภัณฑ์ที่ได้จากการไฮโดรไลซิสด้วยน้ำกึ่งวิกฤติ

ภาควิชา วิศวกรรมเคมี ลายมือชื่อนิสิต .....

สาขาวิชา วิศวกรรมเคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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AMORNRAT MEEDAM: COMPARISON OF HYDROLYSIS METHODS FOR REMOVAL OF GLYCERIDES IN RICE BRAN ACID OIL. ADVISOR: PROF. ARTIWAN SHOTIPRUK, Ph.D., 86 pp.

The objective of this study is to determine the suitable hydrolysis method for removal of glycerides in rice bran acid oil based on the percentage of glycerides conversion, remaining content of γ-oryzanol and antioxidant activity. The experiment was divided into two parts. Firstly, three hydrolysis methods consisting of subcritical water hydrolysis, acid-catalyzed hydrolysis and base-catalyzed hydrolysis were compared. The suitable methods were selected and were evaluated in the second part to determine the suitable hydrolysis conditions. Subcritical water hydrolysis and base catalyzed hydrolysis was found to give higher percentage of glycerides conversion than acid catalyzed hydrolysis, while the antioxidant activities of the reaction products obtained from various methods did not differ significantly. Therefore in suitable conditions were then determined for these two methods. Base on the percentage of glycerides conversion and remaining content of γ-oryzanol, the most suitable temperature and time for subcritical water hydrolysis was 200°C and 30 minutes, respectively. At this condition, the percentage of glycerides conversion and the remaining content of γ-oryzanol were 86.49±1.18 and 80.34±1.80 % respectively. For base-catalyzed hydrolysis, the most suitable condition is 2.5N sodium hydroxide solution at 90 °C, and the reaction time between 5 to 10 minutes. At these conditions, glycerides were completely removed and the content of γ-oryzanol remained in hydrolyzed product were 56.48±1.54 % and 65.88±0.25 % for the reaction time of 5 and 10 minutes, respectively. Moreover, the antioxidant activity of hydrolyzed products obtained from base-catalyzed hydrolysis was found to be the higher than that obtained from subcritical water hydrolysis.

Department: Chemical Engineering Student's Signature .....
Field of Study: Chemical Engineering Advisor's Signature .....
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CHAPTER I
INTRODUCTION

1.1 Motivation

With an approximate of 35 million tons of annual production, Thailand is known as one of the largest rice producer and the world's second leading rice exporter (Thai Rice Exporters Association). Apart from white rice which is a major product, a large amount of by products is produced from paddy milling process, including husk, germ and bran. One hundred kilograms of paddy on milling yield 10 to 20 kg rice bran (Kahlon 2009). Rice bran in particular is highly nutritional, containing oil (20-25 wt.%), protein (13.30 wt. %) and fiber (7.39 wt. %) (Thai Edible Oil CO., Ltd), as well as other phytonutrients such as tocopherol, tocotrienol, phenolic compounds and γ-oryzanol. For this reason, rice bran is used not only as animal feed, but also for the production of rice bran cooking oil. In such process, crude rice bran oil is first extracted from rice bran by solvent and is then passed to the refining process so as to improve the properties of oil. Refining of rice bran oil can be carried out either by chemical or physical refining process. Both physical refining and chemical refining follow roughly the same steps, except for the de-acidification step to remove free fatty acid. Free fatty acids are removed by neutralization with base in chemical refining process, whereas in physical refining process, by distillation (Rodrigues et al., 2014). Giving more desirable characteristics of rice bran oil, chemical refining is more commonly used. From this process, a rather important by-product, namely *soap stock*, is produced, specifically as a result of the de-acidification of crude rice bran oil. Often times, to make it easier to handle, *soap stock* is re-acidified by reaction with sulfuric acid, and after dehydration, *acid oil* is obtained. This by-product contains over 90% of γ-oryzanol originally present in crude rice bran oil (Van Hoed et al., 2006). γ-oryzanol is indeed a mixture of 10 ferulate esters of triterpene alcohol has been shown to have the ability to increase the level of high density lipoprotein (HDL) and decrease low density lipoprotein (LDL) in blood, decrease platelet aggregation (Seetharamaiah et al. 1990) and possesses high antioxidant activity (Xu et al. 2001).

A number of research works have been conducted in attempting to recover and purify γ-oryzanol from rice bran acid oil. Yasuo et al., 1969 proposed a four-step procedure, consisting of esterification, distillation, extraction and crystallization, for the isolation of purified γ-oryzanol from rice bran acid oil. Fatty acid in rice bran acid oil was firstly converted into fatty acid methyl ester through esterification and was removed by distillation. Subsequently, the residue of distillation containing 20.2% of γ-oryzanol was extracted, and was then purified by crystallization. By this process high γ-oryzanol purity (98 wt. %) could be achieved. However, the recovery of γ-oryzanol from the residue obtained by this process was low (33.2 wt. %) as a result of losses during the extraction and purification process. Likewise, Das et al. (1998) proposed an alternative multi-step process that produces high purity (96 %) γ-oryzanol crystals. Firstly, fatty acid was removed by distillation without esterification. Then, the residue of distillation containing 6 % of γ-oryzanol was hydrolyzed with sodium hydroxide solution in order to remove glycerides. The hydrolyzed mixture was dissolved with water, and calcium chloride solution was then added to obtain the precipitate of γ-oryzanol containing calcium soap micelles. Subsequently, the solid particle was subjected to drying, and was subsequently extracted with ethyl acetate. After solvent was evaporated, the residue was further purified by chromatography and was treated with charcoal. By this process, 76 % recovery of γ-oryzanol was reported, which it was higher than that of Yasuo et al (1969). However, the remaining content of γ-oryzanol in the residue after distillation was still low compared to that of Yasuo et al. (6 vs. 20%). This is possibly due to the decomposition of γ-oryzanol during distillation. To address the issue of high energy requirement and loss of γ-oryzanol during distillation, Kittiruangthong et al. (2005) proposed a simpler process for isolation of γ-oryzanol from rice bran acid. The process involves rice bran acid oil pretreatment by hydrolysis with alkali in order to remove glycerides, followed by extraction with ethyl acetate. The author reported that the concentration of γ-oryzanol was increased from 6.61% in the initial rice bran acid oil to 29.1% in the extract of hydrolyzed acid oil (after solvent removal). Subsequently, Anjinta et al. (2013) and employed Kittiloungthong's pretreatment step and investigated the further purification process using a normal-phase column chromatography with silica gel as a stationary phase and a mixture of hexane and ethyl acetate as a mobile phase. It was found that

glycerides were not completely removed from the rice bran acid oil used in their study, employing the alkali pretreatment condition suggested by Kittiruangthong et al. probably due to the different sources of starting acid oil. Anjinta's results also suggested that presence of glycerides in the hydrolyzed acid oil is more problematic than that of free fatty acid when chromatographic purification is used, since the retention time glycerides is closer to that of γ–oryzanol. Although complete separation of glycerides and γ–oryzanol could be achieved by adjusting their chromatographic conditions, it is highly recommended that the optimized hydrolysis pretreatment steps be employed, that removes the highest amount of glycerides while maintaining the highest amount of γ-oryzanol.

Hydrolysis of glycerides usually means the cleavage of ester bonds by the addition of water. A number of studies have been carried out to non-catalytically hydrolyze triglycerides in vegetable oil with subcritical water. At subcritical conditions (100°C < T < 374.2°C), water properties are similar to acid and base catalyst due to increased water ionization constant (K_w) (Shitu et al.,2015). From the literature review, the temperatures required for high conversion of triglyceride (>97%) range between 270-280°C (Holliday et al., 1997; King et al., 1999).

Normally, the process requires use of base catalysts or acid catalyst to reduce the reaction temperature. The catalysts may be one of the three types: biocatalyst, base catalyst and acid catalyst. Lipase is a commonly used biocatalyst for hydrolysis of glycerides to produce fatty acid due to its specificity to the reaction and low reaction temperature (40-60°C) (Zenevicz et al., 2016). Nevertheless, long hydrolysis time is its major drawback. Apart from lipase, strong acid catalysts, i.e. sulfuric acid (H_2SO_4), are used for hydrolysis triglyceride to produce fatty acids at moderate temperature. Other than the homogeneous hydrolysis catalysts, heterogeneous catalysts, which are more easily separable and recyclable, such as SO_3H-functional Brønsted acidic ionic liquids (Luo et al., 2014), solid Fe-Zn double-metal cyanide (Satyarthi et al., 2011), have also been employed for this reaction. In addition to the biocatalysts and the acid catalysts, base catalysts have also been used quite frequently for hydrolysis of triglycerides using alkali base such as NaOH or KOH. In such process, other than free fatty acids, sodium or potassium salts of fatty acids are produced as a result of saponification reaction. Despite the availability of various

catalytic processes described above, only base catalyzed process is usually used for the removal of glycerides before the purification of rice bran acid oil derived γ-oryzanol (Das et al.1999; Jesus et al., 2010; Kittiruangthong et al 2005).

The objective of this study is therefore to evaluate various hydrolysis methods: acid, base catalyzed as well as subcritical water hydrolysis for removal of glycerides in rice bran acid oil prior to purification. The suitable hydrolysis condition will be also determined based on the percentage of glycerides conversion, the percentage of remaining content of γ-oryzanol and antioxidant activity of the hydrolyzed product.

1.2 Objectives

1.2.1 To investigate the effect of hydrolysis methods: acid-catalyzed hydrolysis, base-catalyzed hydrolysis and subcritical water hydrolysis, on percentage of glycerides conversion, the percentage of remaining content of γ-oryzanol and antioxidant activity of the hydrolyzed product.

1.2.2 To determine the suitable method and the suitable conditions for the removal of glycerides in rice bran acid oil, prior to purification, giving high percentage of glycerides conversion, percentage of remaining content of γ-oryzanol and antioxidant activity of the hydrolyzed product.

1.3 Working scopes

1.3.1 Rice bran acid oil was hydrolyzed by subcritical water hydrolysis, base-catalyzed hydrolysis using NaOH solution and acid-catalyzed hydrolysis using H_2SO_4 solution.

1.3.2 The glycerides conversion, remaining content of γ-oryzanol and antioxidant activity of γ-oryzanol in hydrolyzed rice bran acid oil from acid-catalyzed hydrolysis, base-catalyzed hydrolysis and subcritical water hydrolysis were evaluated. The suitable methods for hydrolysis were chosen based on these results.

1.3.3 For selected methods, suitable hydrolysis conditions were determined based on high percentage of glycerides conversion, the percentage of remaining of γ-oryzanol and antioxidant activity.

1.3.3.1 The variables and their ranges for the non-catalytic hydrolysis to be studied are temperature (200 and 220°C) and time (10, 20, 30 and 60 minutes).

1.3.3.2 The variables and their ranges for the catalytic hydrolysis to be studied are concentration (1, 2, 2.5 and 3N), temperature (room temperature; 30, 70, 80, 90 and 100°C) and time (5, 10, 20 minutes).

1.3.4 The antioxidant activity of hydrolyzed product were analyzed using $ABTS^{\bullet+}$ scavenging assay and expressed as TEAC value.

1.4 Expected benefits

This study will provide the information regarding suitable condition for hydrolysis of rice bran acid oil to remove glycerides, prior to further γ-oryzanol purification process. The study would lead to the development of technology for purification of γ-oryzanol from rice bran acid oil.

CHAPTER II
BACKGROUND AND LITERATURE REVIEWS

2.1 Rice bran and rice bran oil

Rice bran is a by-product from the paddy milling process. One hundred kilograms of paddy, upon milling, yields 10 to 12 kg of rice bran (Kahlon 2009). As shown in Figure 2.1, rice bran is an integral part of a whole rice grain and is a moderately oily layer of the seed coat, having light brown color, sweet and slightly toasted nutty flavor (Tao 1989). Due to high content of protein and fiber (Table 2.1), it is often used as a low cost animal feed.

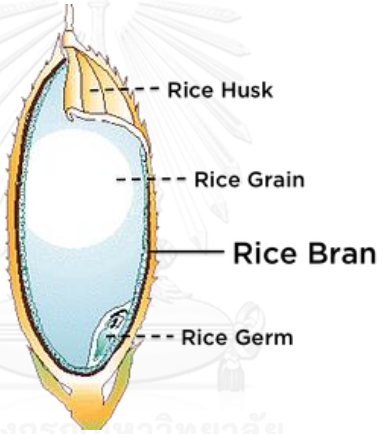


Figure 2.1 Basic structure of rice grain
Reference: https://www.grande-rbo.com

Table 2.1 Composition of rice bran (Thai Edible Oil CO., Ltd)

Component	Percent (%)
Moisture	11.40
Protein	13.30
Oil	19.20
Fiber	7.39
Other	48.71

In addition, due to the high content of oil, rice bran has been used as important raw material in the production of rice bran oil. Depending on the source of rice bran, the oil content differs, but on average, it ranges between 20-25% by weight of the raw rice bran (Lerma-Garcia et al. 2009). As summarized in Table 2.2, 96 % of rice bran oil (RBO) is classified as saponifiable lipids, whereas the remaining 4% consists of unsaponifiable lipids (Ghosh 2007). Saponifiable lipids are composed mostly of triglycerides while unsaponifiable lipids comprise tocopherols, tocotrienol, γ-oryzanol, phytosterols, polyphenols and squalene. Therefore, the saponifiable lipids are rich sources of edible cooking or salad oils, whereas the unsaponfiable lipids are rich sources of antioxidants and micronutrients used for the production of dietary supplements.

Table 2.2 Component of rice bran oil (RBO).

Component	Percent (%)
Saponifiable lipids	
Triacylglycerols	81-84
Diacylglycerols	2-3
Monoacylglycerols	1-2
Free fatty acid (FFAs)	2-6
Waxes	3-4
Glycolipids	0.8
Phospholipids	1-2
Unsaponifiable lipids	4

2.2 Rice bran oil extraction and refining processes

2.2.1 Extraction process

Several methods have been used to extract rice bran oil such as screw press, cold process or extraction with organic solvents or supercritical carbon dioxide (Srikaeo, 2014). Screw compression method gives undesirably low yield and low crude oil quality with high content of impurities: FFAs, wax and bran fines, which cause dark color and foaming during frying. For this reason, industrial processing of

vegetable oils including rice bran oil is generally carried out using solvent extraction (Srikaeo, 2014). Hexane is typically used due to its high capacity for dissolving oil and high stability (Johnson and Lusas 1983). However, hexane poses some hazard concerns due to its toxicity to health and environment. Shot chain alcohols, especially isopropanol, have been considered as alternatives to hexane for rice bran oil extraction. It has been shown that, under optimum condition, isopropanol and hexane extraction from stabilized rice bran oil gave comparable amounts of γ-oryzanol yields (Hu et al. 1996). Being non-toxic, recyclable, cheap, relatively inert, non-flammable and easily separated from the extracts supercritical carbon dioxide has also been investigated as another alternative solvent for oil extraction (Tomita et al. 2014). Imsanguan et al., (2008) compared the efficiencies of three extraction methods: SC-CO_2 extraction, solvent extraction and soxhlet extraction, particularly in terms of the recovery of α-tocopherol and γ-oryzanol in the extracted oil. SC-CO_2 extraction was found to be most favorable at 65°C and 48 MPa for 6h, giving higher rice bran yield and rate of extraction for both α-tocopherol and γ-oryzanol than other methods (Imsanguan et al. 2008). This process also produces oil with a lighter color, less phosphorous, wax and FFA, but more essential fatty acids (EFA) and oryzanol (Ghosh 2007). Despite several advantages, the limitation of supercritical carbon dioxide extraction is the fluctuation in flow rates and pressures, which causes variations in results, as well as the high equipment and installation costs. (Xu and Godber 2000). However, crude rice bran oil however still contains high levels of free fatty acids, wax, bran fines and pigment, which must be eliminated during the subsequent refining process to achieve acceptable quality (Ghosh 2007).

2.2.2 Rice bran oil refining process

To produce edible purified cooking oil, crude rice bran oil must be sent to a refining process in order to remove wax, gum, FFAs, pigment and odor. Normally, refining of crude rice bran oil can be achieved by one of the two means: physical or chemical process. As schematically shown in Figure 2.2, the two processes differ in the de-acidification/ neutralization step, in which FFAs are removed by neutralization with base in chemical refining process, where as in physical refining process, by distillation.

