EXTRACTION OF LIPID AND GAMMA-ORYZANOL FROM RICE BRAN

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรดุษฎีบัณฑิต
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EXTRACTION OF LIPID AND GAMMA-ORYZANOL FROM RICE BRAN

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งานวิจัยนี้ศึกษาวิธีการสกัดลิพิดและแกมมา-โอไรซานอลจากรำข้าว รวมทั้งวิเคราะห์สมบัติทางเคมีกายภาพและลักษณะโครงสร้างระดับจุลภาคของลิพิดและแกมมา-โอไรซานอล โดยสามารถแบ่งงานวิจัยออกเป็น 4 ส่วนดังต่อไปนี้

1) การศึกษาประสิทธิภาพของวิธีการสกัดน้ำมันรำข้าวและผลของวิธีการสกัดต่อคุณภาพน้ำมันรำข้าว โดยวิธีการสกัดได้แก่ การใช้สารเฮกเซน (HE), การบีบด้วยแรงกดโดยไม่ใช้ความร้อน (CE), การปรับสภาพเบื้องต้นด้วยคลื่นเสียงความถี่สูง ตามด้วยการบีบด้วยแรงกดโดยไม่ใช้ความร้อน (UCE) และการปรับสภาพเบื้องต้นด้วยความร้อน ตามด้วยการบีบด้วยแรงกดโดยไม่ใช้ความร้อน (CCE) จากผลการทดลองพบว่า ปริมาณน้ำมันรำข้าวที่สกัดได้แปรผันตรงกับความเข้มและระยะเวลาการใช้คลื่นเสียงความถี่สูงรวมถึงระยะเวลาการให้ความร้อนอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) แต่ระดับความรุนแรงในการปรับสภาพที่เพิ่มขึ้นมีผลต่อการลดคุณภาพของน้ำมันอย่างมีนัยสำคัญทางสถิติ จากการทดลองสรุปได้ว่าการใช้ความร้อนในระยะเวลาสั้นตามด้วยการบีบด้วยแรงกดโดยไม่ใช้ความร้อน (CCE) จะช่วยเพิ่มประสิทธิภาพการสกัดและคงคุณภาพของน้ำมันรำข้าวได้ดี

2) การศึกษาสภาวะที่เหมาะสมของการปรับสภาพน้ำมันให้เป็นกลางด้วยโซเดียมไฮดรอกไซด์ต่อปริมาณน้ำมันรำข้าว (neutralized oil) กรดไขมันอิสระ (free fatty acids) และแกมมา-โอไรซานอล โดยใช้วิธีพื้นผิวตอบสนอง น้ำมันรำข้าวดิบ (CRBO) ถูกปรับสภาพด้วยโซเดียมไฮดรอกไซด์ความเข้มข้น 10-30°Be โดยใช้อุณหภูมิ 60-90°C และระยะเวลา 5-15 min จากการทดลองพบว่าสภาวะที่เหมาะสมที่สุดในการปรับสภาพให้เป็นกลางคือ 19.24°Be, 74.79°C และ 11.18 min ค่าการพยากรณ์ (predicted value) ของปริมาณน้ำมันรำข้าว กรดไขมันอิสระ และแกมมา-โอไรซานอลมีค่า 80.00%, 0.31% และ 1.00% ตามลำดับ สอดคล้องกับค่าที่ได้จากการทดลอง (experimental value) คือ 80.12%, 0.29% และ 1.02% ตามลำดับ จากผลการทดลองสามารถสรุปได้ว่า แบบจำลองพื้นผิวตอบสนองสามารถนำไปใช้พยากรณ์ได้อย่างถูกต้อง

3) การศึกษาสภาวะที่เหมาะสมในการสกัดแกมมา-โอไรซานอลโดยใช้วิธีพื้นผิวตอบสนองและสร้างแบบจำลองจลนศาสตร์ของการสกัดแกมมา-โอไรซานอลจากไขสบู่น้ำมันรำข้าว (DRBS) โดยการสกัดแกมมา-โอไรซานอลจากไขสบู่น้ำมันรำข้าวด้วยเครื่องกำเนิดคลื่นเสียงความถี่สูงในช่วงกำลัง 0.5-4.5 W/g ที่อุณหภูมิ 35-55°C เป็นเวลา 4-26 min จากผลการทดลองพบว่า ปริมาณ

แกมมา-โอไรซานอลสูงสุดที่ได้คือ 98.03% ภายใต้สภาวะที่เหมาะสมของการสกัดที่ 4.0 W/g, 50.0 °C, และ 21.5 min ซึ่งผลการทดลองสอดคล้องกับค่าที่พยากรณ์จากแบบจำลอง

4) การศึกษาการแยกแกมมา-โอไรซานอลจาก gamma oryzanol rich fraction (ORF) ด้วยวิธีการตกผลึก 2 ขั้นตอน จากผลการทดลองพบว่าสภาวะที่เหมาะสมในการเกิดผลึกขั้นที่ 1 เพื่อแยกสารอื่นๆ ออกจาก ORF คือ อัตราส่วนของตัวทำละลาย (30% v/v เอทานอลในเอทิลอะซิเตท) ต่อ ORF เท่ากับ 7.5:1 (v/v) ที่อุณหภูมิ -15°C เป็นเวลา 7.5 hr ซึ่งทำให้แกมมา-โอไรซานอลคงเหลืออยู่ใน ORF เท่ากับ 80.75% สภาวะที่เหมาะสมของการตกผลึกแกมมา-โอไรซานอลในขั้นที่ 2 คือ การตกผลึกที่อุณหภูมิ 5°C เป็นเวลา 6 hr ทำให้ได้ปริมาณแกมมา-โอรีซานอลเท่ากับ 68.89% และมีความบริสุทธิ์เท่ากับ 87.10% นอกจากนี้ภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดชนิดฟิลด์อิมิชชั่น (FESEM) และโครมาโทกราฟีของเหลวสมรรถนะสูง (LC-MS) ได้แสดงให้เห็นว่าอนุภาคแกมมา-โอไรซานอลมีขนาดมากกว่า 150 micron และมีองค์ประกอบคล้ายกับสารแกมมา-โอไรซานอลมาตรฐานตามลำดับ

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชา วิศวกรรมเกษตร
ปีการศึกษา 2561

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RICE BRAN/ULTRASOUND/THERMAL TREATMENT/SOLVENT/GAMMA-ORYZANOL/RESPONSE SURFACE METHODOLOGY/CYRSTALLIZATION

This dissertation attempted to recover lipid and gamma-oryzanol from rice bran and determine their physiochemical properties. The purpose of the first study was to evaluate the effects of four extraction processes on rice bran oil extractability and quality, including: hexane extraction (HE), cold press extraction (CE), ultrasound pretreatment (2.25, 4.50, and 6.75 W/g; and 10, 25, and 40min) as well as thermal cooking (5, 15, 25, and 35min, 100°C) combined with cold press extraction (UCE and CCE). The oil recovery was correlated to ultrasound intensity and to thermal cooking ($p<0.05$). The elevated ultrasonic intensity (all conditions) and extended thermal cooking treatment (10, 15, and 25min) lead to an increase in oil recovery, but the quality of the oil extracted significantly reduced. Meanwhile, the short cooking time (5min) followed by cold press extraction not only improved the recovery but also enhanced the oil quality.

Experiment 2 investigated the effect of the chemical neutralization on the oil recovery, FFA level, and gamma-oryzanol, using response surface methodology. In this study, CRBO was treated with different NaOH concentrations (10-30°Be), process temperatures (60-90°C), and process times (5-15min). The ANOVA results were used to validate the predictive power of the model. The optimal chemical neutralization condition was 19.24°Be, 74.79°C, and 11.18min. Under the optimal condition, the

predicted oil recovery, FFA level, and gamma-oryzanol were 80.00%, 0.31%, and 1.00%. Meanwhile, the corresponding experimental results were 80.12%, 0.29%, and 1.02%, respectively. Thus, the predicted and experimental results were in agreeable and the high predictive ability of the response models.

The aims of the third and fourth studies were (1) to investigate the optimal extraction conditions and the kinetic extraction model of ORF from DRBS, and (2) to determine gamma-oryzanol crystallization conditions. Ultrasonic generator with the power in a range of 0.5-4.5 W/g was used for the extraction. The ORF extraction was conducted at different temperatures (35-55°C) and sonication times (4-26 min). The quadratic response model was generated and statistical analysis was performed to validate the model. The maximum gamma-oryzanol recovery achieved 98.03% at the optimal extraction condition (4.0 W/g, 50.0°C, and 21.5min); and the experimental results fit well with the predicted results. In addition, a kinetic model was also successfully generated to evaluate the effect of different parameters for gamma-oryzanol extraction. In the gamma-oryzanol crystallization, the experimental processes involved a two-step crystallization. The suitable condition for the first step crystallization included 7.5:1 of solvent to ORF ratio, 30% (v/v) of ethanol in ethyl acetate, -15°C, and 7.5 hours. At this condition, the recovery in LP achieved 80.75%. The second crystallization was successfully examined at the temperature of 5°C for 6 hours with the gamma-oryzanol recovery of 58.03% and purity of 87.10%. The FESEM and HPLC results showed that at this condition, almost 75% of gamma-oryzanol particle sizes were larger than 150μm and contained 4 major compounds.

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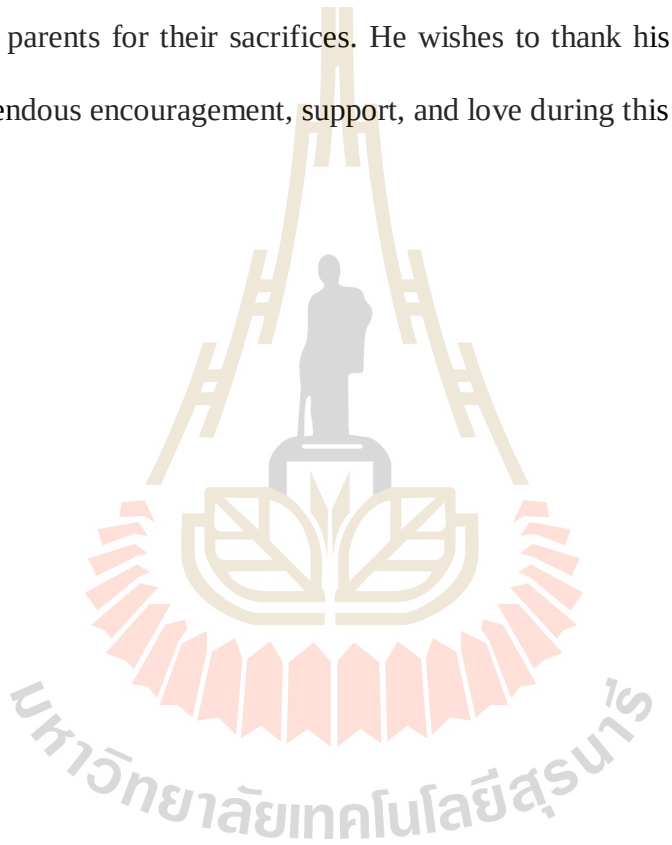

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
AOAC	=	Association of Official Analytical Chemists
AOCS	=	American Oil Chemists' Society
AV	=	Acid value
CRBO	=	Crude rice bran oil
DMI	=	Dry matter intake
DRBS	=	Dried rice bran soap stock
DSC	=	Differential scanning calorimetry
EAE	=	Enzymatic assisted extraction
EE	=	Either extract
FAME	=	Fatty acid methyl esters
FAO	=	Food and Agriculture Organization
FAs	=	Fatty acids
FCCD	=	A face-centered composite design
FESEM	=	Field emission Scanning electron microscope
FFAs	=	Free fatty acids
g	=	gram
GC-FID	=	Gas chromatography-flammable ion detector
GC-MS	=	Gas chromatography-mass spectrometry
HDL	=	High-density lipoprotein cholesterol
IPA	=	Isopropanol

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LIST OF ABBREVIATIONS (continued)

IV	=	Iodine value
L	=	Liters
LDL	=	Low-density lipoprotein cholesterol
LC-MS	=	Liquid chromatography–mass spectrometry (LC-MS)
LP	=	Liquid phase
MAE	=	Microwave assisted extraction
MASE	=	Microwave-assisted solvent extraction
MEE	=	Mixture of ethyl acetate and ethanol
mg	=	Milligram
mL	=	Milliliters
mmol	=	Mill mole
ORF	=	Oryzanol rich fraction
PC	=	Phosphatidyl-choline
PE	=	Phosphatidyl-ethanolamine
PEF	=	Pulsed electric field
PI	=	Phosphatidyl-inositol
PLE	=	Pressurized liquid extraction
PP	=	Phosphorus value
PSD	=	Particle size distribution
PV	=	Peroxide value
SE	=	Soxhlet extraction
SEM	=	Scanning electron microscope

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LIST OF ABBREVIATIONS (continued)

SFE	=	Supercritical fluid extraction
SP	=	Saponification value
SC-CO_2	=	CO_2 supercritical
TLC	=	Thin layer chromatography
UAE	=	Ultrasound assisted extraction
RBO	=	Rice bran oil
RSM	=	Response surface methodology

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CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Rice bran is known as a sub-product from rice milling, mainly used as an animal feed. Rice bran is a good source and inexpensive, and high essential fatty acids (FAs) (Rukmini and Raghuram ,1991). The major fatty acid in oil rice bran included oleic (40-46%), linoleic (30-35%) and palmitic (19-22%), respectively (Luh, 1991; and Prasad, 2006). They play an important role in our daily diet which give us energy and help in the absorption of fat-soluble vitamins like A, D, E and K. It is also required for maintaining a healthy balance between high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol, and decreasing platelet aggregation (Sharma and Rukmini, 1986; Ghosh, 2007).

In addition, rice bran oil is evaluated as one of the important edible oil contained high antioxidant compounds such as gamma-oryzanol, tocopherols, and tocotrienols. In these components, gamma-oryzanol attracts a lot of researches due to the strongest antioxidant and exhibited the highest concentration in rice bran (Joshi et al., 2016; Thanonkaew et al., 2012). Gamma-oryzanol is associated with a decrease in plasma cholesterol, serum cholesterol, and cholesterol absorption and platelet aggregation. Gamma-oryzanol has also been used to treat post-hyperlipidemia syndrome, disorders of menopause and to increase the muscle mass. However, extraction and refining of crude rice bran oil have some special features compared with other vegetable oils (Ahmad Nayik et al., 2015; De and Patel, 2010), which

affected to the oil quality and quantity, especially, the presence of fatty acid profile and gamma-oryzanol in rice bran.

In extraction, extrusion/pressing and solvent extraction (SE) with pure solvent are two popular conventional production methods of rice bran oil (Carrín and Crapiste, 2008; Sayasoonthorn et al., 2012). The extrusion/pressing techniques suffer from low oil extractability although the extracted oil products are of high quality (i.e., high oxidative stability, high gamma-oryzanol, and high unsaturated fatty acids). Meanwhile, the SE method is time-consuming and requires relatively large quantities of environmentally-hostile chemical solvents and low quality, despite the comparatively higher oil extractability. Today, the advanced extraction methods (i.e., the supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), pulsed electric field (PEF), and enzymatic-assisted extraction (EAE)) have been considered and possessed several advantages over the conventional extraction methods, including the short extraction time, low solvent volumes, and high oil extractability (Bjorklund 2000). Nevertheless, these methods still have drawbacks that limit their commercialization potential include high cost operation (MAE, PEF) (Bjorklund, 2000; Zhang et al., 2008), restricted for food process due to high pressure stresses and high thermal (PEF) (Kumar et al., 2015), unsuitable for thermally labile compounds (MAE) (Bjorklund, 2000; Zhang et al., 2011) and lack of knowledge to implement in industry (EAE) (Bjorklund, 2000; Zhang et al., 2011). To date there exists no research on the effect of thermal cooking or ultrasonic pre-treatment combined with cold press extraction on the extractability and quality of rice bran oil.

Rice bran oil refining contained some special features due to crude rice bran oil contents huge phospholipids, soap, and high free fatty acids (FFAs). Refining rice

bran oil can be carried out physically or chemically but the latter being the most common. Physical refining process requires a good-quality raw material used (De and Bhattacharyya, 1998) but commercial rice bran contains high free fatty acid (FFA). Thus, physical refining is unsuitable for rice bran oil refining. The chemical refining comprises the degumming, neutralization, and deodorization (Gunstone, 2002). Hoed et al. (2006) reported that chemical neutralization is more effective in reducing the FFA compared to the physical neutralization. However, this process may affect oil recovery and phytochemicals such as tocopherols, tocotrienols, and gamma-oryzanol. In the chemical refining process, soap and waxes can absorb some neutralized lipid (5-30%), tocotrienols and tocopherols (10-45%) and gamma-oryzanol (93.0 to 94.6%) (Akiya, 1962; Das et al., 1998; Patel and Naik, 2004).

Rice bran soapstock, a by-product from chemical neutralization, is an abundant material, which contains high gamma-oryzanol. From the beneficial effects of gamma-oryzanol on human health, the global interest has been increased in isolation gamma-oryzanol from rice bran soapstock. Until now, there are no simple extraction and isolation methods enabling a fast with the high yield and high pure gamma-oryzanol. Those studies need a lot of steps and contain several tedious steps.

Therefore, this study investigates the effects of ultrasonic and thermal cooking pre-treatments combined with cold press extraction on the recovery and quality (oxidative stability, gamma-oryzanol, and unsaturated fatty acids) of rice bran oil. The optimization conditions of chemical neutralization were conducted to increase oil recovery and preserve high refined rice bran oil quality. Additionally, isolation gamma-oryzanol from rice bran soapstock by solvent mixtures under low temperature was also investigated to replace the advanced technique method.