The first step of the rice bran oil refining process involves dewaxing, in which wax may be removed simply by means of gravity settling followed by decanting, and by filtration or by centrifugation to recover the wax sludge. There are also other ways to recover wax such as cold and hot extraction or crystallization. Second step is the degumming process, in which the content of phospholipids in rice bran oil is to be reduced, by addition of phosphoric acid and water mixture (Tyagi et al. 2012). In the next step, the dewaxed and the degummed oil is sent to the deacidification process, in which, for the chemical refining, FFAs are removed by a neutralization process by alkali treatment process, whereas in the physical process, the FFAs are removed by means of distillation. Specifically, the chemical alkali treatment is carried out by adding sodium hydroxide solution to convert the FFAs to soap. The by-product at this stage is called *soap stock*. The color quality of the rice bran oil can be improved by removing pigments naturally present in the crude oil (including chlorophylls and carotenoids) by adsorption onto bleaching earth. It is noted that the physical refining process typically ends after the dewaxing, degumming, bleaching and deacidification, but the chemical refining process, the bleached deacidified oil is further undergo deodorization process, in which it is heated to 220–270 ºC at low pressures (3–5 mm Hg) to drive off the volatile substances such as ketone aldehyde that are responsible for undesirable odors.

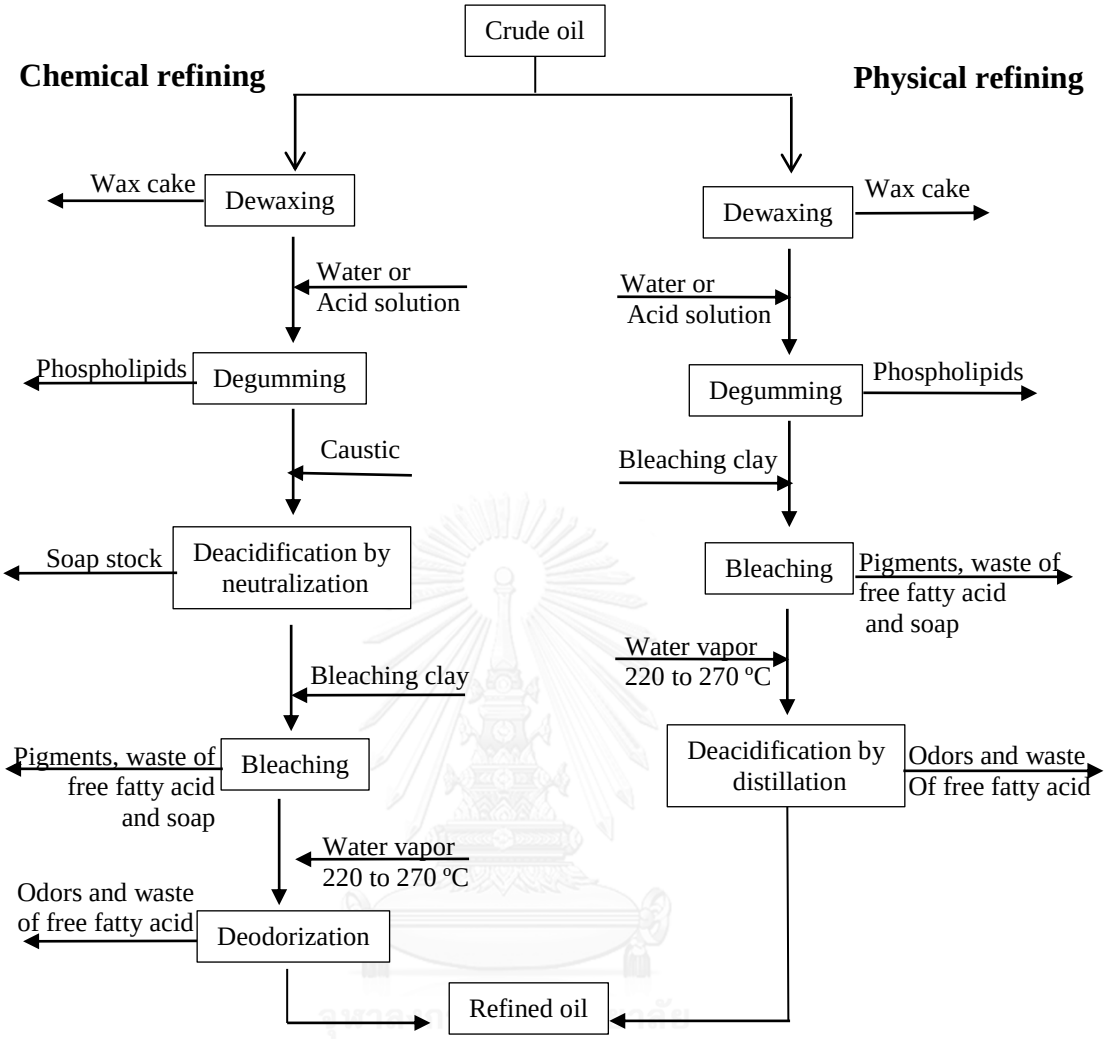


Figure 2.2 Chemical refining process and physical refining process (Rodrigues et al. 2014)

Chemical refining generally produces good quality cooking rice bran oil regarding color and cloud point, and is thus a preferred process over physical refining. As a result it is more widely used industrially at present. However, chemical refining causes significant losses of bioactive components including γ–oryzanols (Lemus et al. 2014). The percentages of γ-oryzanols losses from the original crude oil in degumming step, dewaxing step and alkali treatment or neutralization step have been reported to be 1.1, 5.9 and 93.0–94.6, respectively (Van Hoed et al. 2006). Of these, the maximum loss of γ-oryzanol takes place during the neutralization step into by products, resulting in the rice bran oil with only small content of γ-oryzanol.

2.3 Soap stock and acid oil

Soap stock is one of the by-products of the rice bran oil refining process, and is produced during the deacidification step, in which free fatty acid in dewaxed and degummed crude oil is removed by reaction with NaOH. In this process, 93-94% γ-oryzanol from original crude oil is also removed (Patel and Naik 2004) into the soap stock by-product. Table 2.3 summarizes the composition of rice bran oil soap stock, which consists mainly of water and soap and some amount of unsaponified matter and glycerides. Of the unsaponified matters in soap stock, γ-oryzanol is found to be the major component (up to 20 percent of unsaponified matters), while sterol and fatty acid alcohol are present in smaller amounts (Narayan et al. 2006).

Table 2.3 Component of soap stock.

Component	Percent (%wt.)
water	65–70
Soap	20–22
Glycerides(mainly TG)	2–2.5
Unsaponified matter - Sterol - Oryzanol - Fatty acid alcohol - Hydrocarbon	7–7.5

In a typical oil refining process, soap stock is difficult to handle as it is easily contaminated by the action of lipase in the soap stock that adversely affects the storage quality and the subsequent industrial use. Specifically, hydrolysis of lipids by such enzyme lead to rancidity of the soap stock (Ju and Vali 2005). To extend storage life of this by product, soap stock is generally converted to fatty acid by the reaction with sulfuric acid under relatively low pH (pH 2-3) to ensure that no soap remains (Mag et al. 1983). After the removal of aqueous phase, the resulting dark blown reaction product is called *acid oil*. The composition of acid oil is summarized in Table 2.4.

Table 2.4 Composition of Acid oil. (Thai Edible Oil Co., Ltd)

Component	Percent (%wt.)
Free fatty acid	45
γ-oryzanol	6-7
Natural oil and other	46.5
Moisture	1.5

2.4 γ – oryzanol

2.4.1 Properties of γ – oryzanol

γ-oryzanol was discovered by Kaneko and Tsuchiya in 1954 as white or slightly yellowish tasteless crystalline powder with little or no odor. It has a melting point of 137.5–138.5°C and is highly soluble in diethyl ether and n-heptane, and practically soluble in chloroform but insoluble in water (Bucci et al. 2003).

γ-oryzanol is composed of 10 ferulate esters of triterpene alcohol such as Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campestenyl ferulate, campesteryl ferulate, stigmastenyl ferulate, sitosteryl ferulate, compestanyl ferulate, and sitostanyl ferulate, whose molecular structures are shown in Figure 2.3 (Xu and Godber 1999).

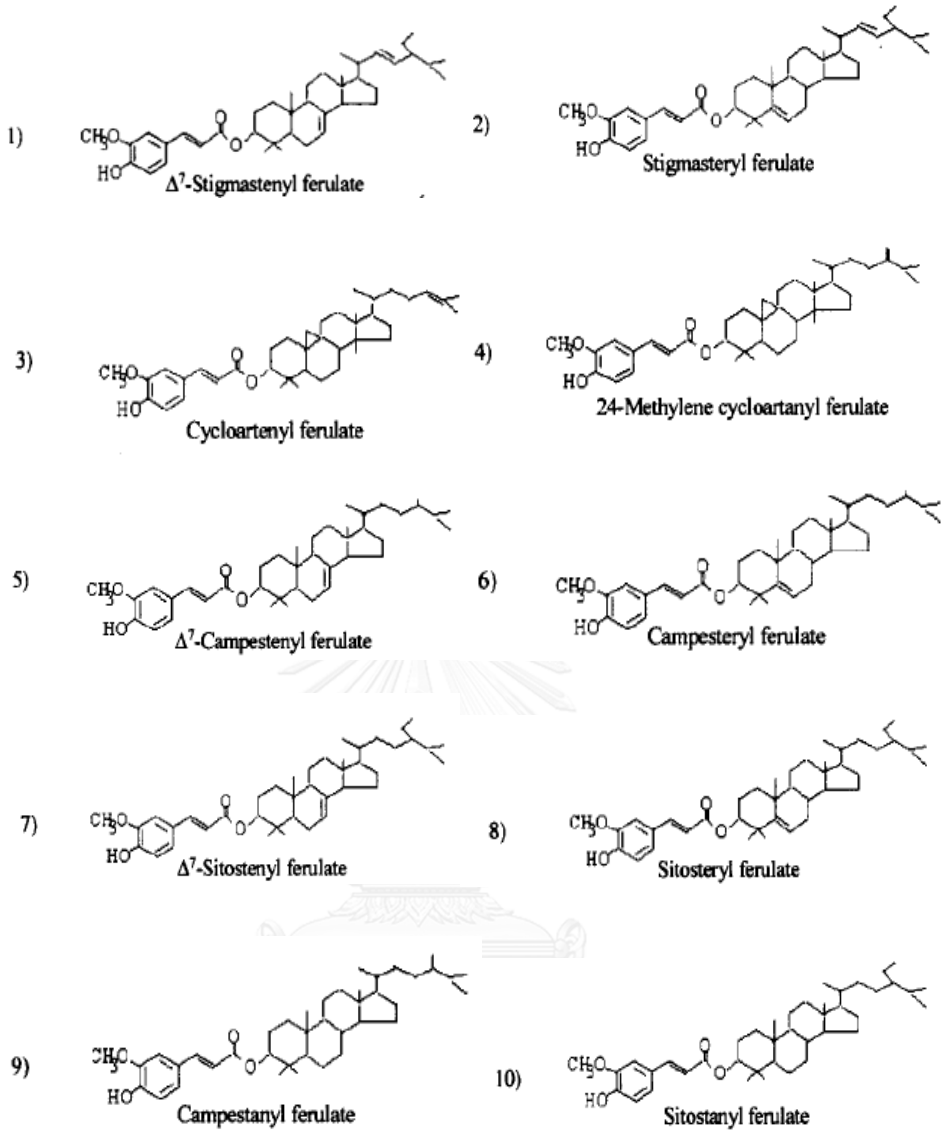


Figure 2.3 Structures of individual components of γ-oryzanol (Xu and Godber, 1999)

2.4.2 Benefits of γ – oryzanol

Owing to a number of benefits of γ-oryzanol, the compound has been widely used in various applications such as in cosmetics, pharmaceutical, and food industries. Antioxidant ability of γ-oryzanol in stopping tissue oxidation has been reported to be more than four time that of vitamin E (Hiramitsu et al. 1991). Because of its role in protecting the skin from oxidative damage from environmental influence, γ-oryzanol is used in many skin products such as sunscreen an anti-aging cream and lotions. Moreover, γ-oryzanol has also been used in hair care products due to the action of

ferulic acid and its ester in stimulating hair growth (Patel et al. 2004). In addition, the anti-oxidation ability γ-oryzanol has also found its application in food industry, as an additive to improve the storage stability and frying quality of foods (Srikaeo, 2014). Other important medicinal benefits of γ-oryzanol include the ability to increase the level of high density lipoprotein (HDL), while reduce the low density lipoprotein (LDL) in blood vessels. High density lipoprotein can prevent accumulation of cholesterol in the arteries by enhancing the conversion of cholesterol to fecal bile acid and sterols, while on the other hand, lack of HDL in the blood increases the chance of heart disease and stroke (Patel et al. 2004). In addition, γ-oryzanol is able to decrease platelet aggregation, thus prevents clogging of the arteries by a blood clot (Seetharamaiah et al. 1990).

2.5 Separation method of γ- oryzanol from soap stock and acid oil

The health benefits of γ-oryzanol that is present in soap stock and acid oil as mentioned in the previous section has drawn increasing interest in recovering and purification of γ-oryzanol from these by-products. Many research articles and patents reported the processes for isolation and purification of γ-oryzanol from soap stock. Typically, the soap stock first needs to be saponified with alkali solution to remove triglycerides that remain in the by-product. Next the aqueous solution is removed, and the saponified soapstock was further oven dried to remove the remaining water. The dried product would then be extracted with an organic solvent such as ethyl acetate, hexane, acetone, ethyl methyl ketone and isopropanol. Of these solvents, ethyl acetate was found the maximum γ-oryzanol recovery (Kumar et al., 2009), and has been used for extraction γ-oryzanol in a number of research studies (Rao et al. 2002; Indira et al. 2005; Kaewboonnum et al., 2010). Then, the extract is further sent to the purification process, which may be carried out by crystallization or chromatography or the combination of both (Narayan et al. 2004; Kaewboonnum et al., 2010). Detailed literature reviews on different processes employed for extraction and purification of γ-oryzanol from soap stock can be found in Kaewboonnum et al., 2007.

Unlike the recovery of γ-oryzanol from soap stock, only four literatures were found on the recovery of γ-oryzanol starting from acid oil, and only two of which were carried out all the way to the purification step. Nevertheless, overall the

procedures were similar, consisting of raw material pretreatment, extraction and purification. In the earliest work, Yasuo *et al*. 1969 applied a four-step procedure for the isolation of purified γ-oryzanol from rice bran acid oil. The first two are pretreatment steps, consisting of esterification of rice bran acid and further distillation to remove the esterified fatty acids of. High boiling fatty acids in rice bran acid oil were converted into methyl esters by esterification with methanol. Having lower boiling temperatures, the fatty acid methyl esters were completely removed by distillation at 280-300 ºC. In the next step, the residue of distillation containing 20.2 % of γ-oryzanol was subjected to extraction using a mixture of hexane and furfural. Because of the different distribution coefficients of γ-oryzanol between hexane and furfural, γ-oryzanol was more concentrated in the furfural. Finally, the furfural layer was separated and was then added with water at half the quality of the furfural. In order to induce crystallization, the solution was incubated at low temperature (lower than -5ºC) and was continuously agitated. Crystals were collected via filtration, and were recrystallized with hexane to obtain purified γ-oryzanol crystals (98.3 % w/w purity). By this process, the recovery from the residue of distilation was 33.2 % (w/w). Although removal of fatty acid methyl ester by distillation has brought about the increase of γ-oryzanol concentration in the residue, the recovery of γ-oryzanol obtained by this process was low due to loss during the extraction and purification process. In this article, the authors did not mention the recovery of γ-oryzanol in extraction step, which make it difficult to identify the step that gives maximum loss of γ-oryzanol. However, crystallization and recrystallization, while increased the purity of γ-oryzanol crystals, may lead to γ-oryzanol loss into mother liquors. Das et al. (1998) proposed a alternative multi-step procedure for purification of γ-oryzanol from rice bran acid oil. In their process, fatty acids were directly removed by distillation at 250-260ºC without prior esterification. The dark residue after the distillation step was found to contain mostly triglycerides, 6 % γ-oryzanol and 9 % fatty acids. In the next step, the dark acid oil residue was then hydrolyzed with 1.8 M of NaOH solution at 90°C for 1 hour. Then by dissolving the hydrolyzed acid oil in water, the resulting sodium soap formed the micellar aggregates with γ-oryzanol trapped inside. Subsequently, $CaCl_2$ solution was added to precipitate the calcium soap micellar containing γ-oryzanol by exchanging the sodium ion with the calcium ions. The

pretreatment step was then completed by drying of the precipitate of the calcium soap with air. The dried precipitate was then extracted with ethyl acetate. At this point the purity of γ-oryzanol in residue after removal of solvent increased to 19 %. To further purify the γ-oryzanol, column chromatography was carried out using silica gel as a stationary phase and chloroform as a mobile phase. Fractions were collected and the solvent was removed. The residue was further purified by activated charcoal treatment in hot methanol solution. The γ-oryzanol crystals were found to have approximately 96 % purity and 76% recovery from the starting dark acid oil residue. It should be noted that this process provided higher recovery of high purity γ-oryzanol, provided that the starting material was the dark acid oil residue obtained after distillation. Nevertheless, the content of γ-oryzanol remained in residue after distillation seemed to be much lower than the earlier mentioned research (6 vs. 20%), and this is possibly due to the decomposition of γ-oryzanol during distillation. Other limitations of this process include requirement of many steps and use of toxic organic solvent (i.e chloroform). Nevertheless, Das's method for the pretreatment of rice bran acid oil has been adapted in some research to use prior to γ-oryzanol extraction. For instance, Jesus et al., (2010) employed the same technique to obtain dry solid γ-oryzanol calcium soap aggregates for their supercritical fluid extraction study. The reported content of γ-oryzanol in the dark residue after distillation was even less than that of Das et al. (4.8 vs. 6% wt). Given the drawback of distillation in terms of high energy requirement and low remaining content of γ-oryzanol, Kittiruangthong et al. (2005) proposed a distillation-free pretreatment process, in which acid oil was hydrolyzed with 2N of NaOH solution at 80 °C for 10 min. This purpose of this process is mainly to completely remove glycerides, but not fatty acids. The hydrolyzed acid oil was then extracted with ethyl acetate. By this simpler process, the author reported higher increase of γ-oryzanol content from 6.48% γ-oryzanol in the original oil to 29.1% γ-oryzanol in the ethyl acetate extract of the hydrolyzed oil. Nevertheless, they did not report further purification process to be carried out onwards to obtain highly purified product. Anjinta et al., 2013 employed Kittiruangthong et al.'s method of pretreatment and further proposed a purification process using preparative chromatography. The composition of the resulting extract were first evaluated were found to contain 5.38 % oryzanol, 13.50% fatty acid and 0.87 % glycerides. Since

fatty acids were not removed by distillation, it is not unusual to find some amount of fatty acids was extracted into ethyl acetate. However, some glycerides were also found despite the 100% glycerides removal claim by Kittiruangthong et al. This is possibly due to fact that the acid oils used in the two studies came from different sources. Nevertheless, when subjected to the normal phase chromatography, with silica gel as a stationary phase and a 75: 25 % (v/v) of hexane and ethyl acetate mixture as a mobile phase, high purity γ-oryzanol (>95%) could still be obtained (Anjinta et al., 2013). Although chromatography seemed to be an effective purification step compared with crystallization, giving high yield and purity of the final product, the process can be rather costly due to the expensive commercial adsorbents. We believe that the hydrolysis pretreatment procedure that maximally removes glycerides, the main impurity in the process, would certainly be a key to the success of γ-oryzanol chromatography purification.