1.2 Research Objectives

1.2.1 To investigate the effects of different extraction methods to recovery and the quality of rice bran oil (i.e., gamma-oryzanol, FFA, PV, IV, and UFAs) (Chapter III).

1.2.2 To determine the optimal conditions of chemical neutralization of rice bran oil by using the response surface methodology (RSM) (Chapter IV).

1.2.3 To evaluate the effects of mixture solvent (ethanol and ethyl acetate) for extraction gamma-oryzanol rich fraction (ORF) from rice bran soapstock. Finding the optimal extraction conditions and kinetic extraction for extraction ORF from rice bran soapstock under ultrasound assisted solvent extraction (Chapter V).

1.2.4 To study the effects of the crystallization conditions (i.e., low temperature, the ratio of solvent and ORF, and crystallization time) on the recovery and purity of gamma-oryzanol (Chapter VI).

1.3 Research hypotheses

1.3.1 In the extraction process, ultrasonic and thermal cooking pretreatment has a strong impact on rice bran tissue and soapstock. Rice bran cell and soapstock fragmentation dramatically increase surface areas, thus the mass transfer rate of target compounds increased.

1.3.2 The response surface methodology was a useful statistical method to evaluate the effect of variable neutralization parameters on the recovery of RBO and the quality of oil.

1.3.3 Gamma-oryzanol absorbed into soapstock is easy to dissolve in ethyl acetate and ethanol. Under ultrasound assisted solvent extraction, it may have higher

efficiency and greater advantage than the traditional solvent extraction in gamma-oryzanol.

1.3.4 During gamma-oryzanol crystallization process, the crystallization parameters such as low temperature, the solvent to samples ratio, and binary solvent mixture are considered to crystal growth and aggregation of crystals.

1.4 Scope and limitation of this study

1.4.1 Rice bran of jasmine variety from *Korat Rice Mill* factory in Thailand's northeastern province of *Nakhon Ratchasima* were used in this study. Experiments were conducted under laboratory.

1.4.2 The cold press machine was used in the lab with low productivity. The isolation of gamma-oryzanol from soapstock needed more processing stages and toxic chemicals.

1.5 Expected results

1.5.1 Ultrasonic and thermal pretreatment combined cold press extraction could increase oil extractability and preserve the quality of crude rice bran oil.

1.5.2 It is estimated the effects of neutralization parameters (i.e., NaOH solution concentration, process temperature, and process time) to the FFA content and the oil quality presenting in response surface methodology (RSM) model.

1.5.3 Ultrasound-assisted solvent extraction and kinetic model extraction could be used to describe the effect of process variables on the ORF extraction from rice bran soapstock.

1.5.4 The effects of crystallization conditions (i.e., temperature, storage time, and solvent to solid ratio) to the gamma-oryzanol recovery and purity successfully

experimented. By applying the knowledge from present study, the producers can guarantee the quality gamma-oryzanol powder and the value-added food products.

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CHAPTER II

LITERATURE REVIEW

2.1 Rice and rice bran production

Rice is the seed of the grass species Oryza sativa (Asian rice) or Oryza glaberrima (African rice) (Casmir, 2003). Rice is a major cereal crop cultivated globally, and is one of the staple foods for human consumption, especially in East and South East Asia, where it supports nearly half of the world population. Rice is one of five grains that has the highest production in the whole world. The world production of the rice paddy in 2015-2016 was 631 million metric tons, and 480.7 million tons of milled rice (FAOSTAT report, 2016). China, India, Indonesia, Bangladesh, Vietnam, and Thailand are the most representative country, producing approximately 30%, 22%, 8%, 7%, 6%, 4% of the world rice crop in 2016 (Figure 2.1a).

Rice bran, a byproduct from rice milling, constitutes from 8 to 9%wt of rice bran and rice germ of 1-2%wt (Singh and Sogi, 2016), with a potential global production of 48 million tons per year (FAO, 2016: http://www.fao.org). In Thailand, more than 640,000 ton of defatted rice bran is remained as low-value agro waste (Donporn et al., 2019). Each year, more than 60% of rice bran is sold cheaply as an animal feed with a low price, and 40% for human food process. Meanwhile, rice bran constitutes about 12-23% of rice bran oil and large amounts of varying nutraceuticals (Saunders, 1985; Nayyar, 1985).

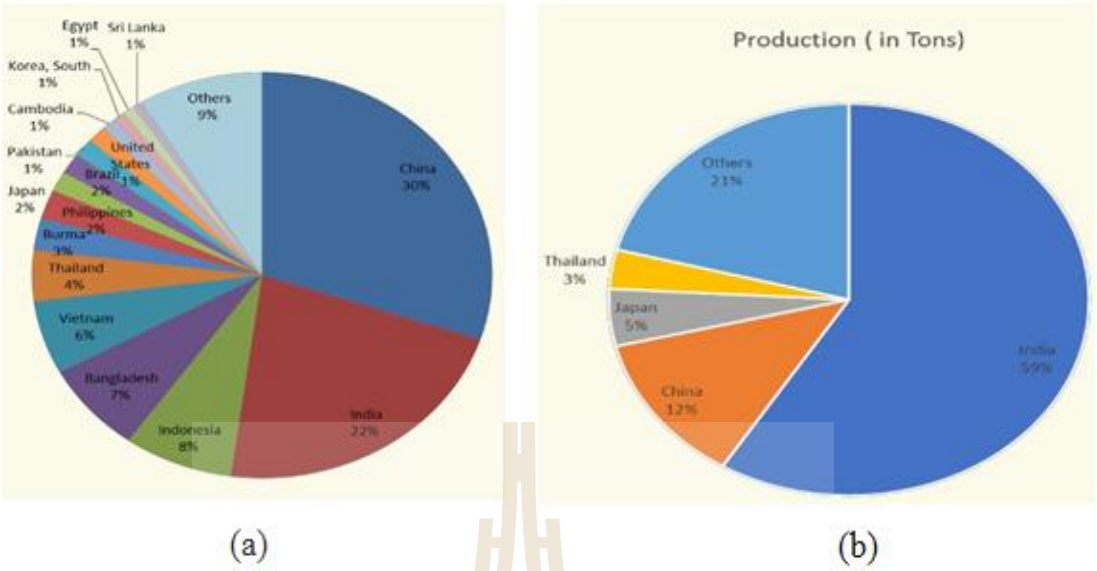

Figure 2.1 Comparison of annual world production of rice paddy (a) and rice bran oil (b) from different countries in 2016 (FAOSTAT: http://faostat.fao.org/).

Currently, there are some markets for rice bran as a value-added health food product such as rice bran oil product, rice bran oil shortening; gamma-oryzanol rich rice bran oil capsule tablet or gamma-oryzanol powder. Among these products, rice bran oil is the most common. India which is the country holds the highest rice bran oil productivity in the world, nearly 59%wt (820,000 metric tons-MT). The next India are China and Japan accounted for 12%wt (90,000 MT) and 5%wt (37,500 MT), respectively (Figure 2.1b). Abundant rice bran availability results in a stable price of rice bran oil market. In addition, increasing consumer health consciousness from edible oils to reduce cholesterol levels supports rice bran oil market growth up. Rice bran oil market size maintains the average annual growth rate of 2.18% from 1,040 million dollars in 2013 to 1,130 million dollars in 2017 with the 1.4 million metric tons of the total annual world production (http://faostat.fao.org/).

Nowadays, ongoing technological innovations along with increasing research and development (R&D) spending is expected to provide significant opportunities for gamma oryzanol production. According to Grand View Research, Inc., the global gamma-oryzanol market demand reached 2.06 billion dollars with 11,520 tons in 2014 (https://www.grandviewresearch.com) and is expected to reach 18,597.8 tons by 2022. The product demands are in sports supplements, pharmaceuticals, cosmetics, and animal feed.

2.2 Rice kernel and rice bran structure

Rice kernel or rice grain (Figure 2.2 and Figure 2.3) is composited of the rice hull, pericarp, aleurone, germ, and endosperms (Saunders, 1985; Nayyar, 1985). The hull, which surrounds each whole grain of rice, is generated of cellulose and fibrous tissues. The hull is to protect the grain during maturation against insect and microorganism. In rice milling, the hull is removed by friction as the paddy grains pass between two abrasive surfaces that move at different speeds. Underneath the hulls are the aleurone layer, germ, and endosperms, which contain valuable nutritional constituents (i.e., lipids, protein, and some active compounds).

Rice bran included aleurone layer, germ, and some endosperms is the most abundant but minor product which is produced in the milling rice. Typically, rice bran contains 15-20% lipid, 12-16% protein, and 34-52% carbohydrate, respectively (Engelmann et al., 2017; Saunders, 1985; Luh, 1991). These components depend on the variety, the degree of mature, milling, and storage time (Roy et al., 2011; Luh, 1991). Especially, the high lipid level in raw rice bran makes it susceptible to oxidation, enhancing to rancidity and an overall deterioration in quality of the rice.

The lipid founds in rice bran are caused by two types of deterioration: hydrolytic and oxidative rancidity (Engelmann et al., 2017; Saunders, 1985).

Lipids in rice bran are divided into two groups involving starch-lipid and non-starch lipid. Starch-lipid presented in rice bran with a small proportion, whereas, non-starch lipid predominantly found in organelles called spherosomes, and more widely as oil bodies (Figure 2.2) (Saunders, 1985). Non-starch lipids are primarily located in the lipid bodies of rice bran (aleurone layer) and germ (embryo) fractions. The major compositions of non-starch lipid in rice bran oil are neutral lipids (triacylglycerols), which comprise 85-90% of body lipids. Triacylglycerols are separated as lipid droplets because of their hydrophobicity and insolubility in water. These triacylglycerols are stored in the adipose tissue as lipid droplets and serve as an energy reserve for the body (Luh et al., 1980; Nantaprapa, 2010).

Oil body has a complex structure and composed of a neutral lipid core surrounded by a monolayer of phospholipids (PLs), and partially embedded protein. The major protein surrounding the lipid body is alkaline proteins called oleosins (Tzen et al., 1993). Oleosins are proteins with low-molecular mass (15-26 kDa), its structure depending on isoforms and plant species (Mohdaly et al., 2017; Qu et al., 1986). Oleosins play important roles in biological functions which relate to oil body synthesis and degradation, act as receptors on the synthesized lipase during germination as well (Huang, 1996). Oleosins comprise three structural domains including: the first term is

N-terminal domain that consists of 40-60 amino acids; next term is a central hydrophobic domain flanked by amphipathic N- and C-terminal regions, contents 68-74 amino acids, and the finally, one serine residue; and an amphipathic C-terminal domain (33-40 amino acids) (Keddie et al., 1993; Tobergte and Curtis, 2013).

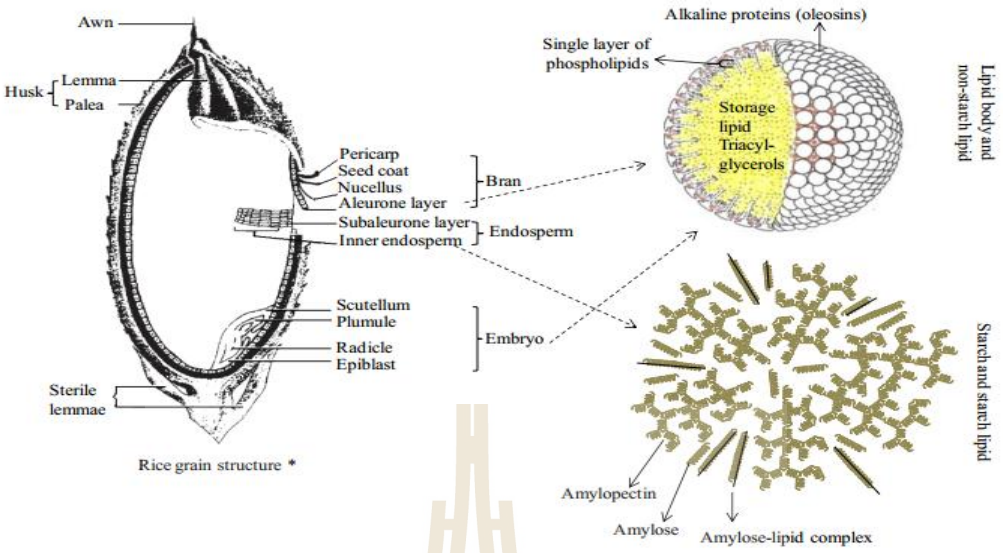

Figure 2.2 Longitudinal section of the rice grain and distribution and illustration of starch and non-starch lipids (Bao, 2012).

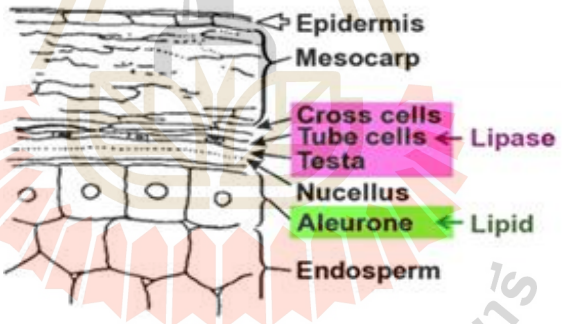

Figure 2.3 Microstructure of the outer layers of rice kernel showing the locations of lipids (cross-testa layer) and lipid aleuron layer (Saunders, 1985).

The single layer of phospholipids (PLs) was esterified with fatty acid via methyl group linkages (Ye et al., 2016; Liu et al., 2013). Phospholipids (PLs) also consist of covalently bound phosphate and lipid (storage in the core) which are a major class of lipid in rice. The major phospholipids in rice bran are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and phosphatidyl inositol (PI) (Liu et al.,

2013). Besides, the starch lipids are lipids on the surface of starch granules in endosperm and are composed mainly of monoacyl lipids. According to Morrison (1988) the starch lipids represent a relatively small proportion of the total lipids but they are involved in starch biosynthesis, starch degradation and are likely to affect starch functionality.

During rice grain development, starch-lipid and non-starch lipid have been changed (Choudhury and Juliano, 1980; Godber and Juliano, 2004). Non-starch lipids can be converted to starch lipids through amyloplast membrane, leading to non-starch lipid reduced in rice bran (Morrison, 1988).

2.3 Rice bran oil composition

The major component of crude rice bran oil is a neutral lipid (Table 2.1). In comparison with other vegetable oil, crude rice bran oil tends to contain high phospholipids, unsaponifiable constituents, waxes, and polar lipids (including glycolipid). Crude rice bran oil differs from other vegetable oils because of its high levels of non-triglyceride components (mono- and di-glycerides) and free fatty acid (FFA). High free fatty acid, mono-glycerides, and di-glycerides are associated with enzymatic hydrolysis (Nantiyakul, 2012). In addition, crude rice bran oil has been known as high phospholipids (4-5%wt) (Nantiyakul, 2012). The high phospholipids content in crude rice bran oil is a major problem during processing of the oil. However, phospholipids can be removed during enzymatic degumming or acid degumming processes.

The relative amount of unsaponifiable matter in crude rice bran oil is more than 5%wt, including tocopherols (0.04%), tocotrienols (0.07%), gamma-oryzanol (1.0-2.0%), and other components (2.5-3%). The concentration of unsaponifiable matter

depends on various factors such as method extraction (Kumar et al., 2009), rice bran stabilization (Nantiyakul et al., 2012), and rice growing environment. Gamma-oryzanol was considered to be an important fraction, along with tocopherols and tocotrienols in the hypocholesterolemic property of crude rice bran oil. High levels of gamma-oryzanol, tocopherols, and tocotrienols are important in protection against oxidation of rice bran oil. This component makes rice bran specialty oil for a niche market. However, the efficiency and mechanism of antioxidant of the gamma-oryzanol, tocopherols, and tocotrienols on rice bran oil have not been well reported. In addition, crude rice bran also is rich in a variety of minerals (phosphorus, calcium, magnesium, copper, and silicon) and vitamin B (thiamin and niacin) (Huang, 1996; Nantiyakul, 2012; Kumar, Tiku, and Prakash, 2009). Thiamin and niacin help the body regulates blood sugar and metabolize carbohydrates, whereas the presence of high phosphorus, calcium, magnesium, copper, and silicon in crude rice bran affected the stabilization of rice bran oil.

Table 2.2 compares fatty acid composition of edible rice bran oil to that of soybean, cottonseed, and peanut oils. It can be seen that unsaturated fatty acids represent more than 80% of the total fatty acid composition. The fatty acid profile of edible rice bran oil revealed that the lipid in rice bran contains oleic acid (42%), linoleic acid (39%), and linolenic acid (1.1%) as unsaturated fatty acids, and palmitic (15%) and stearic acid (1.5%) as saturated fatty acids (Caskill et al., 1999; Nantiyakul, 2012, CODEX std., 1999). The fatty acid composition of rice bran oil is similar to that of peanut oil but slightly higher in saturation level than that of soybean oil. The low linolenic acid content of refined rice bran oil showed that rice bran oil more stable to

Table 2.1 Composition of crude rice bran oil (Nantiyakul, 2012).

Components	% wt of total oil
1. Saponifiable lipids	95
Neutral lipids	85
Triglycerides	71
Diglycerides	3
Monoglycerides	5
Free fatty acids	2
Waxes	3
Phospholipids	4
Glycolipids	6
2. Unsaponifiable lipids	5.0
Phytosterols	1.8
Campesterol	0.51
Stigmasterol	0.27
β-Sitosterol	0.88
4-Methyl sterols	0.40
Triterpene alcohols	1.2
24-Methylene cycloartanol	0.49
Cycloartenol	0.48
Cycloartanol	0.11
Less polar compounds	0.80
Squalene	0.12
Tocopherols	0.04
Tocotrienols	0.07

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oxidation than soybean oil. Furthermore, gamma-oryzanol, tocopherols and tocotrienols in edible rice bran oil are higher than that of peanut oil, cotton, and soybean. The high gamma-oryzanol, tocopherols, and tocotrienols content in rice bran oil found more stable oil under deep-fat frying. As Barrera-Arellano et al., 1999 who documented that gamma-oryzanol, tocopherols, and tocotrienols had a protective action at high temperature.