2.6 Glycerides

Glycerides are esters of fatty acid and glycerol, or more correctly known as acylglycerols. Glycerol has three hydroxyl functional groups, which can be esterified with one, two, or three fatty acids to form monoglycerides, diglycerides, and triglycerides respectively. Vegetable oils and animal fats contain mostly triglycerides, but they can be broken down by hydrolysis reaction into mono and di glycerides, or to free fatty acids and glycerol. Triglycerides are soluble in low polar or nonpolar solvent such as hexane, isopropanol, chloroform and ethyl acetate while mono-glycerides and di-glycerides have lower solubility in these solvent compared with tri-glycerides (Narayan et al., 2006). Structures of acylglycerols are shown in Figure 2.4

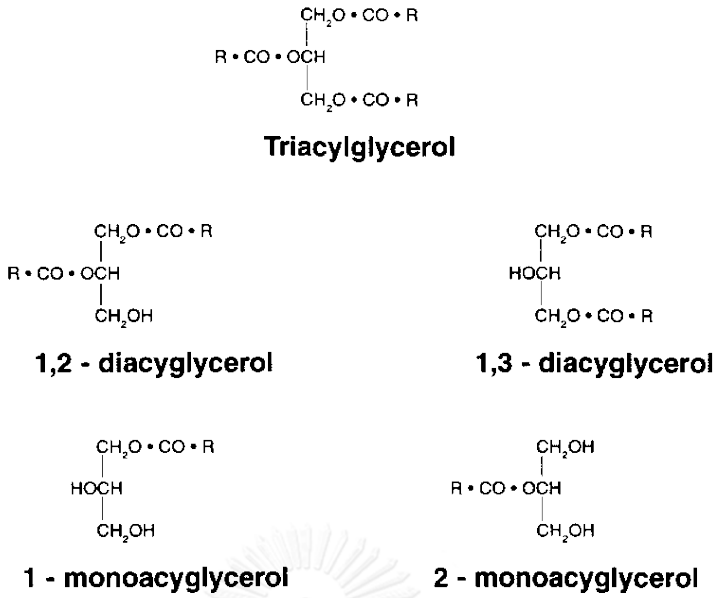


Figure 2.4 Structure of acyglycerols

2.7 Hydrolysis of triglycerides

Hydrolysis defined as a chemical transformation in which an organic molecule, RX, reacts with water. The net reaction is the displacement of X in organic molecule by the hydroxyl (-OH) part of water and cleavage of covalent bond with X in organic molecule.

$$R\text{-}X + H_2O \rightarrow R\text{-}OH + XH$$

For hydrolysis of triglycerides, one molecule of triglycerides requires three molecules of water. Triglycerides can be hydrolyzed into di-glyceride, mono-glyceride and glycerol respectively while each step gives one molecule of fatty acid as shown in Figure 2.5.

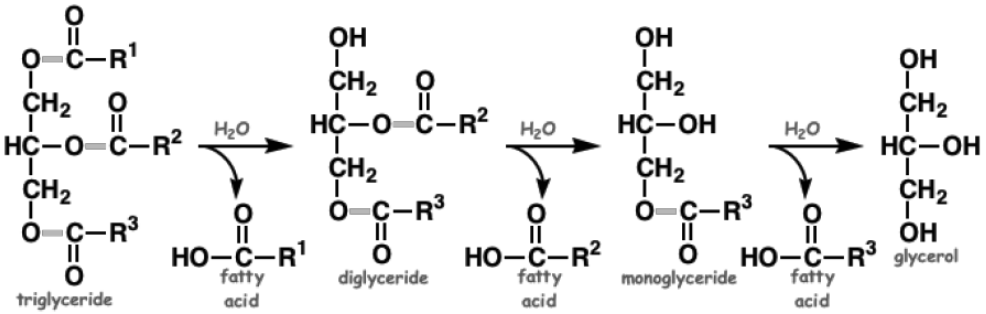


Figure 2.5 hydrolysis of triglyceride

2.7.1 Sub and supercritical water hydrolysis

As described, the hydrolysis of triglycerides requires the presence of hydroxide and hydronium ions from water. Therefore, hydrolysis of triglycerides takes place at elevated temperature. Specifically, high temperature leads to the increase in the concentrations of the hydronium $[H_3O^+]$ and hydroxide $[OH^-]$ ions, indicated by the ionization constant (K_w), which is essentially, an equilibrium constant for the following ionization reaction.

$$2H_2O \leftrightarrow H_3O^+ + OH^- \quad ; \quad K_w = [H_3O^+][OH^-]$$

When water temperature is raised under pressurized conditions (to maintain the liquid state) to above the atmospheric boiling point (100ºC) but lower the critical temperature (374 ºC), the water is called subcritical water or hot pressurized water (Figure 2.6). In this region, value of K_w increases with temperature to a maximum value before it decreases above the critical temperature. For example, as shown in Figure 2.7, at 25 MPa, the K_w value increases from 10^{-14} at 25 °C to about 10^{-11} at 250 °C and then starts to decrease at critical temperature, to 10^{-19} at 390 °C and to 10^{-22} at 500 °C (Sereewatthanawut et al. 2008). For this property, a number of studies have been carried out to non-catalytically hydrolysis using subcritical water without any additional catalyst such as hydrolysis of glycerides in vegetable oil (Holliday et al., 1997; King et al., 1999). For non-catalytic hydrolysis of glycerides in subcritical water condition, one is often interested in the reaction temperatures above 200 ºC.

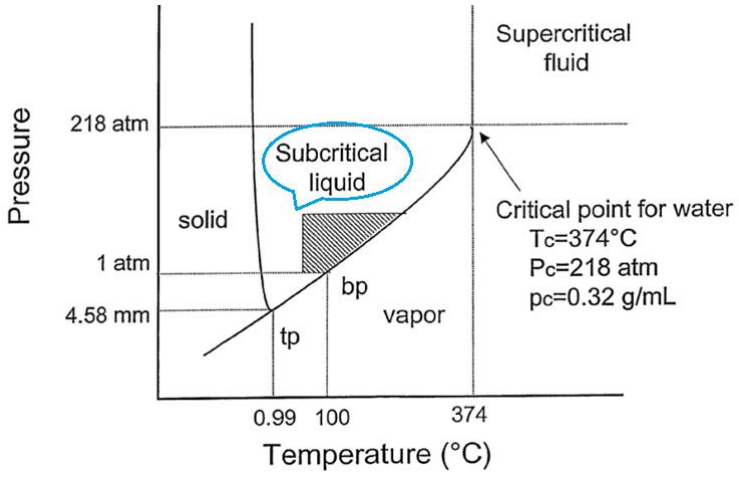


Figure 2.6 Phase diagram for water as a function of temperature and pressure (Shitu, et al. 2015)

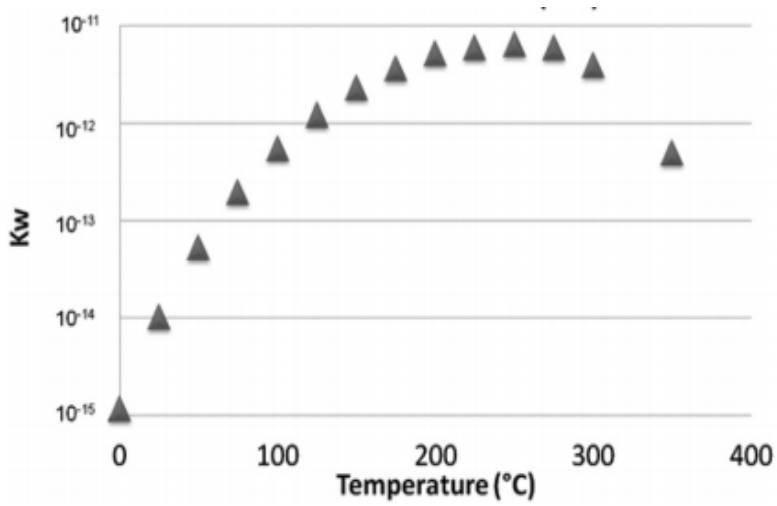


Figure 2.7 Dissociation constant of water as a function of temperature from 0-350 ºC (Pollet et al. 2014)

Actually, hydrolysis reaction can be operating at low temperature by using of catalyst because a catalyst can lower the activation energy of the reaction. Catalyst used for hydrolysis of triglycerides can be divided into two types: acid catalyst and base catalyst as described in the next section.

2.7.2 Acid and base catalyzed hydrolysis

Acid and base are classified as chemical catalyst. For acid catalyst, it refers to proton donor. Many acids are used for a source of proton in acid catalyzed hydrolysis such as hydrofluoric acid, phosphoric acid and sulfuric acid. Acid catalyst hydrolysis is the reverse of esterification. In order to get much hydrolysis as possible, a large excess of water should be used. Conversely, base which is proton acceptor is used as catalyst in hydrolysis reaction such as sodium hydroxide and potassium hydroxide. Base catalyzed hydrolysis is irreversible reaction, so the reactants completely convert into product. In term of final product in hydrolysis of triglycerides, the reaction that used acid as catalyst produces glycerol and fatty acids while using base as catalyst provide salt of fatty acid or soap as a byproduct and glycerol. Mechanism of acid and base catalyzed for hydrolysis of ester are shown in Figure 2.8 and Figure 2.9, respectively.

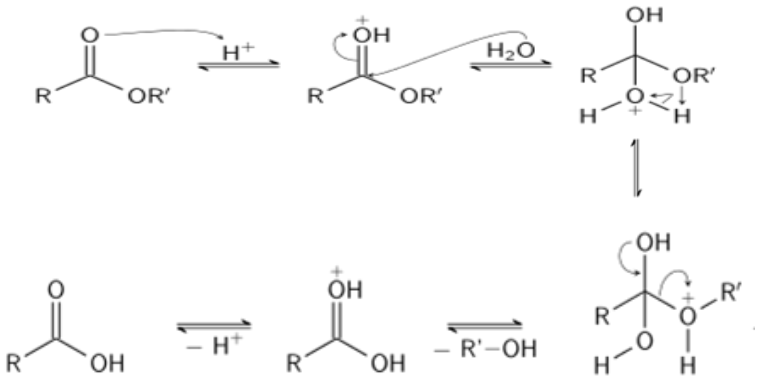


Figure 2. 8 Mechanism of acid catalyzed hydrolysis of ester (Krishnavedala, 2014)

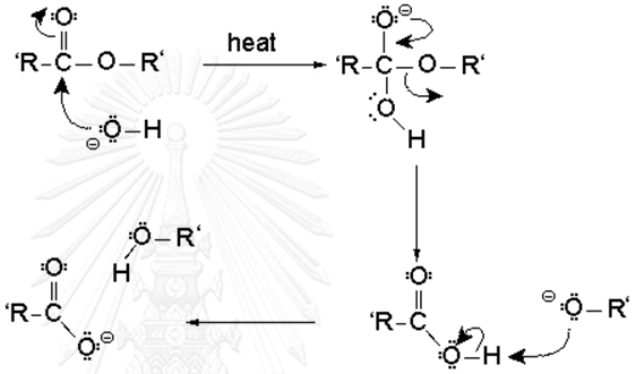
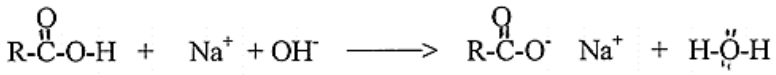


Figure 2. 9 Mechanism of the base hydrolysis of ester

Source: Department of Chemistry, University of Saskatchewan

In case of base catalyzed hydrolysis reaction, neutralization of acids with base may also take place, producing salt of fatty acids or soap as a by-product. In this case NaOH acts as a reactant, rather than a catalyst, and the reaction is called saponification.



2.8 Literature review

In recent years, there are increasing number of publications related to hydrolysis of vegetable oils such as soybean oil, sunflower oil, and olive oil. Hydrolysis of the triglycerides, the main component in these and other vegetable oils, yielded glycerol and fatty acids. Fatty acids are the desired product, which are used in

preparation of a wide variety products, such as soaps, surfactants, lubricants, plasticizers, paints, coatings, pharmaceuticals, foods, agricultural, industrial and personal care products (Satyarthi et al. 2011). As previously described, hydrolysis is achieved by non-catalytic hydrolysis or catalytic hydrolysis.

Without catalysts processes, hydrolysis is usually operated at high temperature and high pressure. Several studies are conducted to investigate the hydrolysis of oil by subcritical water. Holliday et al., 1997 studied optimum condition for the production of the fatty acids from vegetable oil (soy bean oil, linseed oil and coconut oil) with hydrolytic reaction temperatures in the range of 250-375 °C, at fixed volume ratio of oil to water of 4:25 in a batch reactor. The high fatty acid yields (>97%) of three vegetable oils were obtained from the 15-20 minute reactions at subcritical water temperature range of 270-280ºC. These conditions were found not to degrade the oil and fatty acids. When subjected to water near the critical condition of 375ºC for only 8 minutes, the reactants and the fatty acids products were degraded. Likewise, King et al, 1999 conducted hydrolysis of soybean oil using subcritical flow reactor and determined the effect of temperature (270-340 °C), ratio of water to oil and residence time (7-15) on the percentage of fatty acids. Their results suggested that increasing temperature, the ratio of water to oil and the residence time leads to increasing fatty acids yields. High fatty acids yields (96-98%) were obtained at the temperatures between 330-340°C, the ratio of water to oil between 2.5:1-5:1 and the residence time between 10-15 minutes. However, they also observed degradation of the fatty acids at higher temperatures.

Although, regarded as an environmentally benign process, subcritical water hydrolysis requires high temperatures which cause degradation of the desired products. To reduce the operating temperature, one of the three types of catalysts may be used for hydrolysis of triglycerides: biocatalyst, base catalyst, and acid catalyst. Due to the specificity, lipase, an enzyme found in animal, plant and microorganisms, is a type the biocatalyst that is often used for hydrolysis of triglycerides into fatty acids. The lipase catalyzed reaction is carried out at low temperature and is therefore a mild and an energy saving process (Holliday et al. 1997), nevertheless it has a major drawback in terms of long reaction time (Linfield et al. 1984).

Alternatively, chemical catalysts such as acid catalysts can be used in hydrolysis of triglyceride to provide a proton donor to carbonyl group of triglyceride for convert triglyceride into fatty acid and glycerol. Although strong acids such as sulfuric acid are commonly used as homogeneous catalyst for this purpose, many research works have attempted to develop heterogeneous catalyst that are more easily separated and recycled. Satyarthi et al., 2011 proposed to use solid Fe-Zn double-metal cyanide (DMC) catalysts for hydrolysis of soybean oils to produce fatty acid at moderate conditions, and compared the results with commercial acid catalyst such as Amberlyst^{TM70}, SAPO-11, H-β, HY, MoO_x/Al_2O_3 and sulfated zirconia. It was found that DMC show the conversion of triglycerides to fatty acids with selectivity greater than 73 wt. %. However, the catalytic activity is still lower than that of the conventionally used solid acid catalysts. Luo et al., 2014 propose to SO_3H-functional Brønsted acidic ionic liquids catalyst for hydrolysis vegetable oil to fatty acid which was prepared from attachment of anion of sulfonic group (SO_3H-) and cation of ionic liquids. This catalyst can donate a hydrogen ion or proton. The result found that this acidic ionic liquids catalyst gave relatively high yield, of greater than 95 wt. %.

In addition to enzyme and acid catalyzed reaction, hydrolysis of glycerides can be carried out with use of base catalyst as already mentioned in separation method of γ-oryzanol from soap stock and acid oil section 2.5. All above literature review observed that base catalyzed hydrolysis is not the best choice for production of fatty acid since the undesirable side reactions take place and produce soap as a by-product. On the other hand, it usually used for removal of glycerides in rice bran acid oil prior to extraction and purification. This is possibly because formation of sodium soap containing γ-oryzanol, which has been reported by Das et al., 1997, can be prevented degradation of γ-oryzanol. Conversely, under the same purpose, both subcritical and acid catalyst hydrolysis has not appeared despite the ability to hydrolyze glycerides as well.

Therefore, the three hydrolysis methods: subcritical water hydrolysis and acid catalyzed hydrolysis and base catalyzed hydrolysis will be investegestd and determined the suitable condition for hydrolysis of rice bran acid oil in this study.

CHAPTER III
MATERIALS & METHODS

3.1 Materials

Rice bran acid oil was obtained from Thai Edible Oil Co., Ltd., Samutprakarn, Thailand. γ-oryzanol standard was purchased from Santa cruz biotechnology, Japan. Rice bran oil (Thai Edible Oil., Ltd., Bangkok, Thailand) used as the glycerides standard was purchased from a local department store. The oleic acid analytical standard and the solution of concentrated sulfuric acid (98%) were purchased from Sigma-Aldrich, India. Ethyl acetate (99.5%) used as an organic solvent was purchased from Merck, USA. Sodium hydroxide (97%) was purchased from APS fine chem, NSW, Australia.

3.2 Suitability of the hydrolysis methods for the removal of glycerides

To evaluate the possibility of three methods; subcritical water hydrolysis, acid-catalyzed hydrolysis and base-catalyzed hydrolysis on hydrolysis of glycerides in rice bran acid oil, the experiments were divided into two parts. In part I, the effects of three hydrolysis methods on percentage of glycerides conversion, percentage of remaining content of γ-oryzanol and antioxidant activity were investigated and determined the suitable method base on high glycerides conversion. For part II, the chosen hydrolysis methods in first part were also determined the most suitable condition. The procedure of three hydrolysis methods: subcritical water hydrolysis, acid catalyzed hydrolysis, and base catalyzed hydrolysis are described in detail below section 3.2.1.

3.2.1 Effect of the hydrolysis methods on the % glycerides conversion and the % remaining content of γ-oryzanol

3.2.1.1 Subcritical water hydrolysis

According to Holliday et al., 1997 report, the temperature for production of the highest fatty acid yield from vegetable oil using subcritical water hydrolysis was

270-280ºC. Therefore, the operating condition was selected to used herein was at 270ºC for glycerides removal in rice bran acid oil. To investigate subcritical water hydrolysis method, 1 ml of acid oil was mixed with 5 ml of deionized water in a 8.8-ml stainless steel (SUS-316) batch reactor (AKICO Co., Japan). Then, the mixture was heated to 270°C by an electric furnace heater for 10 minutes. The reactor was then suddenly immersed into a cooling water bath for approximately 3 min to allow the mixture to quickly cool down. The mixture of this point consisted of two phases: the oil and the aqueous phases. The upper layer which is the oil phase was simply separated by filtration with paper filter (Whatman NO.1, 90 mm Ø, Retention: 11µm) due to lower density of oil than water. The amount of fatty acids, glycerides and γ-oryzanol in the oil phase were measured using HPLC with an ELSD detector. The antioxidant activity of hydrolyzed products were analyzed using $ABTS^{\bullet+}$ method.

3.2.1.2 Base-catalyzed hydrolysis

The base-catalyzed hydrolysis was carried out using the condition according to Kittiruangthong et al, 2005 report because they has been suggested as the suitable condition for base-catalyzed hydrolysis of glycerides in rice bran acid oil. The procedure consisted of two steps: hydrolysis and extraction. For hydrolysis reaction, 1 ml of acid oil was mixed with 5 ml of 2 N of sodium hydroxide solution. The mixture was agitated using a vortex mixer for 1 minute and then incubated at 80 °C for 10 minutes. Then, the pH of the mixture was adjusted to 9.5 adding 1N hydrochloric acid. Aqueous phase that was produced by addition of acid was separated from hydrolyzed acid oil. Afterward, the hydrolyzed acid oil was extracted by liquid-liquid extraction. For extraction of hydrolyzed acid oil, the hydrolyzed acid oil was added ethyl acetate with volume ratio of 1:1 in test tube. The mixture was agitated using a vortex mixer for 1 minute. The ethyl acetate layer was separated from hydrolyzed acid oil layer using a centrifuge at 4,000 rpm for 5 minutes. Then, the ethyl acetate layer was transferred into another tubes and the hydrolyzed acid oil layer was repeatedly extracted until it appears colorless in ethyl acetate phase. The combined ethyl acetate phases from each extraction were analyzed for the content of γ-oryzanol, glycerides and fatty acids using HPLC with an ELSD detector. Finally, Antioxidant activity of product analyzed using $ABTS^{\bullet+}$ method.

3.2.1.3 Acid-catalyzed hydrolysis

The acid-catalyzed hydrolysis conducted herein was modified from Kittiruangthong et al, 2005 by a change of the catalyst solution into sulfuric acid solution. To start the hydrolysis reaction, 1 ml of acid oil was mixed with 5 ml of 2 N H_2SO_4 solution. The mixture was agitated using a hot-plate magnetic-stirrer at 80°C and 1200 rpm. for 10 minutes. Then, the mixture was poured onto the paper filter (Whatman NO.1, 90 mm Ø, Retention: 11µm) to separate the oil phase from the aqueous phase. The oil layer was then washed several times with hot deionized water (room tempurature) until water is neutral. The oil phase was analyzed for the content of γ-oryzanol, glycerides and fatty acids using HPLC with an ELSD detector. $ABTS^{\bullet+}$ method was also used to measure antioxidant activity of the oil phase.

3.3 Determination of the suitable hydrolysis condition

The conditions of each hydrolysis method as described earlier were used by following previous study. These conditions might not be the most suitable condition for hydrolysis of rice bran acid oil. Because of the different sources of rice bran acid oil leads to the different proportion of composition. Therefore, the chosen suitable methods need to be further determined the suitable condition. For non-catalytic hydrolysis as subcritical water, the effects of reaction temperature, reaction time on percentage of glycerides conversion and percentage of remaining content of γ-oryzanol were investigated. On the other hand, the catalytic hydrolysis, both effect of hydrolysis temperature and time including the concentration of catalyst solution on percentage of glycerides conversion and percentage of remaining content of γ-oryzanol also were determined. The suitable conditions of each method were selected based on high glycerides conversion, remaining content of γ-oryzanol and antioxidant activity.

3.4 Analysis

3.4.1 HPLC Analysis

Quantitative and qualitative analysis of γ-oryzanol, fatty acids and glycerides was carried out using reverse phase high performance liquid chromatography

(HPLC). The HPLC apparatus employed in this study consisted of a pump (All tech model 626, USA), equipped with an ELSD detector (All tech ELSD 2000ES). The detector condition was set as follows: the tube temperature was at 60 °C, the nitrogen gas flow was set at 1.7 L/min, and the impactor was off. The analysis was carried out at room temperature on a uBondapack C18, 300 mm × 4.90 mm I.D. column. The injection volume was 5 μL and the mobile phase consisted of methanol and isopropanol (70:30 v/v, respectively). The mobile phase flow rate was controlled at 1.2 ml/min. The peak area results were calculated into percentage of glycerides conversion and remaining of γ-oryzanol based on an analogous equation with equation 3.1 and 3.2, which are

% Glycerides conversion =

$$\frac{\text{The initial amount of glycerides - The amount of glycerides after hydrolysis}}{\text{The initial amount of glycerides}} \times 100 \quad (3.1)$$

$$\% \text{ Remaining content of } \gamma\text{-oryzanol} = \frac{\text{The amount of } \gamma\text{-oryzanol after hydrolysis}}{\text{The initial amount of } \gamma\text{-oryzanol}} \times 100 \quad (3.2)$$

3.4.2 Determination of antioxidant activity

Antioxidant activities of hydrolyzed products were tested using ABTS method which was modified from Re et al., 1999. Antioxidant activity of samples was indicated with Trolox equivalent antioxidant concentration (TEAC) as compared to Trolox standard. To find this value, the samples were diluted in series with ethanol and each diluted samples were added ABTS•+ solution (the aqueous solution of 7 mM ABTS and 2.45 mM potassium persulfate having the absorbance of 0.70 ± 0.02 at 734 nm) with the volume ratio of 1:2 (sample solution: ABTS solution). The diluted solutions were mixed using a vortex, and then were incubated in the dark at room temperature for 6 minute, after which the absorbance was measured at 734 nm. The mixture of ethanol and ABTS•+ solution (1:2 by volume) was used as a reference absorbance. The value of percent inhibition (PI) was calculated using the following equation:

$$PI(\%) = \left[1 - \frac{A_t}{A_r}\right] \times 100 \quad (3.3)$$

A_t and A_r are the absorbance of samples and the absorbance of the reference, respectively. These values were plotted against sample concentration. Likewise, Trolox standard was dissolved in ethanol with final concentration of 0-25 µM and were added ABTS•+ solution with volume ratio of 1:100 (standard Trolox solution: ABTS solution). After they were incubated in the dark at room temperature for 6 minute, the absorbance of each concentration was measured at 734 nm. The value of percent inhibition (PI) was calculated using equation 3.3, and then was plotted against the Trolox concentration. To express the TEAC value, the gradient of the plot for the sample was divided by the gradient of the plot for Trolox standard.

3.4.3. Statistical analysis

Experimental results were expressed as means ± standard deviation (n=2). Data were evaluated with SPSS (ver.16) for windows. Statistical analysis of the results was conducted using analysis of variance (One-way ANOVA). Difference between means was evaluated by Tukey test at 95% confidence level.

CHAPTER IV
RESULTS AND DISCUSSION

As described in the previous chapter, there were two parts in this study. In Part I, the assessment of the suitability of three different hydrolysis methods for the removal of glycerides from the rice bran acid oil was undertaken. The suitable hydrolysis methods were selected to be further studied based on the observed %glycerides conversion and % γ-oryzanol content remained in the rice bran acid oil as well as the antioxidant activity of the rice bran acid oil after hydrolysis. Part II therefore involves follow-up investigations on determining suitable operating conditions of the select hydrolysis methods. Discussion was made according to the observed results and is shown together with the results in this chapter. Given the experiments were conducted in duplicate, the experimental results shown in all Figures and Tables throughout this section are the average values with error bars (standard deviation).

4.1 Suitability of the hydrolysis methods for the removal of glycerides

The hydrolysis methods of interest included subcritical water hydrolysis, acid-catalyzed hydrolysis and base-catalyzed hydrolysis as described in Chapter 3. Subcritical water hydrolysis was carried out at 270ºC for 10 minutes; acid-catalyzed hydrolysis and base-catalyzed hydrolysis were carried out at 80 ºC for 10 minutes with 2 N of sulfuric acid solution and sodium hydroxide solution, respectively. Their suitability to glycerides removal in the rice bran acid oil was evaluated based on the observed %glycerides conversion and the % γ-oryzanol content remained in the rice bran acid oil after hydrolysis. The suitability evaluation also took into account the antioxidant activity of the rice bran acid oil after hydrolysis.

4.1.1 Effect of the hydrolysis methods on the %glycerides conversion and the %remaining content of γ-oryzanol

As shown in Figure 4.1, it appeared that all glycerides in rice bran acid oil could be converted through subcritical water hydrolysis. Hydrolysis by base catalysis could achieve %glycerides conversion to the same extent as the subcritical water

hydrolysis method (p>0.05). Nevertheless, only around 48% of glycerides were converted by acid-catalyzed hydrolysis, which was significantly lower than the other two methods (p<0.05). In terms of the amount of γ-oryzanol remaining in the hydrolyzed rice bran acid oil, the acid-catalyzed hydrolysis method appeared to cause γ-oryzanol degradation to the less extent than the other two methods given the observed remaining amount of γ-oryzanol of around 38%. γ-oryzanol seemed to be highly affected by the reaction condition of the subcritical water hydrolysis since the observed % remaining amount of γ-oryzanol in the rice bran acid oil after subcritical water hydrolysis was significantly lower (p<0.05). Although it appeared that more γ-oryzanol remained in the rice bran acid oil after hydrolysis by the acid-catalyzed hydrolysis (ca. 38%) than the base-catalyzed hydrolysis (ca. 26%), they was not statistically different (p>0.05) since the standard deviation of the observed % remaining amount of γ-oryzanol was relatively large. It is worth noting that each hydrolysis method was performed with the operating or reaction condition as previously suggested (Kittiruangthong et al, 2005; Holliday et al., 1997) which might not be at an optimum, and therefore the comparison among the hydrolysis methods made here was valid specifically for the herein employed conditions. Given the observed %glycerides conversion, the hydrolysis methods using subcritical water and base catalysis could be selected as the most suitable one for the removal of glycerides. Nevertheless, in terms of the remaining amount of γ-oryzanol after hydrolysis, subcritical water hydrolysis might not be a suitable method for the removal of glycerides comparing to acid-catalyzed and base-catalyzed hydrolysis. Therefore, the effect of these hydrolysis methods was further studied in order to help justify choosing the suitable methods for the removal of glycerides in rice bran acid oil. The following study involved analyzing the antioxidant activity of the hydrolyzed rice bran acid oil by the three hydrolysis methods.

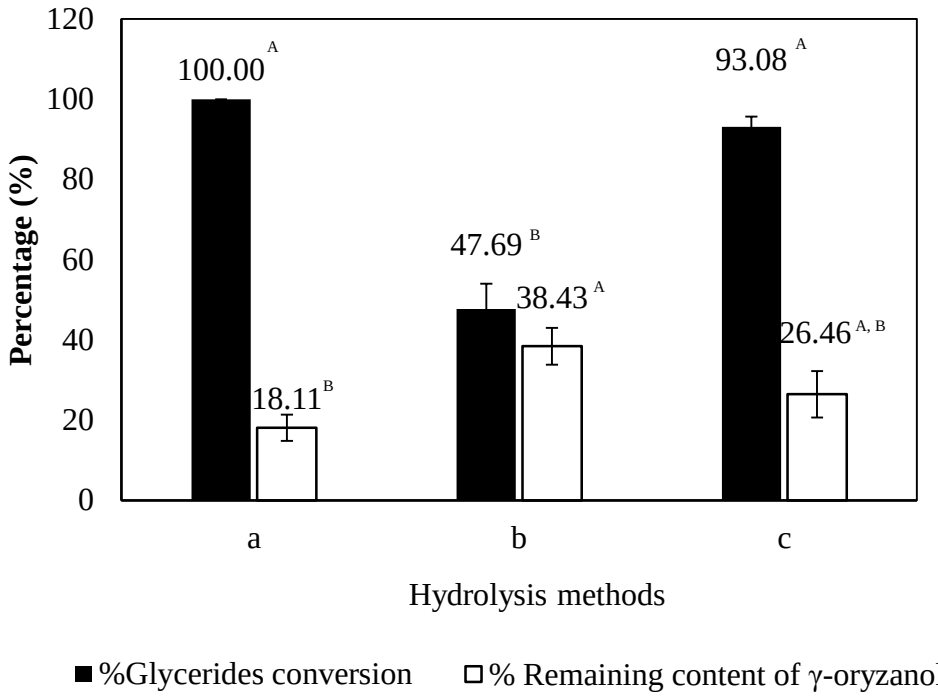


Figure 4.1 Effect of the hydrolysis methods on the percentage of glycerides conversion and the percentage of remaining content of γ-oryzanol; (a) Subcritical water hydrolysis; (b) Acid-catalyzed hydrolysis; (c) Base-catalyzed hydrolysis. "A" indicates being statistically significantly higher, and "B" indicates being statistically significantly lower.