Table 2.2 Composition of selected vegetable oils (Caskill et al., 1999; Nantiyakul, 2012, CODEX std., 1999).

Component	**%**			
	Rice Bran	**Soybean**	**Cottonseed**	**Peanut**
Myristic (14:0)	0.2	0.2	0.80	-
Palmitic (16:0)	15	11	27	8.1
Stearic (18:0)	1.9	3.9	2.0	1.5
Oleic (18:1)	42	23	18	50
Linoleic (18:2)	39	51	50	35
Linolenic (18:3)	1.1	6.8	-	-
Arachidic (20:0)	0.5	0.20	0.30	1.1
Behenic (22:0)	0.20	0.10	-	2.1
Tocopherols (ppm)	49-593	9-535	136-674	-
Tocotrienols (ppm)	142-790	ND-103	ND	ND
Gamma-oryzanol (%)	1.5-1.8	ND	ND	ND

* ND- Non-detectable

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2.4 Rice bran oil processing

2.4.1 Rice bran oil extraction

Today, industrial extraction of rice bran oil is a complex and multistep process and has some special features compared with other vegetable oils. The rice bran oil process can be separated into two parts including extraction crude oil and refining (Figure 2.4). Rice bran is first stabilized to inactivate any lipolytic enzymes and to facilitate pellet formation. The formation pellet increases solvent penetration rate or increases the surface area contact between the screw pressing and material in a mechanical oil expeller press leading to increase oil yield. According to Noppawat et al. (2015), mechanical pressed rice bran oil extraction resulted below 50% of oil recovery compared to soxhlet extraction. Meanwhile, hexane is the industrial favorite for rice bran because of its efficiency (98-99% yield under 4-6 hours extraction) and availability (Capellini et al., 2017; Proctor and Bowen, 1996).

2.4.2 Degumming

Phospholipids in crude rice bran divided into two groups included non-hydratable and hydratable gum. It can be removed by adding water or acid phosphoric into oil, called water or acid degumming. Water degumming is unable to remove non-hydratable in crude rice bran oil and considerable amounts of neutral oil are lost. By contrast, phosphoric acid degumming has a significant efficiency in phosphorus removal but color reaction accelerated, leading to dark-colored refined oil (Rajam et al., 2005). In addition, approximately 15% of tocopherols and tocotrienols were removed in the acid degumming process (Ronal and Junsoo Lee., 2004). Thus, the refined RBO or in preserving its nutritional benefits through an economically viable process. Another way to remove gum now is by using surface active agents such as

sodium oleate, akyl aryl sulfonate (Nash et al., 1984), which had a good efficiency in reducing phosphorus content in comparison with a conventional method. However, degummed oil still contains high phosphorus level and its method damaged oil especially unsaturated fatty acid.

An advanced method for the removal of the hydratable and non-hydratable phospholipids in crude rice bran oil included enzymatic degumming and super-degumming was mentioned. In 2007, Gunstone et al. proposed a method of treating gums by using enzymatic degumming. Phospholipase was used to hydrolyze the ester bond of the phospholipids at the oil-water interface thereby converting non-hydratable gums to fully hydratable gums and FFA. Enzymatic degumming using phospholipase is reported to be successful in reducing the phosphorus content to the level of 5 ppm, but its economic viability has yet to be established. Nowadays, a super-degumming method was developed by Unilever, which converted non-hydratable gum into hydratable form by citric acid for 20 min process. The hydratable gums formed are allowed to crystallize calcium and magnesium salts, waxes and glycerol. The phosphorus content in the degummed oil was less than 15 ppm, and these bio-active compounds (i.e., tocopherols, tocotrienols, and gamma-oryzanol) are stable.

2.4.3 Neutralization

The neutralization stage is aimed at removing the free fatty acids present in the oil and can be performed via chemical or physical neutralization. In the physical neutralization, removal of free fatty acids is carried out using a distillation system, whereas in the chemical neutralization this removal is done by saponification reactions due to the addition of NaOH solution or KOH solution. In physical neutralization, the removal of FFA (low volatility fatty acid) requires high temperature

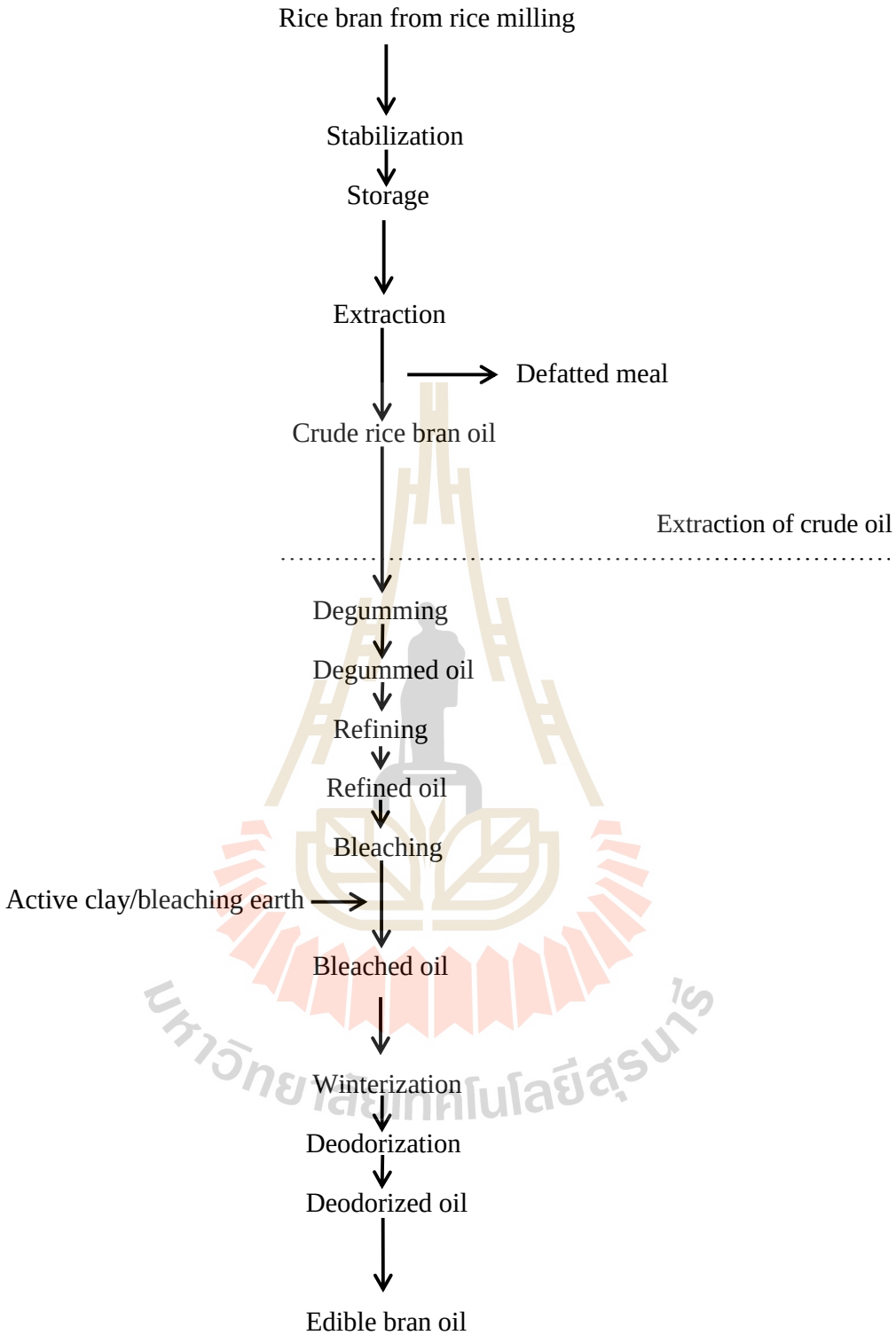

Figure 2.4 Rice bran oil production: crude oil extraction and refining (Nantiyakul, 2012).

(maximum temperature of 240-250°C), resulting of about 0.1-0.2% FFA content in neutralized rice bran oil. However, the phosphatides contained higher 15 ppm (IulianaVintilă, 2009; Antoniassi et al, 1998) and with the presence of iron is the cause of heat darkening during distillation. In physical neutralization, this process has the disadvantage of a high cost of implementation at the industrial level and low oxidative quality of the distillate. Meanwhile, in chemical neutralization, NaOH solution is used in this procedure to remove FFA and other impurities (i.e., phospholipids, proteinaceous, and mucilaginous substances).

In chemical neutralization, FFAs are easily removed during the de-acidification step. Hoed et al. (2006) and Engelmann et al. (2017) reported that chemical neutralization is more effective in reducing the FFA compared to the physical neutralization, leading to improving the quality of rice bran oil. However, chemical neutralization has a major drawback in that it always incurs an oil loss, and nutritional components are destroyed or removed (Ghosh, 2007). Chemical neutralization removed 93.0-94.6% gamma-oryzanol, whereas 1.1-1.74% for physical refined (Akiya, 1962; Das et al., 1998; Patel and Naik, 2004). Tocopherols and tocotrienols reduced 20-45% of total tocols in the chemical neutralization (Nantiyakul, 2012). In chemical neutralization, a strong alkalization breaks the ester linkages of sterol or triterpenol and ferulic (Figure 2.5).

2.4.4 Bleaching and deodorization

Bleaching of rice bran oil is used to remove the pigment chlorophyll, red pigment content, and metallic salts of fatty acid. Generally, bleaching of rice bran oil is done after neutralization by using adsorbent (bleaching earth). In the bleaching process, gum, soap and some oxidations are also absorbed along with pigments

(Bahmaei et al., 2005). The bleaching was conducted under high vacuum at a high temperature of 110-120°C, help to reduce the amounts of oxidation products. At this temperature decomposes hydro-peroxides and the ion exchange properties of bleaching earths promote metal removal. Deodorization is to remove the volatile compounds mainly aldehydes and ketones. A steam distillation process carried out at a temperature between 200 and 220°C. This step, unfortunately, removes to 60-67% of tocopherols and tocotrienols (Ko et al., 2008).

2.5 Rice bran soapstock, gamma-oryzanol, extraction and isolation gamma-oryzanol from rice bran soapstock

2.5.1 Rice bran soapstock compositions

Rice bran soapstock is a byproduct from chemical neutralization process of rice bran oil production. It is obtained after de-acidification by alkali treatment of crude rice bran oil. Soapstock consists mainly of water (55-70%wt) and emulsion of lipids containing water and the dried matter (Gopala Krishna et al., 2001). Lipid (2-15%) present in soapstock in form of mono-glycerides, diglycerides, and triglycerides (Narayan et al., 2006). Especially, it was found that triglycerides are mostly found in soapstock. The amounts of triglycerides in soapstock depend on process condition in the alkali neutralization step. Triglycerides are soluble in low polar or non-polar organic solvents (i.e., hexane, isopropanol, chloroform, and ethyl ether), whereas mono-glycerides and di-glycerides have a lower soluble in these solvents compared with triglycerides. In addition to lipid loss during neutralization process, a significant amount of micronutrients especially, gamma-oryzanol is removed along with soapstock as its sodium salts (15-55g/kg) (Kumar, Tiku, and Prakash, 2009).

Furthermore, rice bran soapstock contents both hydratable and non-hydratable phospholipid (gums), and their proportion depending on the efficiency of the degumming step. Waxes and resinous present in rice bran soapstock, consist of two parts, which are esters of saturated fatty acid and saturated fatty alcohol. In the neutralization step, waxes and resinous precipitated and formed a stable emulsion in soapstock. In solvent extraction of gamma-oryzanol from soapstock, gums, waxes and resinous materials are stable and form micro-emulsion and thereby decrease the rate of phase separation. These impurities also interfere with gamma-oryzanol by disrupting crystal growth. Thus, the effect of degumming of rice bran oil is desirable prior to the alkaline refining step.

2.5.2 Gamma-oryzanol and biological effects of gamma-oryzanol

Gamma-oryzanol is one of the important phytochemicals with high antioxidant activities as well as other beneficial health properties. Gamma-oryzanol was the first extracted from rice bran oil to be a single component (Kaneko et al., 1954). Later it was recognized as a mixture of ferulic acid esterified with phytosterols or triterpene alcohols, comprises 2% of the crude rice bran oil (Figure 2.5). Gamma-oryzanol powder is a white or slightly yellow crystalline powder, which is stable at room temperature. Gamma-oryzanol is soluble in most polar solvents such as acetone, hexane, chloroform, ethyl acetate, and methylene chloride. It has a melting point in a range of 110 to 130ºC (Zullaikah, Melwita, and Ju., 2009), and shows the maximum absorbance of wavelength in 231, 290 and 320nm (Delhi, 2014; Zullaikah, Melwita, and Ju., 2009).

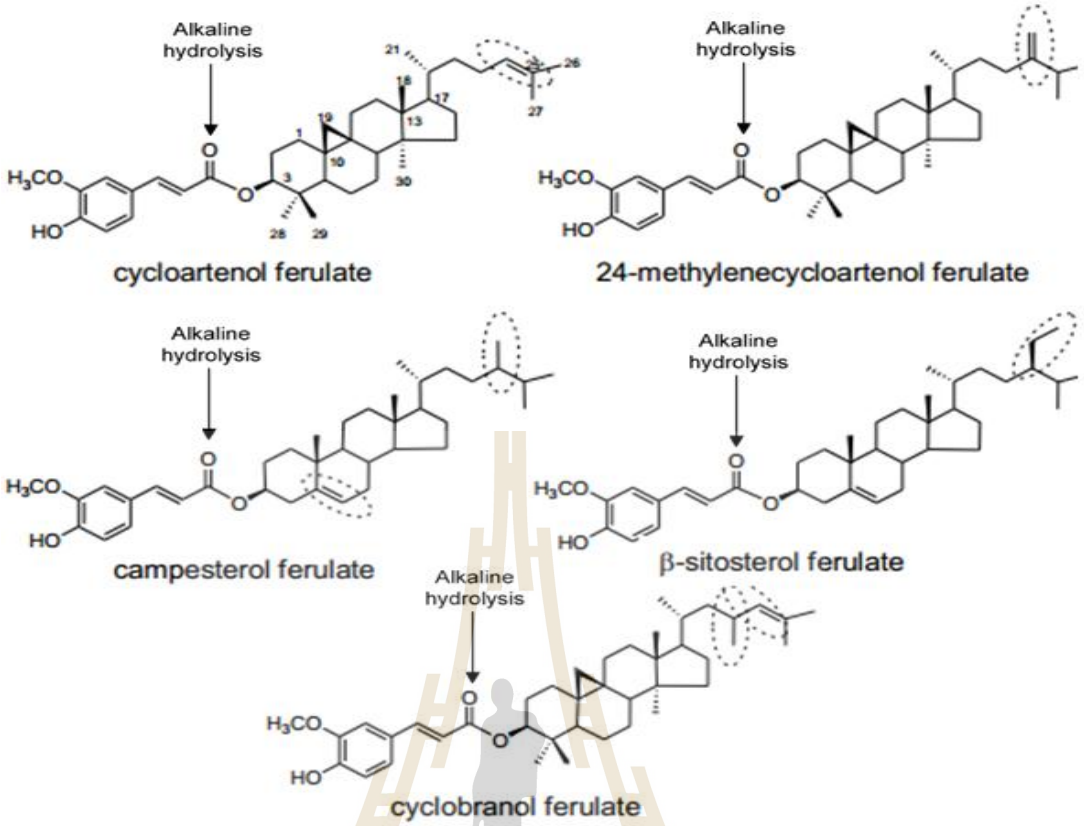

Figure 2.5 Molecular structures of several oryzanols present in rice bran oil. Each oryzanol is an ester of ferulic acid (4-hydroxy-3-methoxy cinnamic acid) with a sterol or triterpenol (Bijay Krishna De and Patel, 2011; Nantiyakul, 2012).

Until now, 10 components of purified gamma-oryzanol were identified by RP-HPLC with the aid of GC-MS (Nantiyakul, 2012; Xu and Godber, 1999). The main components of gamma-oryzanol are cycloartenyl ferulate, campesteryl ferulate, and 24-methylenecycloartanyl ferulate that have an important antioxidant activity and help to reduce plasma cholesterol and low-density lipoprotein (LDL). They occupied more than 70% of the total weight of gamma-oryzanol (Norton, 1995).

In 1962, gamma-oryzanol was initially introduced in Japan by Oguni et al. for the treatment of vegetative neurosis. Gamma-oryzanol compounds were marketed for

symptoms treatment of gastro-intestinal disorder and sequelae induced by head injury (Eslami et al., 2014; Tarnagawa et al**.,** 1992). It exhibited several other pharmacological effects including promotion of skin capillary action (Kamimura et al, 1964) and hyperlipidemia (Tamagawa et al**.,** 1992; Mäkynen et al., 2012). In 1988, Seethararnaiah and Chandrasekhara studied the effects of gamma-oryzanol in hypocholesterolemic of rats and found that levels of total free and esterified cholesterol were significantly reduced in the groups fed gamma-oryzanol as compared to the control group. Similar results were also reported by Lee et al. (1999) in inhibited the development of foci of eosinophil-related necrosis. To date, gamma-oryzanol from rice bran has been popularly marketed as cosmetic, anti-aging supplement, as well as a sport supplement.