4.1.2 Effect of the hydrolysis methods on antioxidant activity

It is well established that γ-oryzanol in rice bran exhibits antioxidant activity and has been used for various pharmaceutical and cosmetic applications (Hiramitsu et al., 1991; Patel et al 2004). Given that it is one of the main components in rice bran acid oil (up to 6%) (Das et al.,1998), evaluating the antioxidant activity of the hydrolyzed rice bran acid oil obtained from three hydrolysis methods could be another way to investigate the effect of these hydrolysis methods on γ-oryzanol and antioxidants. The evaluation was performed using $ABTS^{\bullet+}$ scavenging assay. The antioxidant activity of the samples was expressed as the TEAC value which indicates the antioxidant capacity of the samples as compared to the activity of the standard Trolox. The hydrolyzed products with a high TEAC value therefore possess the high antioxidant activity.

The TEAC values of hydrolyzed rice bran acid oil obtained from the three hydrolysis methods are shown in Table 4.1. Numerically, the hydrolyzed product obtained from subcritical water hydrolysis gave the lowest antioxidant activity while and the hydrolyzed product from the base-catalyzed hydrolysis exhibited the highest antioxidant activity. This could be because of the condition involving in the subcritical water hydrolysis where the high temperature level is required (270ºC). Such the high temperature level could lead the anti-oxidative compounds, such as vitamin E and phenolic compounds, to become more soluble in water (Watchararuji et al., 2008), and thus the amount of antioxidant in the hydrolyzed product was relatively low compared to the products from the other methods. However, the antioxidant activity of the hydrolyzed products from the three methods were not statistically different (P>0.05) since the standard deviation of the measured TEAC values were relatively large. Therefore, more replicates of the experiments would be required in the future studies.

Table 4.1 Effect of the hydrolysis methods: subcritical water hydrolysis, acid-catalyzed hydrolysis, base-catalyzed hydrolysis, on antioxidant activity of the hydrolyzed acid oil.

Hydrolysis Methods	TEAC value [a]
Subcritical water hydrolysis[b]	82.18 ± 3.31*
Acid-catalyzed hydrolysis[c]	92.46 ± 3.65*
Base-catalyzed hydrolysis[d]	97.99 ± 6.55*

[a]the antioxidant activity was expressed as the TEAC value (μmol of Trolox Equivalent /gram of hydrolyzed acid oil)

[b]the subcritical water hydrolysis condition was at 270ºC and 10 minutes

[c]the acid-catalyzed hydrolysis condition was 2 N of sulfuric acid solution, 80ºC and 10 minutes

[d]the base-catalyzed hydrolysis condition was 2 N of sodium hydroxide solution, 80ºC and 10 minutes

*there were no significant difference (p>0.05)

By taking all the above discussed results into consideration, suitable hydrolysis methods for removal of glycerides in rice bran acid oil could be selected. The operating conditions employed in the selected methods and their effects on glycerides conversion as well as γ-oryzanol would be investigated in the following studies. As above discussed, the hydrolysis methods using subcritical water and base catalysis can be ones of the suitable methods for glycerides removal based on the observed high %glycerides conversion (Figure 4.1). Although subcritical water hydrolysis led to more γ-oryzanol degradation than the other two methods as indicated by the observed %remaining content of γ-oryzanol (Figure 4.1), the antioxidant activity of the hydrolyzed product from the subcritical water hydrolysis were on a par with those from the other two methods (Table 4.1). Another aspect to be considered for suitable methods for glyceride removal is the operational condition of hydrolysis. The acid-catalyzed process usually requires a relatively longer reaction time (i.e. 2–10 h), which often causes undesired corrosion of the relevant equipment (Dorado et al., 2004). In addition, the excessive amount of water is needed for improving the conversion of glycerides (Thanh et al., 2012) which in turn leads to the relatively higher cost of the waste water treatment. On the other hand, the base catalytic process normally occurs at a relatively faster rate compared to the acid one if the same amount of acid and base catalysts is used for the reaction (Rashid et al., 2008). In addition, base catalysts are less corrosive than acidic ones, and thus industrial processes usually favor base catalysts, such as sodium hydroxide, potassium hydroxide (Ejikeme et al., 2010). For the environmental aspect, subcritical water hydrolysis is a gaining prominence as more environmentally friendly method. Giving all the above discussed reasons, subcritical water hydrolysis and base-catalyzed hydrolysis were therefore chosen as our model hydrolysis mothods which are, at the best of our knowledge, more suitable ones for glycerides removal in rice bran acid oil. The following section involves further investigations on the operating conditions in the selected methods and their effects on glyceride conversion as well as γ-oryzanol. In this way, optimal conditions for our model methods can be determined.

4.2. Determination of the suitable hydrolysis conditions for the selected hydrolysis method

As suggested in the previous section, base-catalyzed hydrolysis and subcritical water hydrolysis were selected to be the suitable methods for hydrolysis of rice bran acid oil. However, in the previous section both selected methods were perform with the operating, condition that followed previous studies as tentatively described in section 4.1. These conditions might not be the suitable one for hydrolysis rice bran acid oil since the harsh that they negatively affected on the important bioactive compounds especially γ-oryzanol. Therefore in this section, the operating conditions used in both selected hydrolysis methods were further explored. For the subcritical water hydrolysis, rice bran acid oil was hydrolyzed at two different reaction temperatures; 200 and 220°C, and for different reaction times; 10, 20, 30 and 60 minutes. In the case of base-catalyzed hydrolysis, the different concentrations of sodium hydroxide solution; 1, 2, 2.5 and 3N, were used for the hydrolysis reaction at different temperatures; room temperature (30), 70, 80, 90 and 100ºC, and for different reaction times; 5, 10 and 20 minutes. In this way, the effect of these operating parameters on the % glycerides conversion, the % remaining content of γ-oryzanol, and the antioxidant activity of the hydrolyzed product were evaluated, and ultimately suitable operating conditions for each method could be determined.

4.2.1 Subcritical water hydrolysis

4.2.1.1 Effect of the reaction temperature and the reaction time on the % glycerides conversion

The % glycerides conversion of the hydrolyzed products by subcritical water at 200 and 220°C for different reaction times including 10, 20, 30 and 60 minutes is shown in Figure 4.2. Overall, the hydrolysis using subcritical water at 220ºC achieved higher % glycerides conversion than the hydrolysis at 200ºC regardless of the reaction time (p<0.05). However, this was not the case when using the reaction time of 60 minutes where there was no significant in the % glycerides conversion between the hydrolysis at 220ºC and 200ºC (p>0.05). The observed increaced %glycerides conversion as the temperature increased from 200ºC to 220ºC might be the result of

the enhancing water ionization constant (K_w), which indicates the concentration of hydronium and hydroxide ions in the water, as the temperature rose. The K_w value increases from $10^{-11.31}$ at 200ºC to about $10^{-11.24}$ at 220°C (Bundular et al., 2006). As a result, there was an increase in the concentration of hydronium and hydroxide ions. Since hydronium and hydroxide ions can act as an acidic compound and a basic compound, respectively, the increase in these ions would allow the ester bonds of glycerides to be more effectively cleaved (Watchararuji et al., 2008). Considering the effect of the hydrolysis time, it can be seen that there is a significant increase in the %glycerides conversion in the hydrolysis at 200 ºC (p<0.05). Nevertheless, increasing the reaction time had no significant effect on the %glycerides conversion in the hydrolysis at 220ºC (p>0.05). This observed data could indicate that when the hydrolysis reaction was carried out at a high temperature, only a sufficiently short period of the reaction time was required. Based on these above discussed results, suitable operating conditions might not be clearly obtained. Therefore, given the γ-oryzanol content in hydrolyzed product is one of our main concerns, the effect of these operating conditions on γ-oryzanol needs to be take into account.

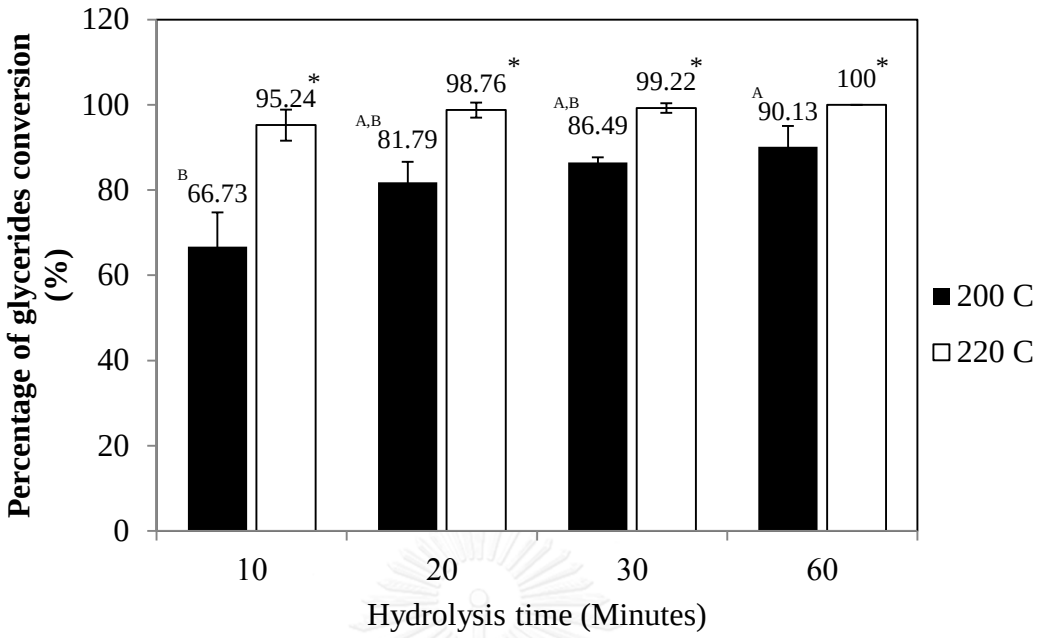


Figure 4.2 Effect of the reaction temperature and the reaction time on the %glycerides conversion, "*" indicates no significant difference, "A" indicates being statistically significantly higher, and "B" indicates being statistically significantly lower. Note that the glycerides conversion of 200 and 220ºC at 60 minutes were not significantly different.

4.2.1.2 Effect of the temperature and the reaction time on the % remaining content of γ-oryzanol

The %remaining content of γ-oryzanol after the hydrolysis using subcritical water at different temperatures and for the different reaction times is shown in Figure 4.3. It appeared that the hydrolyzed acid oil obtained from the hydrolysis at 200 ºC had the higher %remaining content of γ-oryzanol than the hydrolyzed product from the hydrolysis at 220ºC regardless of the reaction time. This suggested that more γ-oryzanol could be degraded as the temperature increased (Srisaipet and Nuddagul 2014). Similarly, a significant decrease in the %remaining content of γ-oryzanol in the hydrolyzed product was observed after the hydrolysis at 220ºC (p<0.05). However, given the observed relatively large standard deviation, it appeared that there was no significant difference in the %remaining content of γ-oryzanol in the hydrolyzed product obtained from the hydrolysis at 200ºC (p>0.05) as the reaction time increased.

Based on the observed result, it could be concluded that the subcritical water hydrolysis at a high temperature and using a long reaction time would have negative effects on the stability of γ-oryzanol.

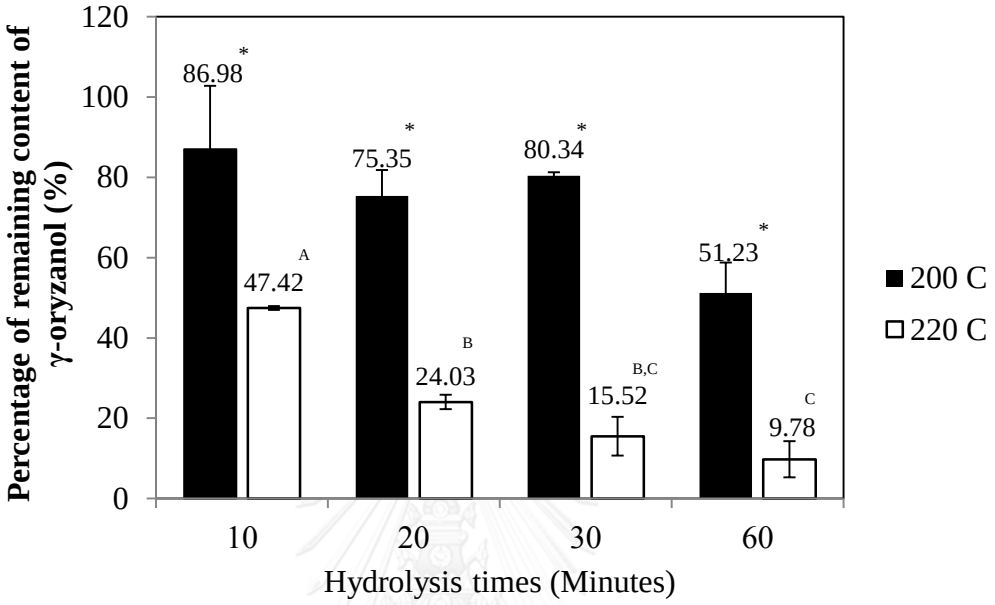


Figure 4.3 Effect of the reaction temperature and the reaction time on the %remaining content of γ-oryzanol, "*" indicates no significant difference, "A" indicates being statistically significantly higher, "B" and "C" indicates being statistically significantly lower.

Considering all the above discussed results, both the %glycerides conversion and the %remaining content of γ-oryzanol were important factors in the selection of suitable conditions of the hydrolysis method, and thus both have be taken into account. It was found that the hydrolysis at the high temperature (220ºC) promoted the removal of glycerides. However, performing the hydrolysis at the high temperature could lead to a decrease in γ-oryzanol content in the hydrolyzed product. Therefore, the subcritical water hydrolysis at 200ºC would be the best chioce for hydrolysis of rice bran acid oil. Considering the reaction time of the hydrolysis at 200ºC, using the hydrolysis time of 20, 30 and 60 minutes achieved the higher %glycerides conversion than using the time of 10 minutes, and showed similar effect on the %remining content of γ-oryzanol. Given this observation, 20, 30 and 60 minutes would be the choices for the suitable reaction times. However, as long as the

similar outcome (both %glycerides conversion and %remaining content of γ-oryzanol) can be obtained from the case with shorter reaction times, the hydrolysis with a significantly longer reaction time (60 minutes) is undesirable due to more energy required and time consuming. In order to choose between 20 and 30 minutes to be the suitable reaction time, we observed that the standard deviation of both the %glycerides conversion and the %remainig content of γ-oryzanol of the hydrolysis with the reaction time of 30 minutes was relatively much smaller than the 20 minutes case. This indicated that performing the hydrolysis with the reaction time of 30 minutes would be much more reliable than doing the reaction for 20 minutes. Therefore, to the best of our knowledge, it could be concluded that the suitable temperature andreaction time for the hydrolysis of glycerides by using subcritical water were 200°C and 30 minutes, respectively.

4.2.2 Base-catalyzed hydrolysis

4.2.2.1 Effect of the concentration of sodium hydroxide solution on the % glycerides conversion and the % remaining content of γ-oryzanol

Figure 4.4 shows the effect of sodium hydroxide concentrations (1, 2, 2.5 and 3N) on the %glycerides conversion and the %remaining content of γ-oryzanol. The reaction condition was as previously described in Chapter 3 (the reaction time of 10 minutes at 80ºC) except that the concentration was varied. As showed in Figure 4.4, there was no improvement in the %glycerides conversion as the sodium hydroxide concentration increased from 1N to 3N ($p>0.05$). Nevertheless, it appeared that there was a significant increase in the %remaining content of γ-oryzanol as the sodium hydroxide concentration increased from 2N to 2.5N ($p<0.05$), but there was no significant difference in the %remaining content of γ-oryzanol when increasing the sodium hydroxide concentration from 1N to 2N and 2.5N to 3N. Based on the observed %glycerides conversion, the sodium hydroxide concentration between 1 and 3N could be the suitable one for the base-catalyzed hydrolysis. However, it was previously reported that apart from glycerides in rice bran acid oil, fatty acids could be reacted with sodium hydroxide to form sodium soap by neutralization (Kittiroungthong et al., 2005), and therefore the hydrolysis using 1N and 2N of sodium hydroxide solution might not be suitable for this study due to insufficient to

catalyze the reaction. Given the observed %remaining content of γ-oryzanol, it seemed that using higher sodium hydroxide concentrations had less negative effect on γ-oryzanol in acid oil. It was possibly because ionization of sodium ions and hydroxide ions in water was enhanced as a result of having more sodium hydroxide. More hydroxide ions would promote the hydrolysis reaction whereas more sodium ions would neutralize fatty acids that were ones of the products obtained from the hydrolysis. As a result, formation of soap surrounding γ-oryzanol molecules (so-called micellar aggregates) would occur more frequently and in turn help prevent γ-oryzanol molecules from degradation by the added base (Das et al., 1999). As above discussed, using sodium hydroxide concentrations of 2.5 N and 3N gave the similar %glycerides conversion and %remaining content of γ-oryzanol. Therefore, in this study 2.5 N of sodium hydroxide solution was selected as the suitable sodium hydroxide concentration since the amount of hazardous waste after the hydrolysis reaction can be reduced. This condition of sodium hydroxide concentration was set as the control variable for the following studies.