2.5.3 Extraction, purification, and quantification gamma-oryzanol

2.5.3.1 Extraction gamma-oryzanol from rice bran soapstock

Gamma-oryzanol is readily dissolved in non-polar solvent typically in hexane, chloroform, and heptane. Besides, all components of gamma-oryzanol contain alcohol group in their ferulate portion, which may also soluble in low polar solvents such as isopropanol, methanol, ethyl acetate, and acetone. The solvent strength of extraction may affect the extractability of gamma-oryzanol from rice bran soapstock. Hexane, petroleum ether, methanol, and dichloromethane are recommended for gamma-oryzanol extraction from rice bran but not for soapstock due to emulsion formation and dissolution of the soapstock (Seetharamaiah and Prabhakar, 1986; Narayan et al., 2006). In 2009, Kumar et al. investigated the extraction of gamma-oryzanol from dried rice bran soapstock using different solvents assisted byconventional extraction (i.e., ethyl acetate, ethyl methyl ketone, acetone, and

isopropanol). The authors recognized ethyl acetate was most suited for gamma-oryzanol extraction with 97-99% yield under 6 hours extraction. In 2010, Kaewboonnum et al. also successfully extracted gamma-oryzanol from rice bran soapstock with ethyl acetate in soxhlet apparatus. The gamma-oryzanol extractability achieved about 99% for 4 hours extraction.

Supercritical fluid extraction (SFE) of gamma-oryzanol has received attention as an alternative to organic solvent extraction. The work on SFE of rice bran soapstock has been done mainly with respect to extractability and to scale-up operations, of various unsaponifiable components in rice bran soapstock such as FFA, triglycerol, and sterols. In 2010, Jesus et al. investigated the recovery of gamma-oryzanol from rice bran soapstock, using SFE. The highest recovery rate and yield achieved 31.3% and 3.2%, respectively at a pressure of 30MPa and temperature of 303K. The limitation of SFE is that the fluctuations in flow rate and pressure cause variations in results, and equipment and installation are expensive.

2.5.3.2 Purification gamma-oryzanol

After solvent extraction, gamma-oryzanol rich fraction contains a large number of various lipids and impurities, besides gamma-oryzanol. In order to purify gamma-oryzanol from interfering lipids in rice bran, a low-pressure distillation is applied. In 1954, Tsuchiya and Kaneko detected and crystalized gamma-oryzanol by using sequence procedure, removed FFA, esterified the fats and removed the fatty acid ester with low-pressure distillation. Kuroda et al. (1977) used a silica gel column to obtain the gamma-oryzanol fraction from rice bran oil, and hexane was used to flush the low-pressure column. Because of low resolution in low pressure silica gel column, gamma-oryzanol only partially purified and an individual component cannot

be isolated. Similar results reported by Van Boven et al. (1997), individual components of gamma-oryzanol could not be isolated because of low resolution in low-pressure silica gel column.

In 2006, Narayanet et al isolated gamma-oryzanol from rice bran soapstock, which was subjected to multiphase fractional crystallization steps. The purity of the gamma-oryzanol obtained at the end of this crystallization process was higher than 98% with a low yield of 1.9%. Gamma-oryzanol recovered from this process has high purity but unsatisfied yield. To improve gamma-oryzanol recovery, Zullaikah et al., (2010) extracted gamma-oryzanol with ethyl acetate and then crystallized with 2 steps. In the first step of crystallization, the temperature reached -22^{O}C and the second crystallization step of -60^{O}C. At the end of the second step, gamma-oryzanol crystals obtained with purity and recovery of 93-95%. Nevertheless, this method performed at a very low temperature, it was difficult to apply in a simple cooling device.

In 2016, Joshi et al. conducted extraction and isolation gamma-oryzanol with a solvent mixture of methanol and acetone, and a cool process was used to form a crystal of gamma-oryzanol. Under the operation conditions, gamma-oryzanol isolated with a low purity and a high recovery were 33.5% and 93-95%, respectively. At the same time, Shimizu et al. mentioned that dissolving soap with ethanol, methanol or isopropanol then precipitated them in the mass extract with sodium dihydrophosphate or sodium EDTA. This method had efficiency in extraction gamma-oryzanol from soap but the low purity (Figure 2.6). Gamma-oryzanol recovered from this has low purity and unsatisfied in a commercial.

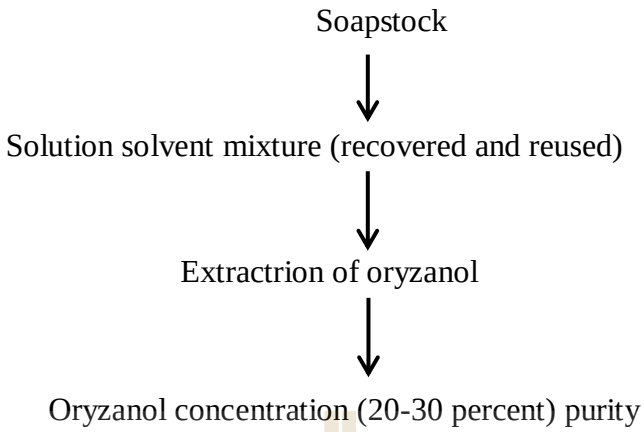

Figure 2.6 Procedure for gamma-oryzanol isolate from dried soap rice bran (Shimizu et al. 2016).

Reverse HPLC or thin layer chromatography (TLC) has been proposed in the purification of gamma-oryzanol (Narayan et al., 2004; Zullaikah et al., 2009). Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been populated in the study of gamma-oryzanol. The gamma-oryzanol purity (95-99%) is higher than low-pressure chromatography (90-95%). Although chromatographic method (TLC and HPLC) is an effective method to achieve high purity and recovery of gamma-oryzanol, it is difficult to recover solvent and scale up. The preparative scale HPLC and TLC are quite similar to analytical HPLC and TLC, except the size of the column and capability of mobile phase pump. Therefore, the preparative scale HPLC and TLC are considered to be a very expensive technique, difficult to be applicable on large scale, and limited to very high added value products (Ganetsos and Barker, 1993). Otherwise, preparative HPLC results in a high cost per product unit.

2.5.3.3 Identification and quantification

The spectrophotometric technique is a simple, practical and inexpensive technique in determination gamma-oryzanol (Joshi et al., 2016; Bucci et al., 2003). However, the results are low accurate because the matrix (sample components) strongly influenced the absorbance reading.

High-performance liquid chromatography (HPLC) has been applied in the determination of gamma-oryzanol. Usually, four fractions can be obtained (Xu and Godber, 1999; Rogers et al., 1993). However, some components of gamma-oryzanol still were not separated individually. This limits the identification and quantification of each of the components of gamma-oryzanol. Gamma-oryzanol can be analyzed via liquid chromatography-mass spectrometry (LC-MS). Various combinations of solvents have been used as the mobile phase of LC-MS and detection of the compounds can be done with mass spectrometry in the mass to charge ratio of charged particles. Due to the high sensitivity and selectivity of mass spectrometry as compared to UV detector, LC-MS is more commonly used in the analysis of gamma-oryzanol. However, quantification of individual gamma-oryzanol components remains as a challenge due to lack of commercially available pure reference standards.

Lately, gas chromatography-mass spectrometry (GC-MS) is a convenient method that is used to identify the structure of the unknown component. From mass spectrometry, the information of molecular weight and structure were described. Xu and Godber, (1999) proposed that gamma-oryzanol have to split into small molecular (triterpine alcohol and ferulic acid) and esterified to reduce the attractive force. Gamma-oryzanol was saponified in an alkali solution to break down ester bond. The triterpine alcohol and ferulic acid were to be derivatized to form volatile trimethylsilyl

(TMS) ether derivative, and then it can be identified their structure by mass spectrum. Using this method, ten components of gamma-oryzanol were identified, and three of these, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate, were major components of gamma-oryzanol. However, the volatilization temperature in GC column is above 400ºC, which affected to the gamma-oryzanol structure.

2.6 Ultrasonic and application

Ultrasound is a sound which is at a frequency beyond the range of human hearing (>20kHz) (Figure 2.7). Ultrasonic devices use transducers to convert electrical energy to sound energy. Based on the level of intensity, ultrasonic treatment could of two types; high intensity (also called power ultrasound) or low intensity. The ultrasound has been utilized for a variety of applications in the food processing industry and high-intensity ultrasound has been proven to be the preferred method for cell disruption and extraction (Piyasena et al., 2003; Zenker et al., 2003).

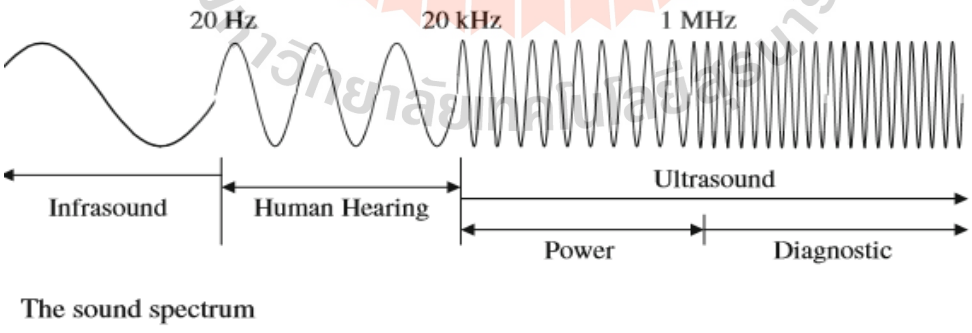

Figure 2.7 Range of sound frequencies (Mason and Lorimer, 2002).

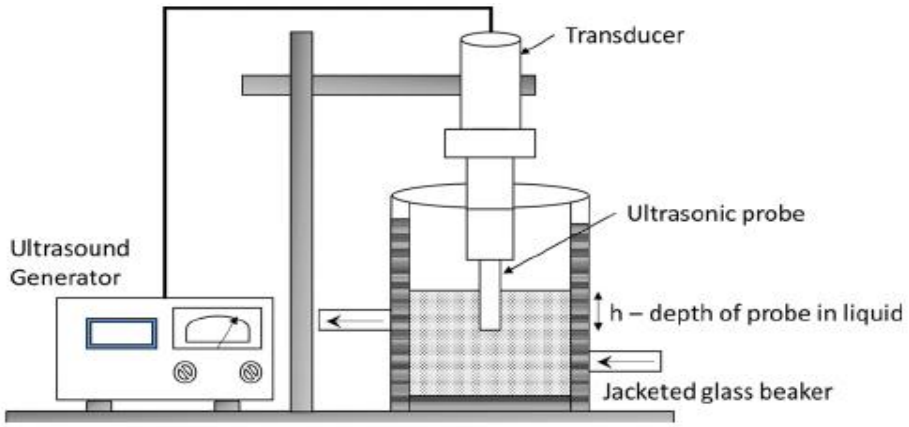

Figure 2.8 A sonic horn, or sonotrode, immersed in a liquid for sonication.

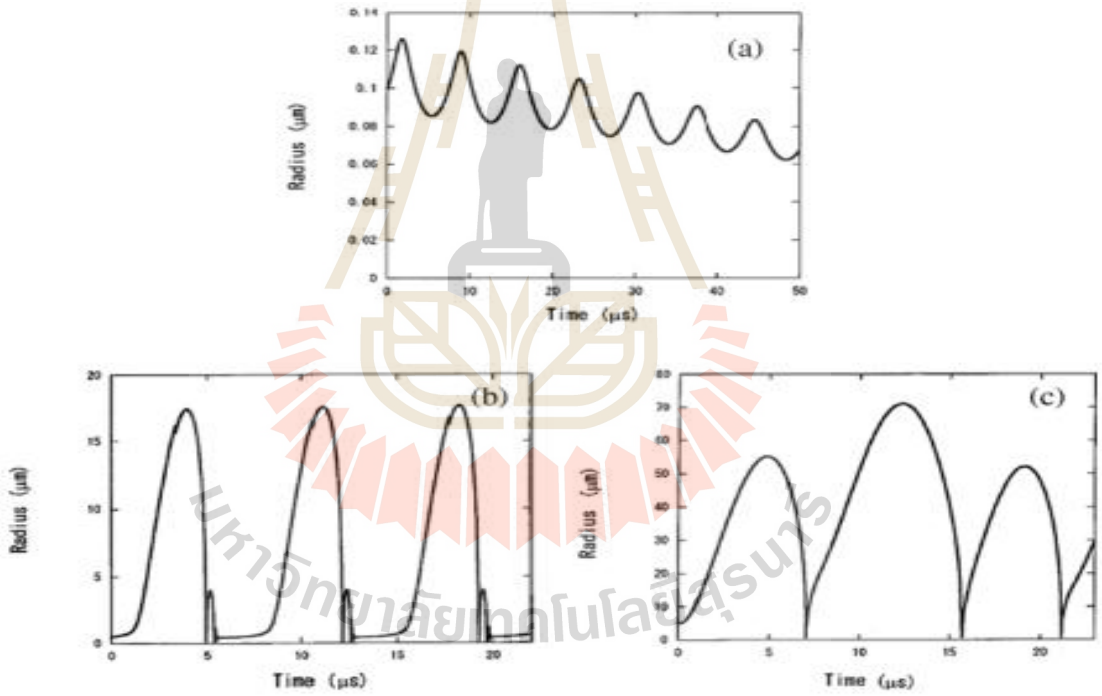

Figure 2.9 Simulated radius time curves at 140 kHz for (a) a dissolving bubble (initial radius 0.1μm and acoustic pressure 500 kPa), (b) a bubble in repetitive transient cavitation (initial radius 0.5μm and acoustic pressure 250 kPa), and (c) a bubble in transient cavitation (initial radius 5μm and acoustic pressure 500 kPa). (Yasui, 2002).

Ultrasound is transmitted to a fluid typically via a sonic horn, or sonotrode, or some other form of a sound transducer. The tip of the horn is immersed in the medium being sonicated (Figure. 2.8) to carry out direct sonication. Direct sonication transfers sonic energy directly to the fluid being processed and is best for processing slurries of disrupt cell and extraction.

During sonication, sound waves propagate through a medium by alternating compression and expansion cycles longitudinally and/or transversely. At the low pressure, the cavitation microbubbles in the liquid are formed and growth (Figure 2.9). Under the compression phase, a collapse of cavitation bubbles causes a rapid rise in local temperature and pressure cavitation bubbles (Hagenson and Doraiswamy, 1998; Lee et al., 2000; Suslick, 1989; and Zhang et al., 2011). Local temperature and pressure may rise to 10,000K and 5,000 bar, respectively (Ashokkumar et al., 2008).

The implosion creates a hot spot in the immediate vicinity, where high temperature and high shear forces work together to bring about the cell disruption of biological cells in the medium. In addition, shock waves produce acoustic streaming, or movement of the fluid in the direction of propagation of the sound wave. Acoustic streaming is caused by the interaction of the sound wave with the molecules of the fluid. Pressure and heat effects may lead to the generation of free radicals to promote certain reactions (Kunaver et al., 2010) (Figure 2.12). Suslick and Grinstaff, (1994) proposed that the superoxide species ($HO_2^{\bullet}$) formed from primary radicals may induce disulfide cross-linkage between proteins. Similarly, Ashokkumar et al 2008 proposed that hydroxyl radicals generated during sonication can be used to enhance the degree of hydroxylation in food materials hence increase the antioxidant activity of foods. Turbulence enhances mixing and mass transfer at the solid-liquid interface (Figure

2.10). Dissipation of acoustic energy may induce motion at the level of molecules, particularly macromolecules, particles and suspended cells. Erosion of solid material is promoted.

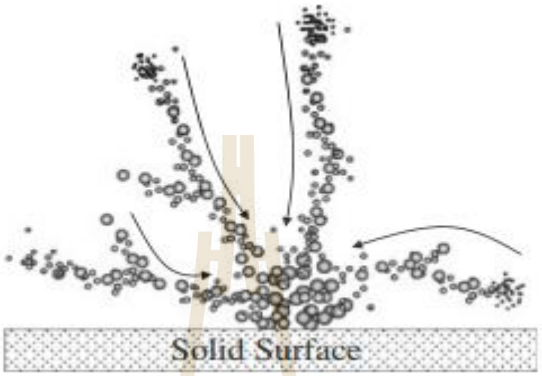

Figure 2.10 The movement of bubbles toward a solid surface acting as a pressure antinode within an acoustic standing wave pattern.

$$H_2O \longrightarrow H\bullet + OH\bullet$$

$$H\bullet + H\bullet \longrightarrow H_2$$

$$OH\bullet + OH\bullet \rightarrow H_2O_2$$

$$H\bullet + OH\bullet \longrightarrow H_2O$$

Figure 2.11 The generation free radical by ultrasound.

The most common known ultrasound is used in fetal imaging; however, it also has applications for detecting cancerous growth within the body (Phull and Mason, 1999). Ultrasound in the MHz range is used in the electronics industry to clean sensitive components such as silicon wafers and disk drive parts without risking the erosive damage that might occur in the cavitation frequency range. Nowadays, in the

food and medicine industry, the use of ultrasound in combination with solvents is also known as ultrasound-assisted extraction (UAE) to extract natural products from plant cells.

UAE is often found to increase the efficiency of the extraction process (Table 2.3). It reduces the amount of solvent and processing time, and efficient in terms of greatly rising components recovery yield (Balachandran et al., 2006; Moulton and Wang, 1982; Riera et al., 2010). There are numerous reports that are available for the ultrasound-assisted extraction of nutraceuticals like gamma-oryzanol, polyphenol, isoflavone and caffeine from different materials such as rice bran, green tea, and coffee, etc. (Gandhi and Bhatnagar, 2015; Garcia-Noguera et al., 2014; Heidtmann-Bemvenuti et al., 2012; Pascual et al., 2013). UAE has also been employed to extract oil from oleaginous crops, soybean, vegetables and seaweed (Heinemann et al., 2008; Moulton and Wang, 1982; Rostagno et al., 2003; Zhang et al., 2008). The advantages and disadvantages of UAE compared with other extraction method were summarized in Table 2.4.

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Table 2.3 List of ultrasound assisted extraction studies from the literature on various food components.

Product	**Ultrasound**	**Solvent**	**Performance**
Olmond oil[1]	Bath, 20 kHz	CO_2 supercritical	30% increased yield or extraction time reduction
Herbal extract[2]	Stirred bath 20 to 2400 kHz	Water and ethanol	Up to 34% increased yield over stirred
Ginseng saponins[3]	Bath, 38.5 kHz	Water, ethanol and n-butanol	3-fold increase of extraction rate
Ginger[4]	Bath	CO_2 supercritical	30% increased yield or extraction time reduction
Soy protein[5]	Continuous, 20 kHz, 3 W per gram	Alkalize and Water	52% and 23% increased yield over equivalent ultrasonic bath conditions
Soy isoflavones[6]	Bath, 24 kHz	Water and solvent	Up to 15% increase in extraction efficiency
Polyphenol, amino acid, caffeine from green tea[7]	Bath, 40 kHz	Water	Increase yield at 65°C, compared with 80°C
Pyrethrines from flower[8]	Bath, 20 kHz and 40 kHz	Hexane	Increase yield at 40°C, compared with 66°C

[1] Riera at al. (2004); [2] Vinatoru et al. (1997); [3] Wu, Lin, and Chau, (2001); [4] Balachandran et al. (2006); [5] Moulton and Wang, (1982); [6] Rostagno et al. (2003); [7] Xia, et al, (2006); [8] Romdhane and Goutine, (2002).

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Table 2.4 Comparison of traditional and recent extraction techniques.

Microwaves assisted extraction (MAE)	**Microwave-assisted solvent extraction (FMASE)**	**Pressurized liquid extraction (PLE)**	**Supercritical fluid extraction (SFE)**	**Soxhlet extraction (SE)**	**Ultrasound assisted extraction (UAE)**
Advantages					
Fast and multiple- extractions [1, 5]	Fast extraction[1]	Fast extraction[1]	Fast extraction[1]	No filtration[1]	Multiple extractions[1, 5]
Low solvent volumes[1]	Low solvent volumes[1]	Low solvent volumes[1, 5]	Minimal solvent volumes[1, 2]	Simple method[2]	Low cost[2, 5]
Elevated temperatures[1]	Low cost[2]	Elevated temperatures[1]	Elevated temperatures[1]		
Low cost[2,5]		No filtration required[1]	Automatic systems [1]		
		Automatic systems[1]	Concentrated extracts[1]		
			Low toxicity[2, 5]		

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Table 2.4 Comparison of traditional and recent extraction techniques (Continue).

Microwaves assisted extraction (MAE)	**Microwave-assisted solvent extraction (FMASE)**	**Pressurized liquid extraction (PLE)**	**Supercritical fluid extraction (SFE)**	**Soxhlet extraction (SE)**	**Ultrasound assisted extraction (UAE)**
Disadvantages					
Extraction solvent must be able to absorb microwaves[1] Waiting time vessel cool down[1] Effect on the phytochemical[2]	Extraction solvent must be able to absorb microwaves Waiting time vessel cool down[1] Effect on the phytochemical[2] Toxicity[2, 3]	Need clean up step[1]	Many parameters to optimize, especially analyte collection[1] High initial cost of equipment [2, 3]	Long time extraction[1] Large solvent volumes[1,2] Toxicity[2]	Large solvent volumes[1,5] Repeated extractions maybe required[1, 2] Effect on the phytochemical [1, 2, 4, 5]

[1] Eskilsson and Bjorklund, (2000), [2] Azwanida Nn, (2015), [3] Ron Self, (2005), [4] Chemat, Grondin, Sing, and Smadja, (2004), [5] Ameer, Shahbaz, and Kwon, (2017)

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CHAPTER III

EFFECTS OF ULTRASONICATION AND THERMAL COOKING PRE-TREATMENTS ON THE EXTRACTABILITY AND QUALITY OF COLD PRESS EXTRACTED RICE BRAN OIL

3.1 Abstract

This research investigates the effects of different extraction processes on the recovery and quality of rice bran oil. The extraction processes under study included hexane extraction (HE), cold press extraction (CE), ultrasonic pre-treatment combined with cold press extraction (UCE), and thermal cooking combined with cold press extraction (CCE*)*. The ultrasonic power and irradiation duration were varied between 2.25, 4.50, and 6.75 W/g; and 10, 25, and 40min. The cooking temperature was 100°C, and the cooking time was varied between 5, 15, 25, and 35 min. The results showed that oil recovery was positively correlated to ultrasonic power and duration and to thermal cooking time, while the oil quality and ultrasonic intensity and thermal cooking period were inversely correlated. Specifically, the oxidative stability, gamma-oryzanol and unsaturated fatty acids of the short-period CCE oil were significantly higher than those of the UCE oil. In addition, similar to the HE and CE oils, oleic, linoleic, and palmitic acids were the dominant fatty acids in the CCE oil. Essentially, the shorter thermal cooking time combined with cold press extraction is optimal for the commercial production of rice bran oil due to improved oil recovery and quality.

Keywords: Rice bran; Cold press extraction; Ultrasonic pre-treatment; Cooking pre-treatment

3.2 Introduction

Rice bran oil is used in food, pharmaceutical, and cosmetic products (Hamm et al., 2013; Lerma-García et al., 2009; Sharma and Rukmini, 1986). Rice bran oil is high in vitamin B, vitamin E (tocopherols and tocotrienols), vitamin K, and gamma-oryzanol (Joshi et al., 2016; Lerma-García et al., 2009). Given a high smoke point of 232°C, rice bran oil is also used in high-temperature cooking, e.g., stir frying and deep frying (Joshi et al., 2016).

Currently, there are two production methods of rice bran oil: extrusion/pressing and solvent extraction (SE) with pure solvent (Sayasoonthorn et al., 2012; Carrín and Crapiste, 2008). Although the extracted oil is of high quality (i.e., high oxidative stability, high gamma-oryzanol, and high unsaturated fatty acids), the extrusion/pressing method suffers from low oil extractability. On the other hand, the SE method achieves high oil recovery but is time-consuming and requires environmentally hostile chemicals. The quality of SE oil is also lower than the mechanically pressed oil.

As a result, advanced extraction methods, including supercritical fluid extraction (SFE), microwave assisted extraction (MAE), pulsed electric field (PEF), and enzymatic assisted extraction (EAE), were proposed for extraction of rice bran oil (Soares et al., 2016; Jegannathan and Nielsen, 2013; Zigoneanu et al., 2008; Zbinden et al., 2013). These advanced extraction methods possess several advantages over the conventional extraction methods, including shorter extraction time, lower solvent requirement, and higher recovery (Bjorklund, 2000). However, their commercialization potential is limited by high operation costs for SFE, MAE, and PEF (Bjorklund, 2000;

Zhang et al., 2011); restricted application to food processing due to high pressure stresses and high temperatures for PEF (Kumar et al., 2015); unsuitability for thermally labile compounds for MAE (Bjorklund, 2000; Zhang et al., 2011); and lack of industrial use for EAE (Jegannathan and Nielsen, 2013).

Other extraction methods were also proposed to improve the recovery and quality of plant-based extract oils. In Sayasoonthorn et al. (2012), screw press was used to improve the rice bran oil recovery. In Taghvaei et al. (2013), the recovery and quality of cotton-seed oil were enhanced by thermal cooking pre-treatment combined with microwave-assisted solvent extraction. In addition, Thanonkaew et al. (2012) experimented with various heating pre-treatment schemes (hot air, roasting, steaming/cooking, microwave) followed by cold press extraction to improve the recovery and quality of rice bran oil.

However, there exists no research on the effect of ultrasonic pre-treatment combined with cold press extraction on the recovery and quality of rice bran oil. In addition, the previous thermal cooking combined with cold press extraction was carried out under high-temperature and long-period condition (130°C and 60min) (Thanonkaew et al., 2012), a condition which is not optimal for the commercial production of rice bran oil.

Thus, this research investigates the effects of ultrasonic and cooking pre-treatments combined with cold press extraction on the recovery and quality (oxidative stability, gamma-oryzanol, and unsaturated fatty acids) of rice bran oil. The study focuses on four extraction processes*:* hexane extraction (HE, Control 1), cold press extraction (CE, Control 2), ultrasonic pre-treatment combined with cold press extraction (UCE), and thermal cooking pre-treatment combined with cold press

extraction (CCE). The ultrasonic power and irradiation times were varied between 2.25, 4.50, and 6.75 W/g; and 10, 25, and 40 min. The cooking temperature was 100°C, and the cooking time was varied between 5, 15, 25, and 35 min.

3.3 Objectives

The objective of the present study was determined the different extraction methods to the oil recovery and oil quality.

3.4 Materials and methods

3.4.1 Materials

Rice bran (RB) of jasmine variety was acquired from Korat Rice Mill factory in Thailand's northeastern province of Nakhon Ratchasima. The RB was passed through a 60-mesh sieve (0.25 mm) and dried at 100°C for 15min to inactivate endogenous lipase. The final moisture content was 6-8% (dry basis, db), determined by a moisture meter (A&D, AD-4714A, Japan). The product was then vacuum-packed and stored below 4ºC for further analysis (Hamm et al., 2013).

Methanol (Mallinckrodt), n-hexane, ethanol and chloroform absolute value (<98.5%) were from Sigma-Aldrich and used without treatment. Standard fatty acid methyl esters (37-component FAME Mix of Supercool, USA) and gamma-oryzanol (Oryza Oil & Fat Chemical Co., Ltd, Japan) were used for quantifications.

3.4.2 Methods

3.4.2.1 Hexane extraction (HE)

A Soxtec solvent extraction system was used for rice bran oil extraction .A 10 g processed RB was packed into a thimble and extracted with 100 mL

n-hexane for 3 hours at 90ºC) Samaram et al., 2014; Zhang et al., 2008(.The n-hexane was then removed and the oil weighed and stored at 4ºC for further analysis .The HE technique yielded 18.50±0.03 g of oil per 100 g of RB (db), which was set as 100% oil recovery (Control 1).

3.4.2.2 Cold press extraction (CE)

A 100 g processed RB was pressed by a cold press machine (Dulong, DL-ZY J02, China) at 35-40ºC, and the extracted oil was collected in a stainless steel container and weighed. The recovery (Control 2) was expressed as the proportion of the cold-press extracted (CE) oil to the HE-treated rice bran oil (Zhang et al., 2008).

3.4.2.3 Ultrasonic pre-treatment combined with cold pressing extraction (UCE)

A 100 g processed RB was dispersed in 300 mL distilled water (1:3) in a conical stainless steel container. The ultrasonic pre-treatment was carried out at 20 kHz using an ultrasonic generator (VCX750 Vibracell; Sonic & Materials, Inc., Newtown, CT, USA). The ultrasonic power and irradiation times were varied between 2.25, 4.50, and 6.75 W/g; and 10, 25, and 40 min, respectively. The ultrasonic generator operated on a cycle of 50 s on and 10 s off (pulse 50⁄10 s) with the temperature maintained at 30ºC. The ultrasonic-treated slurries were filtered with Whatman filter paper no.1. The solid portion was then heated at 100ºC for 1 hour to reduce the moisture content to 6-8%db before cold press extraction. The oil recovery was expressed as the oil extracted by UCE and the oil content in the initial rice bran as determined by HE.

3.4.2.4 Cooking process combined with cold press extraction (CCE)

The processed RB was placed evenly on a 15 cm-diameter aluminum tray and covered with aluminum foil. The tray was then processed in an autoclave (Tommy, SX700, Japan) at 100°C for 5, 15, 25, and 35min. The cooked RB products were oven-dried (Memmert, ULM400, Germany) at 100°C for 1 hour and the moisture content adjusted to 6-8%db. The oil was then extracted using the cold pressing machine, and the yield was calculated as per equation (1).

$$(\%)\ Oil\ recovery = \frac{\text{Weight of oil extracted (g)}}{\text{Total weight of initial rice bran oil extracted by hexane (g)}} \times 100 \qquad (3.1)$$

3.4.2.5 Physicochemical and phytochemical analysis

The American Oil Chemists' Society (AOCS) official methods (1997) were used: Cd 8b-90 for peroxide value (PV), Ca 5a-40 for acid value (AV), Cd 3a-94 for soaponification value, and Cd 1d-92 for iodine value **(**IV**)**. Color was measured by a Minolta CR-300 Chroma Meter (Osaka, Japan) using the la Commission Internationale de l'Eclairage (CIE) L*a*b* (lightness, redness, and yellowness) color system. Density was measured by pycnometer. Gamma-oryzanol in the extracted rice bran oils was analyzed by UV-spectrophotometer/NIR (Shimazu, UV-2600, Japan) (Joshi et al., 2016).

3.4.2.6 Gas Chromatography with Flame-Ionization Detection (GC-FID) analysis

The fatty acids (FAs) were analyzed by GC-FID (Agilent 7890C axis detector, England), following Stanisavljevic et al. (2009) with some modifications. In the analysis, a 20 mg rice bran oil sample was weighed into a test tube and 1 mL 0.5 M KOH added. The solution was heated in a water bath at 90°C for

30min, and then neutralized with 0.6 M HCl before adding 3.0 mL BF_3 in methanol. The mixture was re-heated at 90°C for another 15min in a water bath. The methylated oil was then extracted with n-hexane, and then the solvent was removed by nitrogen gas prior to the FA analysis.

The GC-FID protocol was as follows: the injector temperature was 180°C, the oven temperature started from 50°C to 250°C at a rate of 40°C/min, with the detector temperature at 250°C, and the ion source temperature was 230°C. The FAs were identified by the GC–FID mass fragmentation pattern and spectral and compared against the standards of the National Institute of Standards and Technology.

3.4.2.7 Scanning electron microscope (SEM)

Scanning electron microscopy (SEM JEOL, JSM 6010 LV, Technology Development Ltd., Japan) was used to determine the effect of HE, ultrasonic and thermal cooking pre-treatments on the morphology of the experimental rice bran. In the analysis, the native and treated rice bran samples were placed on the metal stub and gold-sputtered. The shape and surface characteristics of the rice bran samples were determined, and the most representative images selected.

3.4.3 Statistical analysis

All analyses were carried out in triplicate, and the results expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to analyze the differences among group means. Tukey-HSD multiple comparison was used to compare the means, given the 5% significance level ($p<0.05$). The statistical analysis was carried out using Stagraphic Centrution XV (Statsoft Inc., USA).

3.4.4 Experimental site

The experiment was conducted at Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.5 Results and Discussions

3.5.1 Oil recovery

Table 3.1 compares the rice bran oil recovery by different extraction methods (HE, CE, UCE, and CCE) and conditions. The HE method produced the maximum oil recovery (18.5 g oil/100g RB; set as 100%) (Control 1), which is slightly higher than 16.7 g oil/100g RB in Gunawan et al. (2006). The oil recovery of the UCE and CCE methods were in the range of 86.42-95.11% and 93.59-97.93%, respectively.

The ultrasonic (U) and thermal cooking (C) pre-treatments combined with cold press extraction (CE) significantly improved the oil recovery by more than 2% and 10%, respectively, compared with that of cold press extraction (Control 2). Under the UCE, given the same irradiation time, varying ultrasonic powers (2.25, 4.50, 6.75W/g) significantly impacted the oil recovery ($p<0.05$). More specifically, the ultrasonic power was positively correlated to the oil recovery. Similar results were also reported for oils extracted from ground tobacco seeds and flaxseeds pre-treated with ultrasonic waves (Stanisavljević et al., 2009; Zhang et al., 2008).

The ultrasonic pre-treatment enhances the mass liquid transfer by the cavitation forces (Sharma and Gupta, 2006; Zhang et al., 2008). The high shearing force creates micro-bubbles and the bubble collapse generates localized pressures, causing the rupture of cell walls to release the intracellular components into the solvent (Zhang et al., 2008). However, ultrasound contributes to the formation of peroxide radicals in the cavitation bubbles and the subsequent degradation of the

components, resulting in a low quality extracted oil (low oxidative stability, low bioactive components, and low unsaturated fatty acids). Specifically, high ultrasonic intensity induces these radicals to form and accelerates the chemical reactions in the bulk solution and decomposition of components (Chemat et al., 2004).

In the table, the CCE-treated oil extractabilities (i.e., CCE15, CCE25, and CCE35) were significantly higher than those of the UCE-treated ($p<0.05$) (all conditions). The higher CCE oil recoveries could be attributed to the protein denaturation of cell membranes by thermal cooking, enhancing the liquid mass transfer (Taghvaei et al., 2013). On the other hand, sonication (ultrasonic pre-treatment) affected the droplet surface (Hashtjin and Abbasi, 2015), leading to oil oxidation and loss (Lethuaut et al., 2002). This is consistent with Porto et al., 2015; Lou et al., 2010, who documented that high ultrasonic power and an extended sonication period decreased the oil recovery due to the degradation reactions. In this study, the extended thermal cooking pre-treatment followed by cold press extraction (CCE15, CCE25, CCE35) produced higher oil extractabilities than the UCE scheme (all conditions).

The cooking-assisted extraction improved the oil recovery by disruption of tissues (Taghvaei et al., 2013; Thanonkaew et al., 2012). More specifically, thermal cooking at temperatures above 100ºC denatures the enzyme and oil cell's protein to facilitate oil extract from the cell of rice bran (Taghvaei et al., 2013). The high-temperature cooking alters the moisture and porosity of the rice bran, rendering it more brittle to rupture (Thanonkaew et al., 2012). However, the thermal cooking could result in more impurities and oil loss in the refining step (Taghvaei et al., 2013).