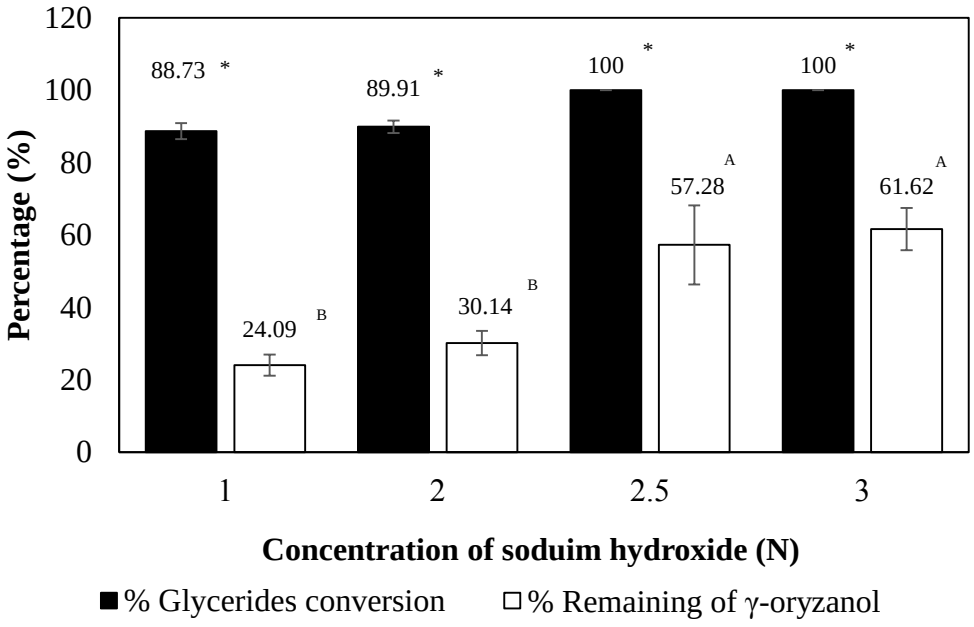


Figure 4.4 Effect of the concentration of sodium hydroxide solution on the %glyceride conversion and the %remaining content of γ-oryzanol, "*" indicates no significant difference, "A" indicates being statistically significantly higher, and "B" indicates being statistically significantly lower.

4.2.2.2 Effect of the temperature on the % glycerides conversion and the %remaining content of γ-oryzanol

To study the effect of the reaction temperature on the % glycerides conversion and the %remaining content of γ-oryzanol, the hydrolysis reaction was carried out at five different temperatures: room temperature (30), 70, 80, 90 and 100ºC. All experiments, rice bran acid oil was hydrolyzed using 2.5 N of sodium hydroxide, as previously selected as one of suitable operating conditions, for 10 minutes. As can be seen from Figure 4.5, it appeared that the complete glycerides conversion (100%) can be achieved by the base-catalyzed hydrolysis at any observed temperature ($p>0.05$). This could indicate that the reaction temperature did not have particular effect on the conversion of glycerides. Considering the %remaining amount of γ-oryzanol in the hydrolyzed rice bran acid oil, there was a significant increase in the %remaining content of γ-oryzanol ($p<0.05$) as the temperature was increased from room temperature to 70ºC. There was no significant difference in the %remaining content of γ-oryzanol ($p>0.05$) when the reaction temperature was set at 70, 80 and 90ºC. Nevertheless, as the temperature was increased from 90 to 100ºC, the %remaining content of γ-oryzanol appeared to significantly decrease ($p<0.05$). This was possibly due to the degradation of γ-oryzanol as a result of the excessively high temperature (Srisaipet and Nuddagul 2014). Since the hydrolysis at any observed temperatures showed no significant difference in the %glycerides conversion, the %remaining content of γ-oryzanol became the important factor in the selection of a suitable temperature for the base-catalyzed hydrolysis in this section. Given that the hydrolysis at 70, 80, and 90ºC appeared to have less negative effect on γ-oryzanol as indicated by the observed relatively high %remaining content of γ-oryzanol, these temperatures can thus be the choices for the suitable reaction temperature of the hydrolysis. However, as showed in Figure 4.5, the outcome obtained from performing the hydrolysis at 70 and 80ºC could be highly varied given the observed relatively large standard deviation of the %remainig content of γ-oryzanol. Therefore, 90ºC was chosen as the suitable temperature for the base-catalyzed hydrolysis and was set as another control variable for the following experiments.

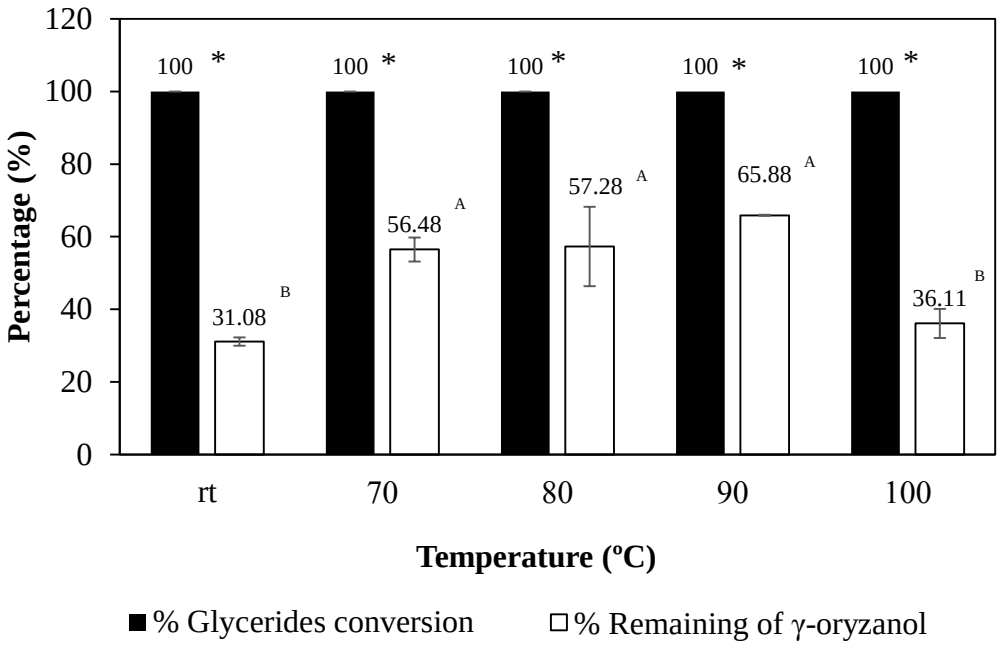


Figure 4.5 Effect of the temperature on the %glycerides conversion and the %remaining content of γ-oryzanol, "*" indicates no significant difference, "A" indicates being statistically significantly higher, and "B" indicates being statistically significantly lower.

4.2.2.3 Effect of the reaction time on the % glycerides conversion and the % remaining content of γ-oryzanol

To investigate the effect of the hydrolysis time on the %glycerides conversion and the %remaining content of γ-oryzanol, the base-catalyzed hydrolysis was carried out using different reaction times: 5, 10, and 20 minutes. As earlier discussed, the sodium hydroxide concentration of 2.5 N and the reaction temperature of 90ºC were set as control variables in this experiment. The %glycerides conversion and the %remaining content of γ-oryzanol obtained from the hydrolysis using those different reaction times are shown in Figure 4.6. It appeared that all glycerides in the rice bran acid oil could be completely converted by the hydrolysis using the reaction time of 5 minutes. As the reaction time was increased to 10 and 20 minutes, there was no significant change in the %glycerides conversion (p>0.05). Considering the effect of the reaction time on γ-oryzanol, around 56% of γ-oryzanol remained in the hydrolyzed product after the hydrolysis with the reaction time of 5 minutes. Like the

%glycerides conversion, there was no significant change in the %remaining content of γ-oryzanol as the reaction time was changed 10 minutes. Nevertheless, when the reaction time was increased to 20 minutes, the %remaining content of γ-oryzanol was significantly decreased to around 23%. This could be due to the degradation as a result of the excessively long reaction time. Given the observed data the base-catalyzed hydrolysis with the reaction time between 5 and 10 minutes would be chosen as suitable operating conditions for glycerides removal

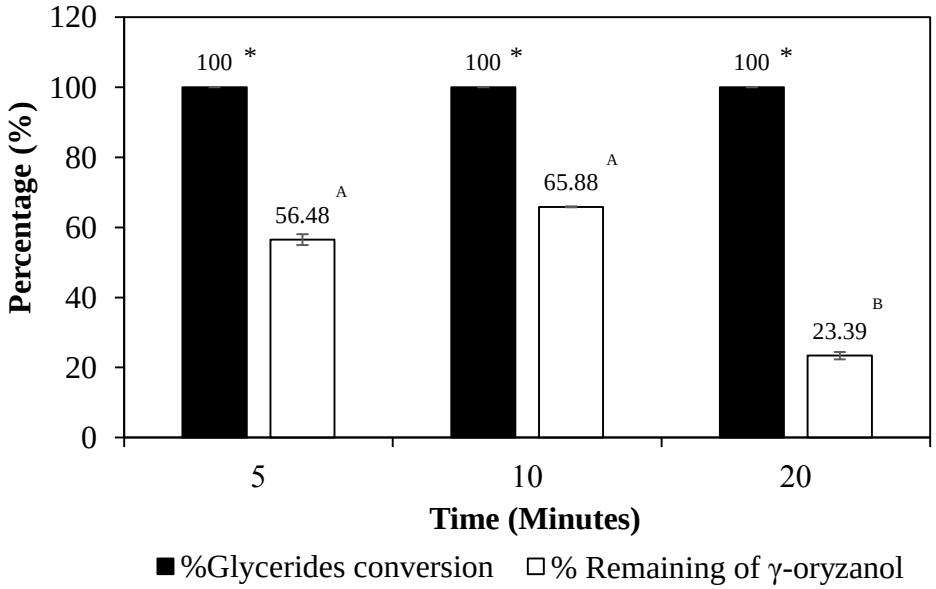


Figure 4.6 Effect of the reaction time on the %glycerides conversion and the %remaining content of γ-oryzanol, "*" indicates no significant difference, "A" indicates being statistically significantly higher, and "B" indicates being statistically significantly lower.

Based on the above discussed results in the base-catalyzed hydrolysis section, overall it appeared that the %glycerides conversion was not significantly affected by the change in sodium hydroxide concentration, the hydrolysis temperature and the hydrolysis time. On the other hand, the change in these operating conditions had significant effect on the %remaining content of γ-oryzanol. Therefore, it could be concluded that the important factor in the selection of suitable hydrolysis conditions of the base-catlyzed hydrolysis is how the operating conditions affect the %remaning content of γ-oryzanol in the hydrolyzed product. It was clear that the hydrolysis at

90ºC with 2.5 N of sodium hydroxide concentration and the reaction time between 5 and 10 minutes gave the higher %remaining content of γ-oryzanol. Therefore, in the following antioxidant activity analysis, the hydrolyzed products obtained from the hydrolysis with the reaction time of both 5 minute and 10 minutes were used.

4.2.3 Antioxidant activity of the hydrolyzed product

In this section, the antioxidant activity of the hydrolyzed products obtained from the subcritical water hydrolysis and the base-catalyzed hydrolysis was measured using the $ABTS^{\bullet+}$ scavenging assay. It should be noted that the hydrolyzed products were obtained from the two hydrolysis methods which were performed at their suitable conditions as suggested according to the above discussed results. As showed in Table 4.2, the antioxidant activity is represented as TEAC value. It appeared that the hydrolyzed product obtained from the subcritical water hydrolysis exhibited the lowest antioxidant activity (ca. 75 μmol of Trolox Equivalent /grams of hydrolyzed acid oil), while the hydrolyzed product with the highest TEAC value (ca. 155 μmol of Trolox Equivalent /grams of hydrolyzed acid oil) was obtained from the base-catalyzed hydrolysis with the reaction time of 5 minutes ($p<0.05$). By taking together the observed antioxidant activity and the observed %remaining content of γ-oryzanol into consideration, it appeared that the hydrolyzed product obtained from the subcritical water hydrolysis, which achieved the lowest antioxidant activity, had the highest % remaining content of γ-oryzanol (ca. 80%), while the hydrolyzed product from the base-catalyzed hydrolysis with the 5 minute reaction time, which had the highest antioxidant activity, contained the relatively lower amount of remaining γ-oryzanol (ca. 56%). This suggested that the antioxidant activity of the hydrolyzed product and the %remaining content of γ-oryzanol in the product might not be proportionally correlated. It should be noted that the observed antioxidant activity also accounted for other antioxidant compounds such as tocopherol and tocotrienol. The observed uncorrelated relation between the antioxidant activity and the amount of γ-oryzanol remaining is probably because these other antioxidants could become more water-insoluble as the temperature rises and thus at a relatively higher temperature the less amount of these antioxidants remained in the hydrolyzed product (Wachararuji et al., 2008).

Table 4.2 Antioxidant activity of the rice bran acid oil after hydrolysis with subcritical water hydrolysis and based-catalyzed hydrolysis

Method	TEAC value [a]
Subcritical water hydrolysis[b]	75.05 ± 0.71[e]
Based-catalyzed hydrolysis[C]	155.09±0.09[e]
Based-catalyzed hydrolysis[d]	119.58±8.80[e]

[a]the antioxidant activity was expressed as TEAC (μmol of Trolox Equivalent /grams of hydrolyzed acid oil)

[b]the subcritical water hydrolysis condition at 200ºC and 30 minutes (80.34 % of remaining γ-oryzanol content)

[c]the base catalyzed hydrolysis condition at 2.5N of NaOH solution, 90 ºC and 5 minutes (56.48 % of remaining γ-oryzanol content)

[d]the base catalyzed hydrolysis condition at 2.5N of NaOH solution, 90 ºC and 10 minutes (65.88 % of remaining γ-oryzanol content)

[e]there were significant different (p<0.05)

To decide which hydrolysis methods would be the most suitable one for the removal of glycerides in the rice bran acid oil, the outcomes from both methods; subcritical water hydrolysis and base-catalyzed hydrolysis, performed with the suggested suitable conditions were compared. Overall, the base-catalyzed hydrolysis with the two suggested operating conditions achieved the complete glycerides conversions (100%) while around 86.5% of glycerides were converted through the subcritical water hydrolysis performed at the suitable condition. Although the amount of γ-oryzanol remaining in the hydrolyzed product obtained from the base-catalyzed hydrolysis was lower than that from the subcritical water hydrolysis, the observed antioxidant activity of the hydrolyzed product from the base-catalyzed hydrolysis was higher. More specifically, the observed antioxidant activity of the product from the base-catalyzed hydrolysis at 90ºC with the reaction time of 5 minutes was two-fold higher than that of the product obtained from the subcritical water hydrolysis. Given these observed outcomes, the base-catalyzed hydrolysis was therefore suggested to be the most suitable method for the removal of glycerides prior to further processes of γ-oryzanol purification from the rice bran acid oil.

CHAPTER V
CONCLUSIONS & RECOMMENDATIONS

5.1 Conclusions

Rice bran acid oil, one of the byproducts from the rice bran oil refinery, was found to contain a relatively high amount of γ-oryzanol, one of antioxidants of importance, and thus has attracted attention to development of γ-oryzanol isolation processes from this byproduct. However, isolation of γ-oryzanol from the rice bran acid oil is not trivial given that the rice bran oil is also comprised of other compounds which can potentially hinder the isolation process. Among those compounds, glycerides were found to be the primary impurities that can affect the purity of the isolated γ-oryzanol. Therefore, prior to the γ-oryzanol isolation process, glycerides must be removed from the rice bran oil. In this study, several methods for glycerides removal were explored. The objective of this study is to identify a suitable hydrolysis method for removal of glycerides in rice bran acid oil based on the observed %glycerides conversion, the observed %remaining content of γ-oryzanol and the measured antioxidant activity of the hydrolyzed product obtained from the hydrolysis methods of interest. Firstly, the preliminary evaluation of suitability of the hydrolysis methods of interest: subcritical water hydrolysis, acid-catalyzed hydrolysis, and base-catalyzed hydrolysis were performed. Secondly, the operating conditions of the methods selected as suitable ones for glycerides removal and their effects on the %glycerides conversion and the %remaining content of γ-oryzanol were then investigated. Finally, the antioxidant activity of the hydrolyzed products obtained from the selected methods performed with the suggested suitable operating conditions was analyzed. By taking all the observed results from the selected methods with the suggested operating conditions into account, the most suitable methods could be herein found.