3.5.2 Physicochemical properties

The peroxide value (PV) and acid value (AV) are respectively used as the indicators of deterioration and edibility of rice bran oils (Li et al., 2014; Akubugo et al., 2007). Peroxide and acid formation indicates the lipid degradation by hydrolysis or oxidation, resulting in rancidity and off-favors of the oil (Chemat et al., 2004; Akabugo et al., 2007; Oluremi et al., 2013).

In Table 3.2, the PV of the CE (Control 2) and CCE5 oils were significantly lower ($p<0.05$) than that of the HE (Control 1), UCE (all conditions), and CCE (15, 25, 35min) oils. The lower PV (5.14±0.02 for CE and 5.37±0.16 meq O_2/kg oil for CCE5) indicated low deterioration and a long shelf life. This is consistent with Akubugwo et al. (2007), who reported that PV should be below 10 meq O_2/kg oil, given that a value between 20 and 40 results in a rancid taste.

The AV of the CE oil (Control 2) was the lowest (3.34±0.17%oleic acid). On the other hand, the lowest AV under the pre-treated conditions (i.e., ultrasound and thermal cooking) of 4.13±0.06 %oleic acid were achieved with the low to moderate ultrasonic intensity (2.25 W/g; 10 and 25min); and short cooking time (CCE5). Both PV and AV increased with increases in the ultrasound intensity and irradiation time as well as the cooking time. The elevated PV and AV were attributed to the degradation of lipids in the presence of high temperatures, oxygen, and ultrasound irradiation (Chemat et al., 2004; Gharby et al., 2016). Similar results were also reported for papaya seed oil (Samaram et al., 2014). In this study, the shorter cooking-time pre-treatment followed by cold press extraction, especially the CCE5, yielded a higher quality rice bran oil in terms of oxidative stability than the UCE scheme (all conditions).

Iodine value (IV) represents the degree of unsaturation or the average number of double bonds of fatty acids (FAs) in oils or fats. A high IV indicates a high degree of unsaturation (Gharby et al., 2016; Oluremi et al., 2013; Samaram et al., 2014). In Table 3.2, the IV of the extracted rice bran oils was in the range of 77.7-103.40 g I_2/100g oil. The IV of the CCE5 oil (101.2 g I_2/100g oil) was similar to that of the CE oil (103.4 g I_2/100g oil, Control 2). The finding indicated that the thermal cooking process slightly altered the degree of unsaturation in the oil. This is consistent with Oluremi et al. (2013), who documented that the IV of rice bran oil was in the range of 85-105 g I_2/100g oil. In addition, the IV of the CCE5 oil was significantly higher (p<0.05) than that of the HE (Control 1), UCE (all conditions), CCE15, CCE25, and CCE35 oils.

The increase in the processing parameters of the UCE and CCE processes adversely affected the IV, especially those of the oils under the high-intensity ultrasound pre-treatment conditions (i.e., high ultrasonic power and long irradiation time), which were considerably reduced. The IV reduction could be attributed to lipid oxidation which altered the chemical structures of UFAs. The collapse of micro-bubbles during the sonication increased temperatures and pressures in the medium, causing the lipid oxidation (Chemat et al., 2004; Kentish et al., 2011). This is consistent with Chemat et al. (2004), who reported that an ultrasonic power of 0.65W/g reduced the UFAs in sunflower oil due to the presence of conjugated dienes. This showed that sonication induced oil oxidation, resulting in a reduction in the degree of unsaturation.

Saponification value is used to check adulteration (Oluremi et al., 2013). The saponification value was found in crude rice bran oil from 175.28-177.23

mg KOH/g. The SV of HE, CE, and CCE were considerable significantly lower than that of UCE at all treatments under high intensity. The increase in saponification value might have occurred due to the hydrolysis of the esters of fatty acids of the oil. It results in improved free fatty acids and glycerol formation with consequential saponification value increases in the oil. Furthermore, these resulted the increase in hydrolytic oxidation of the oil (Haji and Taghian, 2018). Under high-intensity ultrasound pre-treatment, the increase temperature caused lipid to break down, and reduce the average molecular weight of the oil. Our result is in good agreement with Haji and Taghian, 2018 about the effect of ultrasound process on the saponification value increase. Meanwhile, the relative density index, phosphorus matter, and saponification value of the oils were not significantly affected ($p<0.05$) by different extraction means and ultrasound assisted extraction. Similar results were reported by Latif, Diosady and Anwar.

Carotenoids and chlorophylls are the main pigments present in vegetable oils. During the thermal processing, chlorophyll decomposes into pheophytins which cause the oil to become opaque and dark-colored (Sabah, 2007). Table 3.3 demonstrates the effects of different extraction methods and conditions on the color of the rice bran oil.

Table 3.1 The rice bran oil recovery from different extraction methods and conditions.

Extraction method[1]	Condition	Sample code	Oil recovery (%)[2]
HE	-	HE	100^{a}±0.00
CE	-	CE	84.97^{h}±1.06
UCE	2.25 W/g, 10min	UCE2.25-10	86.42^{hg}±0.92
	4.50 W/g, 10min	UCE4.50-10	89.89^{f}±1.06
	6.75 W/g, 10min	UCE6.75-10	94.78^{cd}±0.59
	2.25 W/g, 25min	UCE2.25-25	92.52^{e}±0.54
	4.50 W/g, 25min	UCE4.50-25	93.93^{d}±0.71
	6.75 W/g, 25min	UCE2.25-25	95.07^{c}±1.03
	2.25 W/g, 40min	UCE4.50-40	92.54^{e}±0.56
	4.50 W/g, 40min	UCE6.75-40	93.91^{d}±0.63
	6.75 W/g, 40min	UCE6.75-40	95.11^{c}±0.23
CCE	100ºC, 5min	CCE5	93.59^{d}±0.14
	100ºC, 15min	CCE15	96.81^{b}±0.31
	100ºC, 25min	CCE25	97.73^{b}±0.50
	100ºC, 35min	CCE35	97.93^{b}±0.45

[1] HE, CE, UCE, and CCE denote the hexane extraction, cold press extraction, ultrasonic pre-treatment combined with cold press extraction, and CCE cooking process combined with cold press extraction, respectively. [2] Different letters denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications ± standard deviation.

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Table 3.2 Physicochemical properties of the rice bran oils under different extraction methods and conditions.

Oil extract sample code	Peroxide Value (meq O_2/kg oil)[1]	Acid Value (%oleic acid)[2]	Iodine Value (g I_2/100 g oil)[3]	Saponification value (mg KOH/g oil)[4]	Relative Density[5]	Phosphorus content (ppm)[6]
HE	9.11^{e}±0.37	5.09^{de}±0.09	98.42^{bc}±1.34	175.28^{a}±0.42		
CE	5.14^{a}±0.02	3.34^{a}±0.17	103.40^{a}±3.03	175.47^{a}±0.76		
UCE2.25-10	5.41^{b}±0.34	4.13^{b}±0.06	100.20^{b}±1.39	175.51^{a}±0.47	0.91-0.92	317.1-318.5
UCE4.50-10	8.53^{d}±0.21	4.87^{d}±0.27	98.34^{bc}±0.54	176.69^{b}±0.60		
UCE6.75-10	12.87^{h}±0.08	6.27^{f}±0.08	86.26^{d}±0.39	177.53^{c}±0.61		
UCE2.25-25	9.27^{e}±0.51	4.13^{b}±0.06	97.11^{c}±0.34	175.53^{a}±0.19		
UCE4.50-25	9.82^{f}±0.12	4.85^{d}±0.22	96.08^{c}±0.03	176.50^{b}±0.30		
UCE6.75-25	13.78^{i}±0.28	6.27^{f}±0.07	79.97^{e}±0.24	175.96^{ab}±0.62		
UCE2.25-40	10.14^{g}±0.41	4.40^{c}±0.02	88.25^{d}±0.45	176.30^{b}±0.16		
UCE4.50-40	13.16^{h}±0.12	5.24^{e}±0.06	80.99^{e}±1.39	177.11^{bc}±0.33		

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Table 3.2 Physicochemical properties of the rice bran oils under different extraction methods and conditions (Continued).

Oil extract sample code	Peroxide Value (meq O_2/kg oil)[1]	Acid Value (%oleic acid)[2]	Iodine Value (g I_2/100 g oil)[3]	Saponification value (mg KOH/g oil)[4]	Relative Density[5]	Phosphorus content (ppm)[6]
UCE6.75-40	15.53^{k}±0.32	6.34^{g}±0.09	77.68^{f}±0.25	177.16^{c}±0.42		
CCE5	5.37^{ab}±0.16	4.13^{b}±0.10	101.20^{ab}±0.69	175.53^{a}±0.27		
CCE15	6.68^{c}±0.16	4.35^{c}±0.15	100.01^{b}±0.90	175.95^{a}±0.53	0.91-0.92	317.1-318.5
CCE25	9.06^{e}±0.17	4.91^{d}±0.02	99.12^{bc}±0.38	175.51^{a}±1.01		
CCE35	10.05^{fg}±0.17	5.16^{e}±0.02	98.56^{bc}±0.38	175.5^{a}±1.01		

[1,2,3,4,5,6] Different letters in each column denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications ± standard deviation.

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The HE (Control 1), CE (Control 2), CCE5, and CCE15 oils had the high lightness (L*) values (22.61±0.05, 22.59±0.05, 22.59±0.05, and 22.59±0.08, respectively) ($p<0.05$). In this study, the high-intensity ultrasound (i.e., high ultrasonic power and extended irradiation time) and longer thermal cooking time pre-treatments reduced L* of the extracted rice bran oils.

The CE (Control 2), CCE5, and CCE15 oils had the low yellow (b*) values (3.53±0.10, 3.55±0.07, and 3.76±0.09, respectively) ($p<0.05$). The CE oil (Control 2) possessed the lowest red (a*) value (4.23±0.11) ($p<0.05$). The extended extraction time at elevated temperatures facilitated the mass transfer of pigments and other impurities from the sample matrix to the extracted oil (Mizukoshi et al., 1999; Samaram et al., 2014a). Moreover, the Maillard products contributed to the red color of oils under high temperatures (Severimi et al., 1994; Negroni et al., 2001). By comparison, the CCE5 and CCE15 oils had L* comparable to the HE (Control 1) and CE (Control 2) oils, given that L* is commonly used to indicate the oil quality (Shahidi et al., 2006).

3.5.3 Phytochemical property

Gamma-oryzanol plays an important role in the quality of extracted oil. The gamma-oryzanol contents of the HE, CE, UCE, and CCE oils were 1.79%, 1.83%, 0.53-1.79%, and 1.78-1.83%, respectively (Table 3.4), suggesting the effect of different extraction processes on the gamma-oryzanol contents. According to Chotimarkorn et al. (2008); Pengkumsri et al. (2015), the recovery of gamma-oryzanol is subject to extraction methods.

In this study, the CE and CCE5 oils had the highest gamma-oryzanol contents (1.83±0.02% and 1.83±0.03%, respectively). The longer thermal cooking

time (CCE15, CCE25, CCE35) slightly decreased the gamma-oryzanol content. However, the elevated ultrasonic power (4.50 and 6.75 W/g) accelerated the degradation of gamma-oryzanol. According to Kentish et al. (2011), high ultrasonic power generated high pressure, resulting in higher temperatures or severe turbulence in the medium and disintegration of the microstructures. The finding is consistent with Khuwijitjaru and Taengtieng, 2004, who reported that the degradation of gamma-oryzanol was accelerated as the temperature increased from 132°C to 222°C. In Table 3.4, the gamma-oryzanol content of CCE5, CCE15, CCE25, and CCE35 were significantly higher than the UCE with 4.50 and 6.75 W/g ultrasonic power ($p<0.05$).

3.5.4 GC analysis for fatty acid composition

Table 3.5 illustrates the fatty acid (FA) composition of the HE, CE, UCE, and CCE rice bran oils. The dominant FAs were oleic, linoleic, and palmitic acids, while myristic, dihomo gamma-linolenic, heneicosylic, and stearic acids were present in smaller amounts. The FA composition of the HE (Control 1) and CE (Control 2) oils were similar. The FAs of the UCE and CCE oils varied, depending on the pre-treatment conditions.

The saturated (myristic, palmitic, stearic, and heneicosylic acids) and unsaturated (oleic, linoleic, and dihomo gamma-linolenic acids) FAs of the HE, CE, and CCE oils were in the range of 27-30% and 70-73%, respectively. The unsaturated fatty acids (UFA) of the UCE oil decreased by 16.17% when the ultrasonic power and irradiation time increased from 2.25 W/g, 10min (72.98%) to 6.75 W/g, 40min (61.18%). Meanwhile, linoleic acid decreased by 31.70%, and oleic acid by only 2.2%. Dihomo gamma-linolenic acid also slightly decreased under the high ultrasonic intensity (i.e., high ultrasonic power and extended irradiation time), while the

saturated fatty acids (SFA) increased from 27.02 (2.25 W/g, 10min) to 38.82% (6.75 W/g, 40min). The finding indicated that high ultrasonic intensity exerted more influence on UFAs than SFAs, consistent with Stanisavljević et al. (2009), who reported that ultrasonic intensity had a strong effect on UFAs, particularly linolenic acid in rice bran oil when the ultrasonic power increased from 0.048 to 0.481 W/g. This was attributable to the ultrasonic-induced cavitation, which altered the structural and functional components of UFAs (Chemat, Grondin, Costes, et al., 2004; Samaram et al., 2014).

Under the CCE, the unsaturated fatty acids (UFAs) slightly decreased while the saturated fatty acids (SFAs) minimally increased as the cooking time increased. Specifically, the thermal cooking pre-treatment had a smaller impact on the FA composition of the extracted oils. Moreover, the unsaturated compounds of the CCE5 oil closely resembled those of the CE oil (Control 2).

3.5.5 Microstructural analysis

SEM was used to investigate the microstructures of the native and treated (i.e., HE-processed, ultrasonic- and cooking-pretreated) rice bran. In Fig. 3.1a, the native rice bran had a relatively complete structure, regular or compact shape and smooth surface, rendering it less ideal for oil extraction. In Fig. 3.1b, the micro-fractures and cracks appeared on the HE-treated rice bran as the solvent penetrated, resulting in the expanded cellular structure and partially dragging lipids and starches (Alcázar-alay et al., 2015).

The ultrasonic- and cooking-pretreated rice bran (prior to cold press extraction) exhibited numerous micropores with fissured and rough surface (Figs.3.1c, d, e, f, g, h, k). The results are consistent with (Zhang et al., 2008; Kurian et al., 2015;

Yi et al., 2016), who reported that the ultrasonic and cooking pre-treatments induced the structural changes, fissures, and cavities in the samples. The ultrasonic and thermal cooking pre-treatments disrupt the tissues and cell walls of rice bran, enhancing the mass transfer and the subsequent increased oil extractabilities.

3.6 Conclusions

This study comparatively investigates the effects of four extraction methods of rice bran oil on the extractabilities. and quality*:* the hexane extraction (HE), cold press extraction (CE), ultrasonic pre-treatment combined with cold press extraction (UCE), and cooking process combined with cold press extraction (CCE). The results showed that elevated ultrasonic intensity (i.e., higher ultrasonic power and longer irradiation time) and extended cooking time increased the rice bran oil extractabilities; but lowered the oil quality (i.e., oxidative stability, gamma-oryzanol, phosphorus content and unsaturated fatty acids). The results also indicated that the oil quality of the rice bran oil extracted under the short-time thermal cooking combined with cold press extraction (CCE5) was higher than the ultrasonic-pretreated UCE rice bran oils (all conditions). The SEM images illustrated that the ultrasonic and cooking pre-treatments induced the structural changes, fissures, and cavities in the rice bran. Overall, the short cooking time followed by cold press extraction (CCE5) is highly efficient and commercially viable for the rice bran oil extraction because of the improved recovery (increased oil yield) and good oil quality.

Table 3.3 Color of the rice bran oil under different extraction methods and conditions.

Oil extract sample code	L*[1]	a*[2]	b*[3]
HE	22.6^{a}±0.05	4.31^{i}±0.09	3.57^{g}±0.08
CE	22.59^{a}±0.05	4.23^{k}±0.11	3.53^{h}±0.10
UCE2.25-10	22.43^{b}±0.02	4.38^{h}±0.09	4.38^{f}±0.09
UCE4.50-10	22.23^{c}±0.02	4.50^{g}±0.05	4.50^{e}±0.05
UCE6.75-10	22.25^{cd}±0.03	4.61^{ef}±0.07	4.61^{d}±0.07
UCE2.25-25	22.39^{b}±0.01	4.66^{e}±0.05	4.66^{d}±0.05
UCE4.50-25	22.12^{d}±0.03	4.59^{f}±0.06	4.59^{d}±0.06
UCE6.75-25	22.05^{e}±0.05	4.72^{d}±0.10	4.72^{c}±0.10
UCE2.25-40	22.38^{b}±0.02	4.85^{c}±0.06	4.85^{b}±0.06
UCE4.50-40	22.07^{e}±0.10	4.98^{ab}±0.09	4.98^{a}±0.09
UCE6.75-40	22.06^{e}±0.03	4.93^{b}±0.08	4.93^{a}±0.08
CCE5	22.59^{a}±0.05	4.32^{i}±0.07	3.55^{h}±0.07
CCE15	22.59^{a}±0.08	4.31^{i}±0.08	3.76^{gh}±0.09
CCE25	22.10^{d}±0.03	4.67^{e}±0.10	4.02^{g}±0.11
CCE35	22.61^{a}±0.05	5.01^{a}±0.05	4.48^{e}±0.08

[1,2,3] Different letters in each column denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications ± standard deviation.

Table 3.4 Gamma-oryzanol content of rice bran oils under different extraction processes.

Oil extract sample code	Oryzanol level (%)[1]
HE	1.79^{ab}±0.37
CE	1.83^{a}±0.02
UCE2.25-10	1.79^{ab} ±0.35
UCE4.50-10	1.38^{c} ±0.21
UCE6.75-10	0.89^{e} ±0.08
UCE2.25-25	1.77^{ab} ±0.51
UCE4.50-25	1.37^{c} ±0.12
UCE6.75-25	0.98^{d} ±0.28
UCE2.25-40	1.64^{b} ±0.41
UCE4.50-40	1.05^{d} ±0.12
UCE6.75-40	0.53^{f} ±0.32
CCE5	1.83^{a}±0.03
CCE15	1.79^{ab} ±0.16
CCE25	1.78^{ab} ±0.17
CCE35	1.78^{ab}±0.17

[1] Different letters in column denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications ± standard deviation.

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Table 3.5 Fatty acid compositions (%) of the rice bran oils extracted by HE, CE, UCE, and CCE.

Extraction method	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Dihomo gamma-linolenic acid	Heneicosylic acid
	C14:0	C16:0	C18:0	C18:1	C18:2	C20:3	C21:0
HE	4.40^{l}±0.09	14.52^{m}±1.21	3.61^{k}±0.10	44.86^{b}±2.09	23.16^{b}±1.35	4.64^{c}±0.21	4.81^{i}±0.21
CE	4.39^{l}±0.05	14.50^{m}±1.11	3.42^{j}±0.12	44.90^{a}±1.74	23.19^{a}±1.00	4.77^{a}±0.15	4.82^{i}±0.25
UCE2.25-10	4.40^{l}±0.08	14.67^{l}±1.45	5.20^{f}±0.09	43.04^{c}±1.61	23.09^{c}±0.94	4.70^{b}±0.17	4.90^{h}±0.30
UCE4.50-10	5.52^{h}±0.09	19.40^{h}±1.89	3.57^{i}±0.17	41.45^{e}±1.69	19.05^{g}±1.36	4.50^{d}±0.23	6.51^{a}±0.27
UCE6.75-10	7.00^{d}±0.04	22.34^{d}±1.49	5.90^{d}±0.31	39.92^{h}±2.01	15.75^{j}±0.94	3.62^{k}±0.12	5.47^{e}±0.38
UCE2.25-25	5.78^{f}±0.04	17.22^{k}±0.98	3.57^{i}±0.35	41.48^{d}±2.19	21.77^{d}±1.03	4.27^{h}±0.10	5.91^{c}±0.19
UCE4.50-25	5.62^{g}±0.10	20.10^{g}±1.12	5.58^{e}±0.64	41.46^{e}±1.63	18.05^{h}±1.71	4.08^{i}±0.22	5.11^{g}±0.93
UCE6.75-25	8.10^{c}±1.01	22.25^{e}±2.01	6.30^{c}±0.89	39.92^{h}±1.39	14.17^{k}±0.97	3.31^{k}±0.09	5.95^{b}±0.56
UCE2.25-40	6.69^{e}±0.13	18.45^{i}±1.04	6.80^{b}±0.09	40.68^{f}±1.24	17.51^{i}±0.94	4.42^{e}±0.14	5.45^{e}±0.24
UCE4.50-40	8.39^{b}±0.15	22.77^{b}±1.65	6.30^{c}±0.19	40.02^{g}±1.04	13.47^{l}±1.01	3.78^{j}±0.15	5.27^{f}±0.31

Table 3.5 Fatty acid compositions (%) of the rice bran oils extracted by HE, CE, UCE, and CCE (Continued).

Extraction method	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Dihomo gamma-linolenic acid	Heneicosylic acid
	C14:0	C16:0	C18:0	C18:1	C18:2	C20:3	C21:0
UCE6.75-40	9.03^{a}±0.21	25.12^{a}±2.02	9.50^{a}±0.12	34.24^{k}±1.09	13.30^{m}±0.89	3.15^{l}±0.10	5.66^{d}±0.43
CCE5	4.39^{l}±0.07	14.51^{m}±0.94	3.42^{j}±0.06	44.90^{a}±2.00	23.20^{a}±0.99	4.76^{a}±0.24	4.82^{i}±0.46
CCE15	4.46^{k}±0.10	19.37^{j}±0.99	4.55^{i}±0.05	41.03^{g}±1.16	21.46^{d}±1.05	4.31^{f}±0.12	4.82^{i}±0.29
CCE25	4.93^{i}±0.14	21.88^{f}±1.67	4.90^{h}±0.10	39.03^{i}±1.42	20.14^{e}±0.95	4.31^{f}±0.11	4.81^{i}±0.05
CCE35	4.78^{j}±0.12	22.56^{c}±2.11	5.10^{g}±0.07	39.00^{j}±1.67	19.49^{f}±0.97	4.29^{g}±0.18	4.78^{j}±0.18

Different letters in column denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications ± standard deviation.

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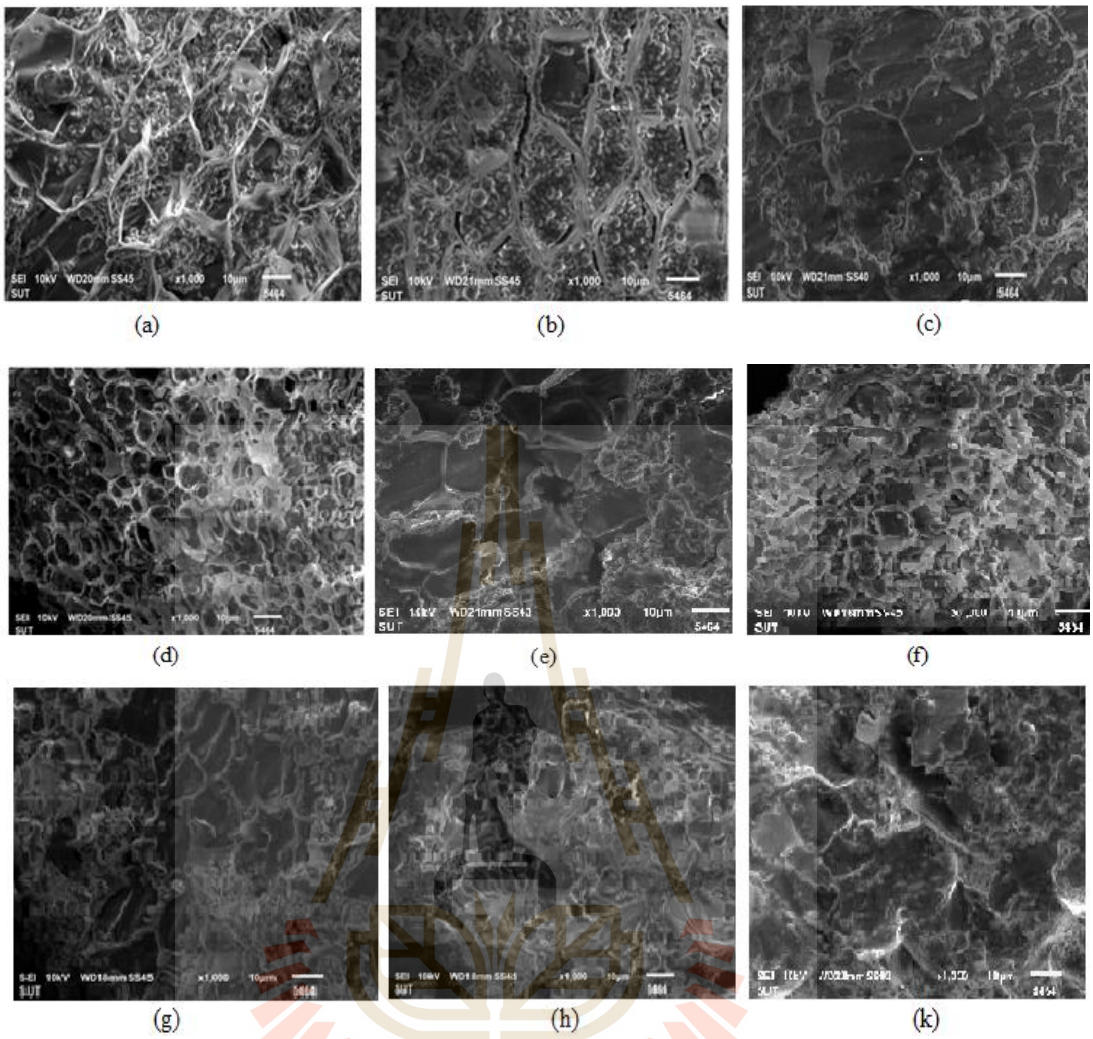

Figure 3.1 SEM images (1000x) of: (a) native rice bran (RB), (b) HE-treated RB, (c) ultrasonic pre-treated (2.25 W/g, 10 minutes) RB, (d) ultrasonic pre-treated (6.75 W/g, 10 minutes) RB, (e) ultrasonic pre-treated (2.25 W/g, 25 minutes) RB, (f) ultrasonic pre-treated (6.75 W/g, 25 minutes) RB, (g) ultrasonic pre-treated (2.25 W/g, 40 minutes) RB, (h) ultrasonic pre-treated (6.75 W/g, 40 minutes) RB, (k) cooking pre-treated (5 minutes) RB

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CHAPTER IV

RESPONSE SURFACE METHODOLOGY OPTIMIZATION OF CHEMICAL NEUTRALIZATION PARAMETERS FOR ENHANCED RICE BRAN OIL RECOVERY AND QUALITY

4.1 Abstract

This research investigates the effect of variable chemical neutralization parameters on the oil recovery, free fatty acid (FFA) level, and gamma-oryzanol content of neutralized rice bran oil, using response surface methodology. The neutralization parameters under study included NaOH concentrations (10-30°Be), temperatures (60-90°C), and process time (5-15min). The quadratic response models were generated and statistical analysis performed to validate the models. The optimal neutralization condition was 19.24°Be, 74.79°C, and 11.18min. Under the optimal condition, the predicted oil recovery, FFA level, and gamma-oryzanol were 80.00%, 0.31%, and 1.00%, respectively, while the corresponding experimental results were 80.12%, 0.29%, and 1.02%, suggesting good agreement between the predicted and experimental data and high predictive ability of the models. The response models could thus be applied to optimizing the neutralization parameters for enhanced oil yield and quality.

Keywords: Response surface methodology, rice bran oil, neutralization, free fatty acids, gamma-oryzanol.

4.2 Introduction

Rice bran is cuticle between the paddy husk and rice grain which obtained as a byproduct during rice processing (Amarasinghe et al., 2004). Rice bran contains lipids, proteins, minerals, and vitamins (Oluremi et al., 2013) and is a rich source of unsaturated fatty acids (UFAs), including oleic, linoleic, and linolenic (Oluremi et al., 2013). UFAs help lower cardiovascular risks, such as hyperlipidemia, hypercholesterolemia, and hypertriglyceridemia (Grundy, 1975).

According to Jung et al. (2017); Ghasemzadeh et al. (2015), polyphenols, gamma-oryzanol, tocopherols, and tocotrienols are found in abundance in rice bran oil (RBO). These bioactive compounds are beneficial for decreasing plasma cholesterol (Wilson et al., 2007), platelet aggregation, and cholesterol absorption (Sharma et al., 1986; Seetharamaiah et al., 1986). RBO is thus commonly used in food, cosmetic, and pharmaceutical products (Lerma-García et al., 2009).

In RBO production, the main challenge lies in the refining process of crude rice bran oil (CRBO) to remove impurities, such as free fatty acids (FFAs), phospholipids, pigments, and waxes, to improve the flavor, odor, appearance, and shelf life of the oil (Gunstone, 2011). According to De et al. (2011), CRBO poses the greatest refining challenge in order to meet the standards of edible oil.

There are two CRBO refining processes: physical and chemical. The physical refining technique involves degumming and distillation (steam refining and deodorization) (Jiri et al., 2000). The advantages of the physical refining included low oil loss and preserved gamma-oryzanol (De and Bhattacharyya, 1998; Rajam et al., 2005). However, the disadvantages are high implementation costs at industrial level

(Engelmann et al., 2017) and sensitivity to the quality of input crude oil (Kövari et al., 2000; Engelmann et al., 2017).

The chemical refining involves degumming, neutralization, bleaching, and deodorization (Gunstone, 2002). Neutralization is crucial to remove FFAs in CRBO by saponification using sodium hydroxide (NaOH) solution (De and Patel, 2010). Chemical neutralization is more effective in removing FFAs and impurities (waxes and mucilage), contributing to improved RBO quality (Hoed et al., 2006). However, chemical neutralization suffers from excessive loss of gamma-oryzanol, in which as high as 90% was lost through soapstock (Das et al., 1998; Gopalakrishna et al., 2001).

Previous research on improved neutralization of RBO focused on low oil recovery (Chumsantea et al., 2012; Gopalakrishna et al., 1988), darkening of color (Hoed et al., 2010; De et al., 1998), or low gamma-oryzanol content (Chumsantea et al., 2012; Das et al., 1998; Gopalakrishna et al., 2001). There is currently no study on chemical neutralization optimization specific to RBO to improve FFA removal and oil and gamma-oryzanol recovery.This study thus investigates the effect of variable neutralization parameters on the recovery of RBO, FFA removal, and preservation of gamma-oryzanol, using response surface methodology. The neutralization parameters under study included NaOH concentrations, temperature, and process time, which were varied between 10, 20, and 30°Be; 60, 75, and 90°C; and 5, 10, and 15min, respectively.

4.3 Objectives

In order to study the effect of neutralization condition to optimize the neutralization parameters of rice bran oil to reduce the oil loss during alkaline

neutralization and improve the oil quality by lowering free fatty acids while preserving gamma-oryzanol.

4.4 Materials and methods

4.4.1 Materials

Rice bran (RB) of jasmine variety was acquired from *Korat Rice Mill* factory in Thailand's northeastern province of *Nakhon Ratchasima*. The RB was passed through a 60-mesh sieve (0.25 mm) and pretreated according to Sun et al. (2017), with minor modifications. Rice bran was dried at 100°C for 15min to inactivate endogenous lipase. Dried samples were thermally cooked at 100°C for 5min (pretreated), followed by cold press extraction (Phan et al., 2018). The crude rice bran oil was centrifuged at 10,000 rpm for 10min and the CRBO stored in amber plactic container at -20°C for further analysis.

Methanol (Mallinckrodt), *n*-hexane, ethanol, chloroform absolute value (<98.5%), hanus reagent, ammonium molybdate, and ammonium metavanadate were from Sigma-Aldrich. Citric acid (99%) was acquired from Acros Organics (New Jersey, USA). Standards of fatty acid methyl esters (37-component FAME Mix of Supercool, USA) and gamma-oryzanol (Oryza Oil and Fat Chemical Co., Ltd, Japan) were used for quantification.

4.4.2 Experimental methods

4.4.2.1 Degumming of crude rice bran oil (CRBO)

The degumming was carried out according to Ringers (2000) with some modifications. In degumming, 50 g of CRBO was heated to 70°C in water bath, and then hydrated by adding 0.3%wt of 50% citric acid solution. The degumming was conducted at 70°C for 30min with continuous slow agitation.

Degummed rice bran oil (DRBO) was then centrifuged at 10,000 rpm for 10min to remove the gum and decanted for further refining.

4.4.2.2 Neutralization of degummed of crude rice bran oil (DRBO)

Free fatty acids in DRBO were neutralized following the procedure in Engelmann et al. (2017) with some modifications. NaOH concentrations were varied between 10, 20, and 30°Be, and the mixtures heated at different temperatures (60, 75, and 90°C) and durations (5, 10, and 15min). The oil was stirred vigorously and heated up to detemine temperature for the addition of aqueous NaOH, and then 15%v/v of hot ultra-pure water sprayed on (Andersen et al., 1962). The oil was left to settle and centrifuged at 10,000 rpm for 10min to precipitate the sludge before neutralized rice bran oil (NRBO) collected. Residual water in NRBO was vacuum-evaporated prior to physicochemical analysis. The NRBO recovery was calculated by

$$\text{NRBO recovery (\%)} = \frac{\text{Weight of neutralized oil}}{\text{Weight of inital degummed rice bran oil}} \times 100 \quad (4.1)$$

4.4.3 Experimental design for oil neutralization

In this study, response surface methodology (RSM) with central composite face-centered (CCF) design was used to optimize the neutralization parameters for RBO refining. Statistical software (Modde version 5.0, Sweden) was used to analyze the experimental data. Three independent variables included NaOH concentration (°Be, X_1), temperature (°C, X_2), and time (min, X_3). The responses (Y) were the NRBO recovery, FFA level, and gamma-oryzanol content. Each independent variable was coded -1, 0, and +1 (Table 4.1), and the design of experiment consisted of 17 experimental runs (Table 4.2). The experiments were carried out in triplicate and averaged.

Table 4.1 Experimental levels of the three independent variables.

Symbol	Variables	Levels		
		-1	**0**	**+1**
$\mathbf{X_1}$	NaOH concentration (°Be)	10	20	30
$\mathbf{X_2}$	Temperature pretreatment (°C)	60	75	90
$\mathbf{X_3}$	Time pretreatment (min)	5	10	15

Multiple regression analysis was utilized to derive second-order polynomial equation to predict the oil recovery (Y_1), FFA (Y_2), and gamma-oryzanol content (Y_3):

$$f(Y) = \beta_0 + \sum_{n=1}^{k} \beta_i X_i + \sum_{n=1}^{k} \beta_{ii} X_i^{\,2} + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j \qquad (4.2)$$

where Y is the predicted response; β_0 is a constant; X_i, and X_j are the independent variables, β_i is the linear coefficient, β_{ij} is the interactive coefficient, and β_{ii} is the quadratic coefficient.