Among the three hydrolysis methods, the subcritical water hydrolysis and the base-catalyzed hydrolysis provided the relatively high %glycerides conversions (100±0.0 and 93.08±2.55%, respectively), and thus they were chosen to be the suitable methods for glycerides removal. For the subcritical water hydrolysis, it was

found that performing the hydrolysis reaction at the reaction temperature of 200ºC and with the reaction time of 30 minutes could achieve around 86.5±1.18% glycerides conversion and 80.3±1.80% remaining content of γ-oryzanol. Given these observed data, the reaction temperature of 200ºC and the reaction time of 30 minutes were suggested as the suitable conditions of the subcritical water hydrolysis. In the base-catalyzed hydrolysis case, the complete glycerides conversion (100±0.00%) and the relatively high %remaining content of γ-oryzanol could be achieved from the hydrolysis performed using 2.5 N of sodium hydroxide solution at 90 ºC and with the reaction time between 5 to 10 minutes. Therefore, these operation conditions of the base-catalyzed hydrolysis were also selected as suitable ones. The antioxidant activity of the hydrolyzed product obtained from the subcritical water hydrolysis performed with its suggested suitable condition was around 75±0.71 μmol Trolox Equivalent per gram of hydrolyzed acid oil while that of the hydrolyzed product from the base-catalyzed hydrolysis with its suggested suitable condition were around 119.58±8.80 to 155±0.09 μmol Trolox Equivalent per gram of hydrolyzed acid oil. Given all the observed results, the base-catalyzed hydrolysis was suggested to be the most suitable method for hydrolysis of rice bran acid oil.

5.2 Recommendations

Recommendations for further study are as follows.

1. It should be noted that one of the products from the hydrolysis is fatty acids. Therefore, the amount of fatty acids in the hydrolyzed products might be increased compared to the initial fatty acid amount. This remaining fatty acid in the hydrolyzed rice bran acid oil might be later problematic for the following γ-oryzanol purification by column chromatography. Removal of fatty acids from the hydrolyzed acid oil could be therefore required prior to the purification process. This can be accomplished through vacuum distillation (Das et al., 1999).
2. For the catalytic hydrolysis reaction, it appeared that in this study the acid-catalyzed hydrolysis showed the lowest %glycerides conversion (ca. 47.7 %). This was possibly due to the operating condition used herein being not

the suitable one. Therefore, in the future study, the condition used with this method should be further investigated.

3. According to the procedure of the acid catalyzed hydrolysis, the unreacted acid catalyst must be removed from the hydrolyzed acid oil by washing. This process is time-consuming and requires the large amount of water to neutralize the hydrolyzed product. As a possible alternative, the heterogeneous acid catalyst could be used instead of the homogenous one since it allows easier separation and recycling. This could be an interesting topic that might be further evaluated in the future study.

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APPENDIX

APPENDIC A
EXPERIMENTAL DATA & ANALYSIS

A-1 Standard calibration curve of γ-oryzanol HPLC analysis

Concentration of γ-oryzanol (mg/ml)	Peak area
1.6	17373.227
1.2	12881.593
1	9781.363
0.24	754.123
0.1	279.000

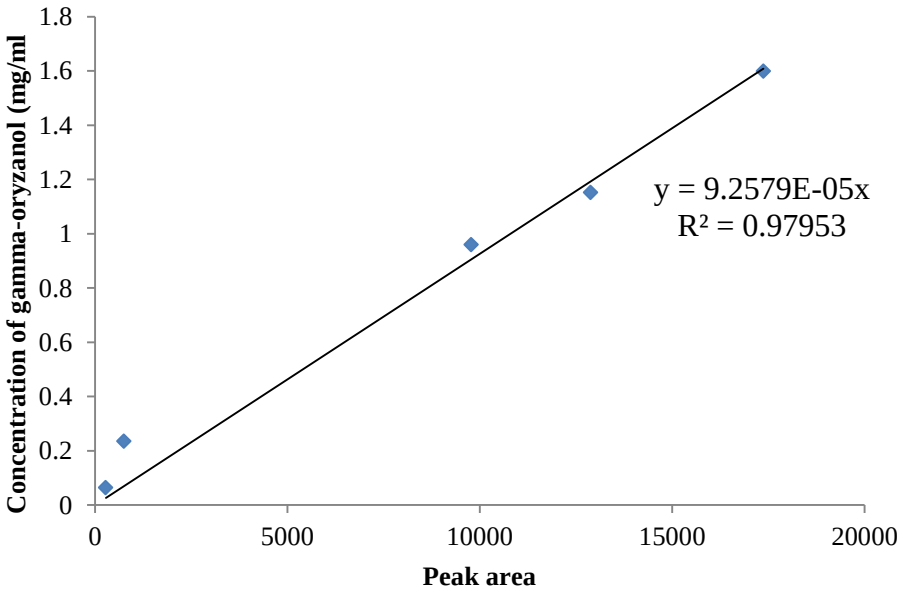

Figure A-1.1 Standard calibration curve of γ-oryzanol standard analyzed by HPLC

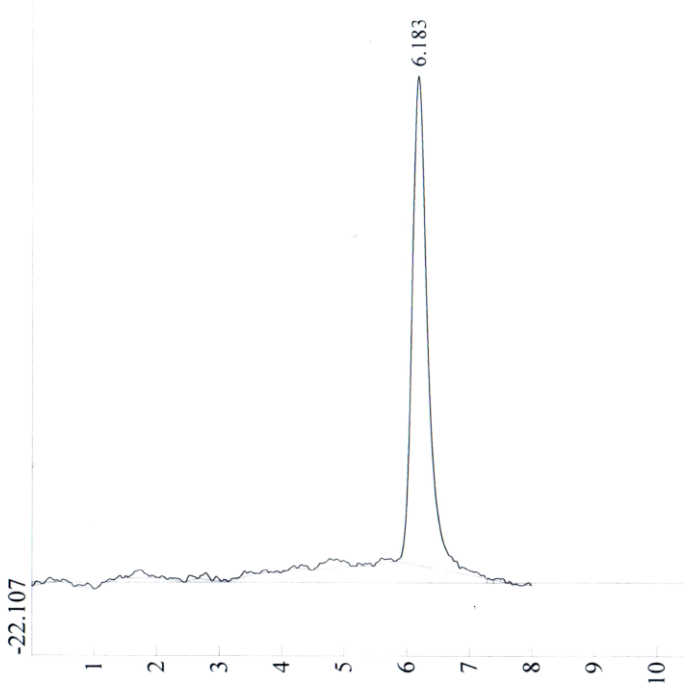

Figure A-1. 2 Chromatogram of γ-oryzanol standard

A-2 Standard calibration curve of glycerides HPLC analysis

Concentration of glycerides (mg/ml)	Peak area
0.10	1016.960
0.06	594.707
0.03	274.967
0.023	232.684
0.01	98.218
0.006	61.064

*Peak area of glycerides was calculated from the four main peaks appeared in Figure A-2.1

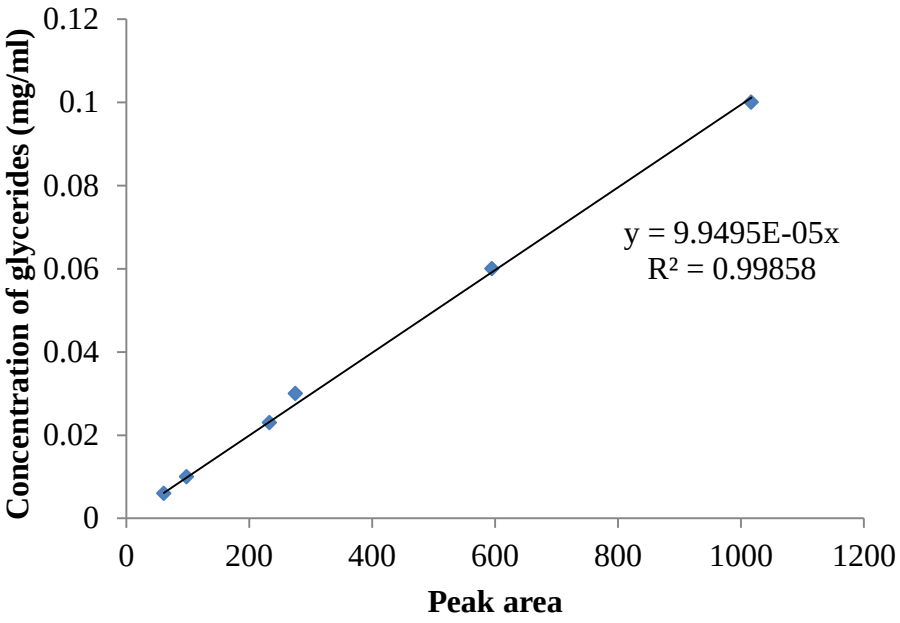

Figure A-2.1 Standard calibration curve of glycerides standard analyzed by HPLC

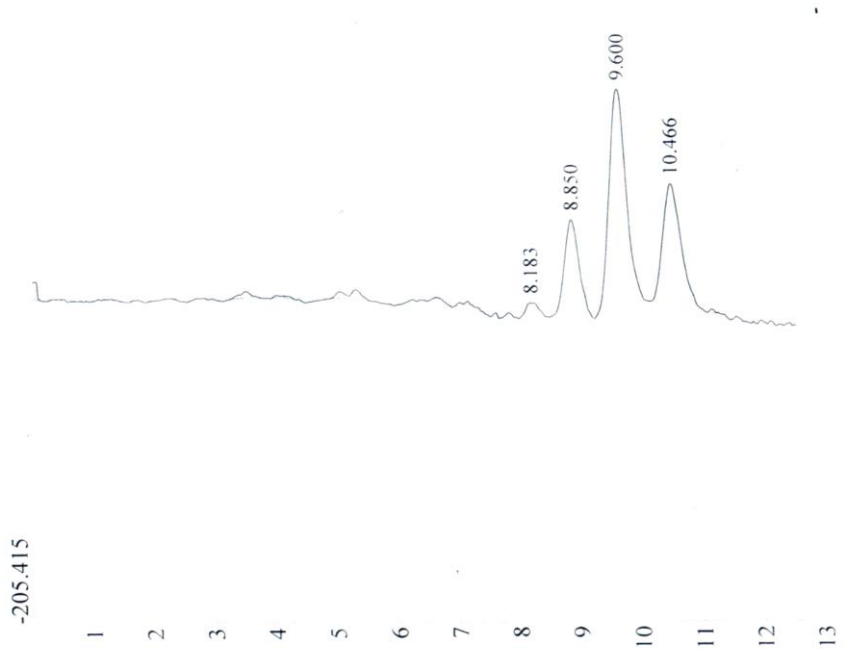

Figure A-2. 2 Chromatogram of glycerides standard

A-3 Standard calibration curve of fatty acid HPLC analysis

Concentration of fatty acid (mg/ml)	Peak area
10	82592.68
8	81949.5
6	64823.19
4	49482.82
2	25370.71

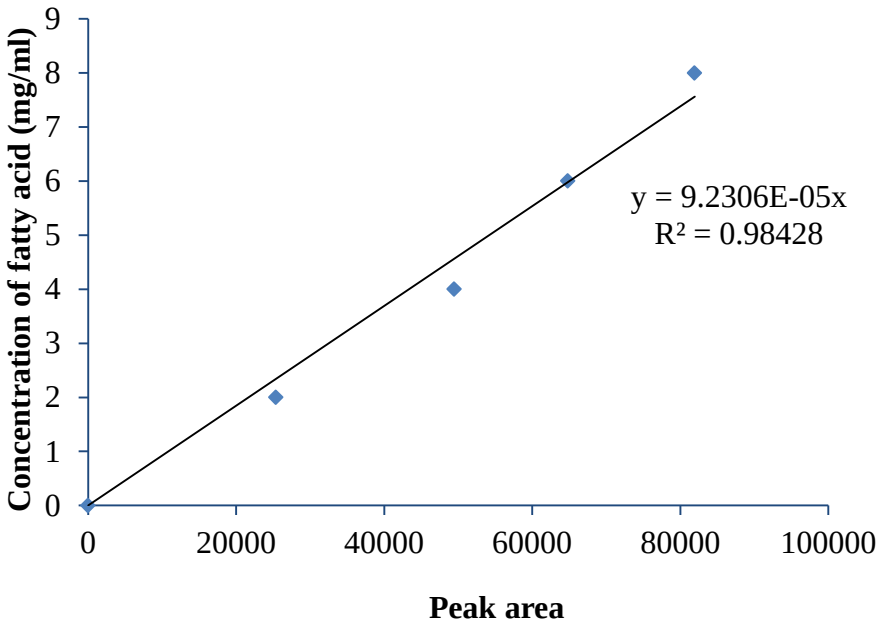

Figure A-3.1 Standard calibration curve of fatty acid standard analyzed by HPLC

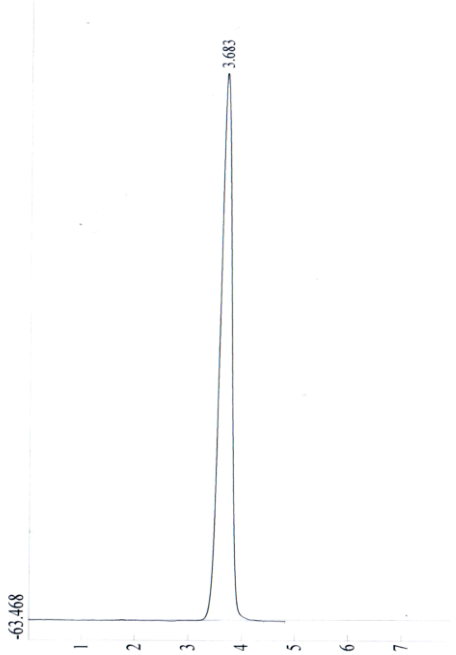

Figure A-3. 2 Chromatogram of fatty acid standard

A-4 Standard calibration curve of Trolox by UV- spectrophotometer analysis

Concentration of Trolox (μM Trolox)	% Inhibition
0	0
5	9.25
10	20.81
15	31.98
20	44.89
25	55.88

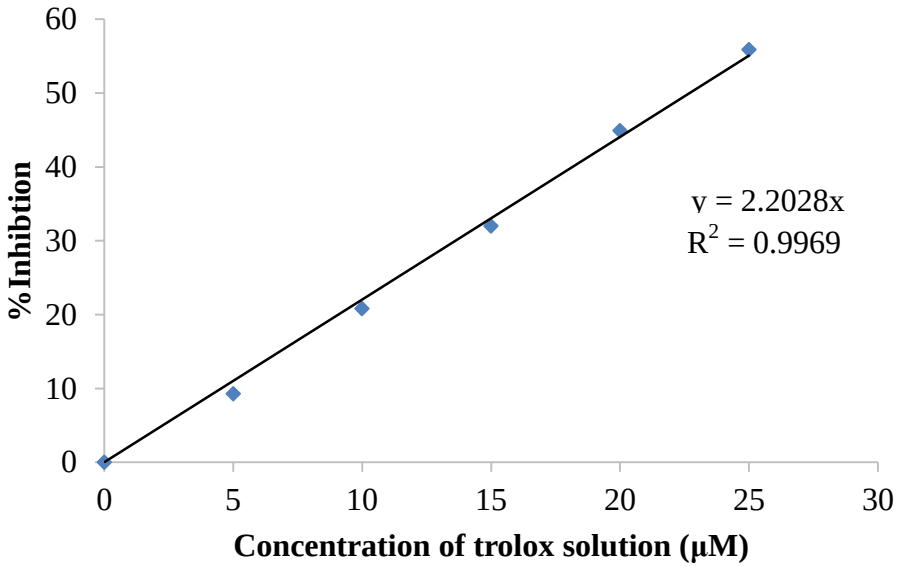

Figure A-4.1 Standard calibration curve of fatty acid standard analyzed by UV-Spectrophotometer

APPENDIX B
EXPERIMENTAL DATA

B-1 Equation of percentage of glycerides conversion and percentage of remaining content of γ-oryzanol

$$\% \text{ Glycerides conversion} = \frac{\text{The initial amount of glycerides - The amount of glycerides after hydrolysis}}{\text{The initial amount of glycerides}} \times 100$$

$$\% \text{ Remaining of } \gamma\text{-oryzanol} = \frac{\text{The amount of } \gamma\text{-oryzanol after hydrolysis}}{\text{The initial amount of } \gamma\text{-oryzanol}} \times 100$$

B-2 Experimental data of rice bran oil hydrolysis with different methods

Table B-2.1 Percentage of glycerides conversion of rice bran acid oil from subcritical water hydrolysis, acid-catalyst hydrolysis, and base-catalyst hydrolysis

Hydrolysis method	Glycerides conversion (%)			SD
	Exp.1	Exp.2	Average	
Subcritical water hydrolysis	100	100	100	0.00
Acid catalyst hydrolysis	43.23	52.15	47.69	6.31
Base catalyst hydrolysis	91.28	94.88	93.08	2.55

Table B-2.2 Percentage of remaining content of γ-oryzanol from subcritical water hydrolysis, acid-catalyst hydrolysis, and base-catalyst hydrolysis

Hydrolysis method	Remaining content of γ-oryzanol (%)			SD
	Exp.1	Exp.2	Average	
Subcritical water hydrolysis	20.42	15.81	18.11	3.26
Acid catalyst hydrolysis	41.68	35.19	38.43	4.59
Base catalyst hydrolysis	30.54	22.38	26.46	5.77

Table B-2.3 Antioxidant activity of hydrolyzed acid oil from subcritical water hydrolysis, acid-catalyst hydrolysis, and base-catalyst hydrolysis

Hydrolysis method	TEAC (μmol Trolox Equivalent /gram of hydrolyzed acid oil)			SD
	Exp.1	Exp.2	Average	
subcritical water hydrolysis	79.84	84.52	82.18	3.31
acid catalyst hydrolysis	95.04	89.88	92.46	3.65
base catalyst hydrolysis	102.61	93.36	97.99	6.55

B-3 Experimental data of rice bran oil hydrolysis with subcritical water hydrolysis

Table B-3.1 Percentage of glycerides conversion of rice bran acid oil from subcritical water hydrolysis

Temperature (°C)	Time (min)	Glycerides conversion (%)			SD
		Exp.1	Exp.2	Average	
200	10	72.42	61.04	66.73	8.04
	20	78.39	85.20	81.79	4.82
	30	87.33	85.65	86.49	1.18
	60	86.67	93.59	90.13	4.90
220	10	92.67	97.81	95.24	3.63
	20	100.00	97.52	98.76	1.75
	30	98.44	100.00	99.22	1.10
	60	100.00	100.00	100.00	0.00

Table B-3.2 Percentage of remaining content of γ-oryzanol from subcritical water hydrolysis

Temperature (ºC)	Time (min)	Remaining content of γ-oryzanol (%)			SD
		Exp.1	Exp.2	Average	
200	10	77.31	96.65	86.98	13.68
	20	66.22	84.49	75.35	12.91
	30	81.62	79.07	80.34	1.80
	60	40.60	61.86	51.23	15.03
220	10	47.08	47.76	47.42	0.48
	20	22.76	25.31	24.03	1.80
	30	18.94	12.09	15.52	4.85
	60	12.96	6.60	9.78	4.50

B-4 Experimental data of rice bran oil hydrolysis with base-catalyzed hydrolysis

Table B-4.1 Percentage of glycerides conversion of rice bran acid oil from base-catalyzed hydrolysis

Sodium hydroxide concentration (N)	Temperature (ºC)	Time (min)	Glycerides conversion (%)			SD
			Exp.1	Exp.2	Average	
1	80	10	85.59	91.87	88.73	4.44
2			85.97	91.88	89.91	3.43
2.5			100.00	100.00	100.00	0.00
3			100.00	100.00	100.00	0.00
2.5	rt (30ºC)	10	100.00	100.00	100.00	0.00
	70		100.00	100.00	100.00	0.00
	80		100.00	100.00	100.00	0.00
	90		100.00	100.00	100.00	0.00
	100		100.00	100.00	100.00	0.00
2.5	90	5	100.00	100.00	100.00	0.00
		10	100.00	100.00	100.00	0.00
		20	100.00	100.00	100.00	0.00

Table B-4.2 Percentage of remaining content of γ-oryzanol from base-catalyzed hydrolysis

Sodium hydroxide concentration (N)	Temperature (ºC)	Time (min)	Remaining content of γ-oryzanol (%)			SD
			Exp.1	Exp.2	Average	
1	80	10	28.24	19.94	24.09	5.87
2			37.87	26.27	30.14	6.7
2.5			41.80	72.77	57.28	21.90
3			53.34	69.90	61.62	11.71
2.5	rt (30ºC)	10	32.71	29.45	31.08	2.31
	70		61.13	51.82	56.48	6.58
	80		41.80	72.77	57.28	21.90
	90		65.70	66.06	65.88	0.25
	100		41.80	30.42	36.11	8.05
2.5	90	5	49.36	63.60	56.48	1.54
		10	65.70	66.06	65.88	0.25
		20	24.86	21.92	23.39	2.08

B-5 Experimental data of antioxidant activity of hydrolyzed acid oil by subcritical water hydrolysis and base-catalyzed hydrolysis at suitable condition

Table B-5.1 Antioxidant activity of hydrolyzed acid oil from subcritical water hydrolysis and base-catalyst hydrolysis

Hydrolysis method	TEAC (μmol Trolox Equivalent /gram of hydrolyzed acid oil)			SD
	Exp.1	Exp.2	Average	
subcritical water hydrolysis	75.55	74.55	75.05	0.71
homogeneous base catalyst hydrolysis	113.36	125.80	119.58	8.80

APPENDIX C

EXPERIMENTAL DATA ANALYSIS

C-1 Experimental data analysis of rice bran oil hydrolysis with different methods

Table C-1.1 Experimental data analysis of glycerides conversion from subcritical water hydrolysis, acid-catalyst hydrolysis, and base-catalyst hydrolysis

(I) method	(J) method	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Subcritical water hydrolysis	Base catalyzed hydrolysis	6.92	.320	-9.50	23.33
	Acid catalyzed hydrolysis	52.31*	.002	35.90	68.72
Base catalyzed hydrolysis	Subcritical water hydrolysis	-6.92	.320	-23.33	9.50
	Acid catalyzed hydrolysis	45.39*	.003	28.98	61.80
Acid catalyzed hydrolysis	Subcritical water hydrolysis	-52.31*	.002	-68.72	-35.90
	Base catalyzed hydrolysis	-45.39*	.003	-61.80	-28.98
*. The mean difference is significant at the 0.05 level.					

Table C-1.2 Experimental data analysis of remaining content of γ-oryzanol from subcritical water hydrolysis, acid-catalyst hydrolysis, and base-catalyst hydrolysis

(I) method	(J) method	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Subcritical water hydrolysis	Base catalyzed hydrolysis	-8.35	.312	-27.79	11.10
	Acid catalyzed hydrolysis	-20.32*	.045	-39.77	-.87
Base catalyzed hydrolysis	Subcritical water hydrolysis	8.35	.312	-11.10	27.80
	Acid catalyzed hydrolysis	-11.98	.158	-31.42	7.47
Acid catalyzed hydrolysis	Subcritical water hydrolysis	20.32*	.045	.87	39.77
	Base catalyzed hydrolysis	11.98	.158	-7.47	31.422
*. The mean difference is significant at the 0.05 level.					

Table C-1.3 Experimental data analysis of antioxidant activity from subcritical water hydrolysis, acid-catalyst hydrolysis, and base-catalyst hydrolysis.

(I) Method	(J) Method	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Subcritical water hydrolysis	Base catalyzed hydrolysis	-15.81	.087	-35.56	3.95
	Acid catalyzed hydrolysis	-10.28	.222	-30.03	9.47
Base catalyzed hydrolysis	Subcritical water hydrolysis	15.81	.087	-3.95	35.56
	Acid catalyzed hydrolysis	5.53	.544	-14.23	25.28
Acid catalyzed hydrolysis	Subcritical water hydrolysis	10.28	.222	-9.47	30.03
	Base catalyzed hydrolysis	-5.53	.544	-25.28	14.23

C-2 Experimental data analysis of rice bran oil hydrolysis with subcritical water hydrolysis

Table C-2.1 Experimental data analysis of glycerides conversion from subcritical water hydrolysis at 200ºC

(I) Time: min	(J) Time: min	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
10	20	-15.07	.145	-36.73	6.60
	30	-19.76	.067	-41.43	1.90
	60	-23.40*	.039	-45.07	-1.73
20	10	15.07	.145	-6.60	36.73
	30	-4.70	.815	-26.36	16.97
	60	-8.33	.484	-30.00	13.33
30	10	19.76	.067	-1.91	41.43
	20	4.70	.815	-16.97	26.36
	60	-3.64	.898	-25.31	18.03
60	10	23.40*	.039	1.73	45.07
	20	8.34	.484	-13.33	30.00
	30	3.64	.898	-18.03	25.31

*. The mean difference is significant at the 0.05 level.

Table C-2. 2 Experimental data analysis of remaining content of γ-oryzanol from subcritical water hydrolysis at 200ºC

(I) Time:min	(J) Time:min	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
10	20	11.63	.777	-37.53	60.78
	30	6.64	.942	-42.52	55.79
	60	35.75	.129	-13.40	84.90
20	10	-11.63	.777	-60.78	37.53
	30	-4.99	.973	-54.14	44.16
	60	24.13	.323	-25.03	73.28
30	10	-6.64	.942	-55.79	42.52
	20	4.99	.973	-44.16	54.14
	60	29.12	.216	-20.04	78.27
60	10	-35.75	.129	-84.90	13.40
	20	-24.13	.323	-73.28	25.03
	30	-29.12	.216	-78.27	20.04

Table C-2.3 Experimental data analysis of glycerides conversion from subcritical water hydrolysis at 220ºC

(I) Time: min	(J) Time: min	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
10	20	-3.52	.435	-12.04	4.99
	30	-3.98	.353	-12.50	4.54
	60	-4.76	.247	-13.28	3.76
20	10	3.52	.435	-4.99	12.04
	30	-.46	.996	-8.98	8.06
	60	-1.24	.929	-9.76	7.28
30	10	3.98	.353	-4.54	12.50
	20	.46	.996	-8.06	8.98
	60	-.78	.980	-9.30	7.74
60	10	4.76	.247	-3.76	13.28
	20	1.24	.929	-7.28	9.76
	30	.78	.980	-7.74	9.30

Table C-2. 4 Experimental data analysis of remaining content of γ-oryzanol from subcritical water hydrolysis at 220ºC

(I) Time:min	(J) Time:min	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
10	20	23.39*	.008	9.41	37.36
	30	31.91*	.003	17.93	45.88
	60	37.64*	.001	23.66	51.62
20	10	-23.39*	.008	-37.36	-9.41
	30	8.52	.202	-5.46	22.50
	60	14.26*	.047	.28	28.23
30	10	-31.91*	.003	-45.88	-17.93
	20	-8.52	.202	-22.50	5.46
	60	5.74	.440	-8.24	19.71
60	10	-37.64*	.001	-51.62	-23.66
	20	-14.26*	.047	-28.23	-.28
	30	-5.74	.440	-19.71	8.24

*. The mean difference is significant at the 0.05 level.

Table C-2.5 Experimental data analysis of glycerides conversion of rice bran acid oil from subcritical water hydrolysis with different temperature at 10 min

200-220 (10)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	-4.37	2	.048	-29.61	-58.73	-.48

Table C-2.6 Experimental data analysis of glycerides conversion of rice bran acid oil from subcritical water hydrolysis with different temperature at 20 min

200-220 (20)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	-4.78	2	.041	-17.11	-32.50	-1.72

Table C-2. 7 Experimental data analysis of glycerides conversion of rice bran acid oil from subcritical water hydrolysis with different temperature at 30 min

200-220 (30)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	-12.00	2	.007	-11.49	-15.61	-7.37

Table C-2.8 Experimental data analysis of glycerides conversion of rice bran acid oil from subcritical water hydrolysis with different temperature at 60 min

200-220 (60)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	-2.85	2	.104	-9.87	-24.76	5.02

Table C-2.9 Experimental data analysis of remaining content of γ-oryzanol from subcritical water hydrolysis with different temperature at10 min

200-220ºC (10 min)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	4.07	2	.055	39.37	-2.25	80.98

Table C-2.10 Experimental data analysis of remaining content of γ-oryzanol from subcritical water hydrolysis with different temperature at 20 min

200-220ºC (20 min)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	2.59	2	.122	40.10	-26.52	106.71

Table C-2.11 Experimental data analysis of remaining content of γ-oryzanol from subcritical water hydrolysis with different temperature at 30 min

200-220 ºC (30 min)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	16.97	2	.003	58.22	43.46	72.98

Table C-2.12 Experimental data analysis of remaining content of γ-oryzanol from subcritical water hydrolysis with different temperature at 60 min

200-220 ºC (60 min)						
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Equal variances assumed	3.74	2	.065	41.45	-6.29	89.19

C-3 Experimental data analysis of rice bran oil hydrolysis with base-catalyzed hydrolysis

Table C-3.1 Experimental data analysis of percentage of glycerides conversion from base-catalyzed hydrolysis at different concentration

(I) Concentration: N	(J) Concentration :N	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
1	2	-.20	1.000	-12.61	12.22
	2.5	-11.27	.068	-23.68	1.14
	3	-11.27	.068	-23.68	1.14
2	1	.195	1.000	-12.22	12.61
	2.5	-11.08	.071	-23.49	1.34
	3	-11.08	.071	-23.49	1.34
2.5	1	11.27	.068	-1.14	23.68
	2	11.08	.071	-1.34	23.49
	3	.00	1.000	-12.41	12.41
3	1	11.27	.068	-1.14	23.68
	2	11.08	.071	-1.34	23.49
	2.5	.00	1.000	-12.41	12.41

Table C-3. 2 Experimental data analysis of remaining content of γ-oryzanol from base-catalyzed hydrolysis at different concentration

(I) Concentration: N	(J) Concentration:N	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
1	2	-7.98	.584	-45.19	29.23
	2.5	-33.20	.068	-70.40	4.01
	3	-37.53*	.049	-74.74	-.32
2	1	7.98	.584	-29.23	45.19
	2.5	-25.22	.133	-62.42	11.99
	3	-29.55	.092	-66.76	7.66
2.5	1	33.20	.068	-4.01	70.40
	2	25.22	.133	-11.99	62.42
	3	-4.34	.763	-41.54	32.87
3	1	37.53*	.049	.32	74.74
	2	29.55	.092	-7.66	66.76
	2.5	4.34	.763	-32.87	41.54

*. The mean difference is significant at the 0.05 level

Table C-3.3 Experimental data analysis of percentage of remaining content from base-catalyzed hydrolysis at different temperature

(I) Temperature: ºC	(J) Temperature: ºC	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
rt : room temperature (30)	70	-25.40	.067	-53.39	2.60
	80	-26.21	.061	-54.20	1.79
	90	-34.80*	.024	-62.80	-6.80
	100	-5.03	.664	-33.03	22.97
70	rt (30)	25.40	.067	-2.60	53.39
	80	-.81	.944	-28.81	27.19
	90	-9.41	.427	-37.40	18.59
	100	20.37	.120	-7.63	48.36
80	rt(30)	26.21	.061	-1.79	54.20
	70	.81	.944	-27.19	28.81
	90	-8.60	.466	-36.59	19.40
	100	21.18	.109	-6.82	49.17
90	rt(30)	34.80*	.024	6.80	62.80
	70	9.41	.427	-18.59	37.40
	80	8.60	.466	-19.4005	36.59
	100	29.77*	.041	1.7745	57.77
100	rt(30)	5.03000	.664	-22.9655	33.02

	70	-20.37	.120	-48.36	7.63
	80	-21.18	.109	-49.17	6.82
	90	-29.77*	.041	-57.77	-1.77

*. The mean difference is significant at the 0.05 level

Table C-3. 4 Experimental data analysis of percentage of remaining content of γ-oryzanol from base-catalyzed hydrolysis at different hydrolysis time

(I) Time: min	(J) Time:min	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
5	10	-9.40	.377	-34.21	15.41
	20	33.09*	.023	8.28	57.90
10	5	9.40	.377	-15.41	34.21
	20	42.49*	.011	17.68	67.30
20	5	-33.09*	.023	-57.90	-8.28
	10	-42.49*	.011	-67.30	-17.68

*. The mean difference is significant at the 0.05 level

C-4 Experimental data analysis of antioxidant activity from subcritical water hydrolysis and base-catalyzed hydrolysis at suitable condition

Table C-4.1 Experimental data analysis of antioxidant activity from subcritical water hydrolysis and base-catalyst hydrolysis at suitable condition

(I) method	(J) method	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Subcritical water hydrolysis	Base-catalyzed hydrolysis (10 min)	-44.53*	.006	-65.82	-23.24
	Base-catalyzed hydrolysis (5min)	-80.04*	.001	-101.33	-58.74
Base-catalyzed hydrolysis (10 min)	Subcritical water hydrolysis	44.53*	.006	23.24	65.82
	Base-catalyzed hydrolysis (5min)	-35.51*	.012	-56.80	-14.21
Base-catalyzed hydrolysis (5min)	Subcritical water hydrolysis	80.04*	.001	58.74	101.33
	Base-catalyzed hydrolysis (10 min)	35.51*	.012	14.21	56.80

* The mean difference is significant at the 0.05 level

VITA

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List of publication;

Amornrat Meedam, Artiwan Shotipruk, "Effect of hydrolysis Methods on key components of rice bran acid oil" The 26th National Thai Insititute of Chemical Engineering and Applied Science Conference (TIChE2016) and The 6th International Thai Insititute of Chemical Engineering and Applied Science Conference (ITIChE2016): 27-28 October 2016, Bangkok, Thailand.

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