The model adequacy was evaluated by lack of fit, F-test, and regression coefficient of determination (R^2) from analysis of variance (ANOVA), and the 3D response surface plots generated. Model validation was subsequently carried out. The significance level was based on the 5% significance level ($p<0.05$).

4.4.4 Analytical methods

4.4.4.1 Fatty acid analysis

Fatty acids of CRBO, DRBO, and optimized NRBO were analyzed by GC-FID (Agilent 7890C axis detector, England), following Stanisavljević et al. (2009) with some modifications. The fatty acids were transformed into methyl esters in BF_3-methanol at 90°C for 15min in water bath. The methylated oil was then extracted with *n*-hexane, and the solvent removed by nitrogen gas.

The temperature scheme used was as follows: the injector temperature was 180°C, the oven temperature started from 50 to 250°C at a rate of 40°C/min, and the ion source temperature was 230°C. The injection volume was 1 μL in splitless injection mode with Luer Tip syringe (LT) (Merck KGaA, Darmstadt, Germany). The FA methyl esters were identified by comparing the retention time of oil components against the standards of the National Institute of Standards and Technology.

4.4.4.2 α-tocopherol chromatography conditions

The HPLC analysis was carried out using the Agilent 1200 series equipped with an Agilent 1200 series with a pump (LPG 3X00), auto-sampler (ACC-3000), and diode-array UV/VIS detector (DAD). The mobile phase was consisted of acetonitrile (50%) and methanol (50%) at a flow rate of 1.0 mL/min. The quantitative estimation of *α*-tocopherol is performed on reversed phase C18 column (1.5 mm × 150 mm, 1.75μm).The injection volume was 20 μL. The detection UV wavelength was set at 330 nm, and the column temperature was set at 25°C.

Table 4.2 Experimental and predicted oil recovery (Y_1), FFA level (Y_2), and gamma-oryzanol of neutralized rice bran oil (NRBO) (Y_3) under variable NaOH concentrations (°Be, X_1), temperature (°C, X_2), and time (min, X_3).

Run	Code variable						Experimental			Predicted		
	X_1	X_2	X_3	X_1	X_2	X_3	Y_1 (%)	Y_2 (%)	Y_3 (%)	Y_1 (%)	Y_2 (%)	Y_3 (%)
1	-1	-1	-1	10	60	5	80.13±1.21	0.71±0.05	1.00±0.06	80.22±1.05	0.68±0.03	1.02±0.09
2	-1	1	-1	10	90	5	80.20±1.13	0.76±0.08	0.90±0.07	80.15±1.13	0.78±0.05	0.89±0.08
3	1	-1	-1	30	60	5	79.13±1.41	0.48±0.06	0.65±0.05	79.03±1.12	0.48±0.02	0.64±0.07
4	1	1	-1	30	90	5	79.61±1.39	0.60±0.05	0.63±0.08	79.55±1.19	0.59±0.04	0.63±0.08
5	-1	-1	1	10	60	15	79.82±1.34	0.69±0.06	0.99±0.08	79.83±1.09	0.72±0.05	0.98±0.09
6	-1	1	1	10	90	15	80.12±1.15	0.79±0.08	0.88±0.07	80.14±1.05	0.81±0.05	0.88±0.05
7	1	-1	1	30	60	15	79.11±1.29	0.25±0.03	0.49±0.05	79.12±1.06	0.23±0.05	0.50±0.09
8	1	1	1	30	90	15	80.08±1.20	0.31±0.04	0.55±0.05	80.02±1.15	0.32±0.05	0.52±0.05
9	0	-1	0	20	60	10	79.69±1.09	0.33±0.03	0.96±0.08	79.72±1.15	0.35±0.04	0.93±0.09
10	0	1	0	20	90	10	80.06±1.08	0.45±0.03	0.89±0.09	80.14±1.04	0.44±0.04	0.87±0.09

Table 4.2 Experimental and predicted oil recovery (Y_1), FFA level (Y_2), and gamma-oryzanol of neutralized rice bran oil (NRBO) (Y_3) under variable NaOH concentrations (°Be, X_1), temperature (°C, X_2), and time (min, X_3) (Continued).

Run	Code variable						Experimental			Predicted		
	X_1	X_2	X_3	X_1	X_2	X_3	Y_1 (%)	Y_2 (%)	Y_3 (%)	Y_1 (%)	Y_2 (%)	Y_3 (%)
11	-1	0	0	10	75	10	80.12±1.21	0.68±0.05	1.02±0.05	80.11±1.10	0.64±0.03	1.02±0.07
12	1	0	0	30	75	10	79.23±1.19	0.28±0.03	0.63±0.08	79.45±1.06	0.30±0.04	0.65±0.09
13	0	0	-1	20	75	5	80.01±1.05	0.31±0.03	0.90±0.08	80.01±1.05	0.39±0.05	0.91±0.09
14	0	0	1	20	75	15	80.05±1.21	0.30±0.02	0.81±0.04	80.05±1.17	0.28±0.05	0.84±0.08
15	0	0	0	20	75	10	80.02±1.27	0.33±0.05	0.95±0.09	79.99±1.03	0.32±0.03	0.93±0.09
16	0	0	0	20	75	10	80.09±1.09	0.30±0.05	0.90±0.07	79.99±1.45	0.32±0.05	0.93±0.07
17	0	0	0	20	75	10	80.09±1.10	0.35±0.03	0.95±0.05	79.99±1.04	0.32±0.04	0.93±0.06

Different letters in column denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications ± standard deviation.

4.4.4.3 Total phenolic content

The total phenolic content (TPC) was determined according to the Folin-Ciocalteau method (Slinkard and Singleton, 1977), with slight modifications. A 0.1 mL sample was mixed with 2 mL Folin-Ciocalteau, and 0.6 mL Na_2CO_3 (20.0%). The test tubes were vortexed and allowed to stand for 30min in the dark before measurement at absorbance of 765 nm. The results were expressed as mg of Gallic acid per g of powder (mg GA/g powder).

4.4.4.4 Thermal analysis

Differential scanning calorimetry (DSC 7, Perkin Elmer Corp., Norwalk, CT, USA) was utilized to characterize the thermal behavior. In the analysis, the baseline was first established using two empty sealed aluminum crucibles under a thermal program in which the initial temperature was 30°C and cooled down to -60°C before heating to 60°C at a rate of 10°C/min. Then, 3-5 mg of CRBO, DRBO, and optimized NRBO were individually transferred into one of the crucible (i.e., sample crucible) and the analysis carried out and the melting curve plotted.

4.4.4.5 Other analytical methods

The American Oil Chemists' Society (AOCS) official methods (1997) were used: Cd 1b-87 for iodine value, Aa 516-01 for peroxide value, Ca 5a-40 for free fatty acid, Ca 12-55 for phosphorus value, and Cd 3a-94 for saponification value. The relative density of oil was measured by density bottle. Gamma-oryzanol was analyzed by UV-spectrophotometer/NIR (Shimazu, UV-2600, Japan) (Joshi et al., 2016).

4.4.5 Statistical analysis

All experiments were carried out in triplicate and the results expressed as mean±standard deviation. One-way analysis of variance (ANOVA) was used to

assess the effects of degumming and neutralization on the fatty acid profile and physicochemical properties of DRBO and NRBO, CRBO. Tukey-HSD comparison was used to compare means, given $p<0.05$. The statistical analysis was carried out using Stagraphic Centrution XV (Statsoft Inc., USA) and Modde 5.0 (Umea, Sweden).

4.4.6 Experimental site

The experiment was conducted at Suranaree University of Technology, Nakhon Ratchasima, Thailand.

4.5 Results and Discussion

4.5.1 Evaluation of progress for neutralization parameters

Experimental data based on RSM with CCF were used to optimize the neutralization parameters; and evaluate the relationships between three independent variables: NaOH concentration (°Be, X_1), temperature (°C, X_2), and time (min, X_3) and the responses: oil recovery (Y_1), FFA (Y_2), and gamma-oryzanol content (Y_3). Table 4.2 tabulates the experimental and predicted values. Specifically, the oil recovery, FFA level, and gamma-oryzanol from the experiments were 79.11-80.20%, 0.25-0.76% oleic acid, and 0.49-1.02%, respectively.

The statistical significance of quadratic model terms was determined by ANOVA, and the significance of regression coefficients by F-value, p-value, and lack of fit. The insignificant terms ($p>0.05$) were excluded from the quadratic models. Tables 4.3a-c respectively present the ANOVA results for the fitted quadratic models for the oil recovery, FFA, and gamma-oryzanol of NRBO. Specifically, there existed statistically significant relationships between the independent and response variables, with high F-values and very low p-values ($p<0.05$), indicating that the model terms were significant. According to Quanhong and Caili (2005); Yolmeh et al. (2014), larger

F-values and smaller p-values indicated a strong relationship between independent variables and the response. In this research, the p-values of lack of fit were greater than 0.05, indicating that the models satisfactorily fitted the experimental data.

The robustness and predictive ability of the models were evaluated by coefficient of determination (R^2), adjusted R^2, and model predictive ability (Q^2). The high R^2 (0.952, 0.976, 0.987) and adjusted R^2 (0.891, 0.944, 0.971) of the oil recovery (Y_1), FFA (Y_2), and gamma-oryzanol (Y_3) models suggested good agreement between the experimental and predicted data. Meanwhile, Q^2 of Y_1, Y_2, and Y_3 models were 0.575, 0.863, and 0.826, respectively, indicating that the variability of responses was well explained by the models with small prediction error. According to Veerasamy et al. (2011), high Q^2 (>0.5) indicates good predictive ability of a model. In this research, the oil recovery (Y_1) model possessed a high R^2 (0.952) but low Q^2 (0.575), suggesting that the predictive ability of the Y_1 model was not as high as the FFA (Y_2) and gamma-oryzanol (Y_3) models. This was probably attributable to over-fitting (Makanjuola et al., 2015).

The fitted quadratic relationships between the independent variables (X_1 = NaOH concentration, X_2 = temperature, X_3 = time) and responses (Y_1 = the oil recovery, Y_2 = FFA, and Y_3 = gamma-oryzanol content) were as follows:

$$Y_1 = 79.9908-0.2577X_1+0.1652X_2+0.0911X_1*X_2+0.0775X_1*X_3-0.1310X_1^2 \quad (4.3)$$

$$Y_2 = 0.3157-0.1343X_1+0.0386X_2-0.0437X_3-0.0462X_1*X_3-0.0967X_1^2+0.0493X_2^2 \quad (4.4)$$

$$Y_3 = 0.9280-0.1462X_1-0.0207X_2-0.0283X_3+0.0195X_1*X_2-0.0156X_1*X_3-0.0556X_1^2-0.0331X_3^2 \quad (4.5)$$

Table 4.3a ANOVA results of the response surface quadratic model on the recovery of neutralized rice bran oil.

Oil yield	Degree of freedom	Sum of squares	Mean square	F-value	p-value
Total Corrected	16	2.1797	0.1362		
Regression	9	2.0744	0.2305	15.3227	0.0010
Residual	7	0.1053	0.0150		
Lack of Fit	5	0.1022	0.0205	13.4089	0.0760
Pure Error	2	0.0031	0.0016		

R^2 = 0.9520; Adjusted R^2 = 0.8901; Q^2 = 0.5750; p-value<0.05 indicates statistical significance.

Table 4.3b ANOVA results of the response surface quadratic model on FFA of neutralized rice bran oil.

FFA	Degree of freedom	Sum of squares	Mean square	F-value	p-value
Total Corrected	16	0.6008	0.0376		
Regression	9	0.5862	0.0652	31.059	0.0000
Residual	7	0.0147	0.0021		
Lack of Fit	5	0.0134	0.0027	4.235	0.2020
Pure Error	2	0.0013	0.0006		

R^2 = 0.9760; Adjusted R^2 = 0.9440; Q^2 = 0.8630; p-value<0.05 indicates statistical significance.

Table 4.3c ANOVA results of the response surface quadratic model on gamma-oryzanol of neutralized rice bran oil.

Gamma-oryzanol level	Degree of freedom	Sum of squares	Mean square	F-value	p-value
Total Corrected	16	0.4635	0.0289		
Regression	9	0.4578	0.0509	63.25	0.000
Residual	7	0.0056	0.0008		
Lack of Fit	5	0.0041	0.0008	1.0848	0.5700
Pure Error	2	0.0015	0.0008		

R^2 = 0.9870; Adjusted R^2 = 0.9710; Q^2 = 0.8260; p-value<0.05 indicates statistical significance.

Table 4.4 Regression coefficients and p-values for the recovery of neutralized rice bran oil after backward elimination.

Source [1]	Regression coefficients	p-value [2]
Constant	79.988	0.0000
X_1	-0.2577	0.0010
X_2	0.1652	0.0010
X_1*X_2	0.0911	0.0120
X_1*X_3	0.0775	0.0261
X_1^2	-0.1310	0.0266

[1] X_1, X_2, and X_3 denote NaOH concentration (°Be), temperature (°C), and time (min), respectively.

[2] p<0.05 indicates statistical significance

Table 4.5 Regression coefficients and p-values for FFA in neutralized rice bran oil after backward elimination.

Source [1]	**Regression coefficients**	**p-value** [2]
Constant	0.3157	0.0000
X_1	-0.1343	0.0000
X_2	0.0386	0.0119
X_3	-0.0437	0.0066
X_1*X_3	-0.0462	0.0026
X_1^2	0.0967	0.0009
X_2^2	0.0493	0.0255

[1] X_1, X_2, and X_3 denote NaOH concentration (°Be), temperature (°C), and time (min), respectively. [2] $p<0.05$ indicates statistical significance.

Table 4.6 Regression coefficients and p-values for gamma-oryzanol in neutralized rice bran oil after backward elimination

Source [1]	**Regression coefficients**	**p-value** [2]
Constant	0.9280	0.0000
X_1	-0.1462	0.0000
X_2	-0.0207	0.0183
X_3	-0.0283	0.0046
X_1*X_2	0.0195	0.0015
X_1*X_3	-0.0156	0.0186
X_1^2	-0.0556	0.0005
X_3^2	-0.0331	0.0084

[1] X_1, X_2, and X_3 denote NaOH concentration (°Be), temperature (°C), and time (min), respectively. [2] $p<0.05$ indicates statistical significance.

4.5.2 Effect of neutralization parameters on oil recovery

The removal of FFAs causes the loss of neutralized rice bran oil. In the neutralization process, FFAs are removed as sodium soaps but some neutralized oil is entrapped in the emulsion and removed during centrifugation. Table 4.4 presents the effects of neutralization parameters on the oil recovery. The oil recovery was negatively affected by X_1 (NaOH concentration) and X_2 (temperature). The linear terms X_1 and X_2, the quadratic terms X_1^2, and the interaction terms X_1X_2, X_1X_3 had significant effects on the oil recovery ($p<0.05$). On the other hand, X_3, X_2^2, X_3^2, and X_2X_3 had no significant effect on the oil recovery ($p>0.05$).

In Figure 4.1 (A), the maximum oil recovery was achieved under the NaOH concentration (X_1) of 19.25°Be and temperature (X_2) of 78.36°C. In Figure 4.1 (B), lower NaOH concentrations (5.0-19.05°Be) and shorter times (5.0-11.28min) yielded greater oil recovery, while increased NaOH concentrations and time reduced the oil recovery. Specifically, increased NaOH concentrations accelerated the formation of rice bran soap and trapped oil, reducing oil discharge (Gunstone et al., 1994; Casimir, 2003; Sayre et al., 1985). Similar results were also reported on neutralization of sun flower and soybean oils due to higher alkali-refining loss (Lasztity, 1999).

In Figure 4.1 (C), the maximum recovery was achieved under 75°C and 10.63 min condition. Specifically, elevated temperatures (from 60 to 90°C) and extended time (from 5 to 15min) had a positive effect on oil recovery. However, high temperatures (90ºC) were ineffective in neutralizing FFA, contributing to gamma-oryzanol loss (Table 4.2). This is consistent with Wei et al. (2015), who documented that acids value slightly increased while bioactive compounds (sterols, α-

tocopherol, and Vitamin D3) were significantly reduced at high neutralization temperatures (85ºC) after 30min. According to Lee et al. (2014); Chew et al. (2017), neutralization was normally undertaken between 60-75°C and 10-50min to allow for the complete reaction of FFA with NaOH solution.

4.5.3 Effect of neutralization parameters on FFA

FFA content is used to determine the edibility and deterioration of oils and fats. The presence of FFA and other fatty materials in oil led to offensive odor and taste caused by enzymatic hydrolysis during processing and storage (Chemat et al., 2004; Oluremi et al., 2013). In this research, the variables that affected the removal of FFA were the linear terms X_1, X_2, X_3, the quadratic terms X_1^2, X_2^2, and the interaction term X_1X_3 (Table 4.5).

In Figure 4.2 (A), FFA was reduced from 0.79 to 0.28% when NaOH concentration (X_1) and temperature (X_2) were increased to 19.26°Be and 78.36°C, given X_3 of 10min. The neutralization efficiency nevertheless was lowered beyond the 19.26°Be (X_1) and 78.36°C (X_2) threshold. In Figure 4.2 (B), elevated NaOH concentrations (X_1, from 10 to 19.29°Be) and extended time (X_3, from 5 to 11.19min) decreased FFA to 0.32%. Specifically, NaOH concentrations (X_1) had more impact on the neutralization of rice bran oil. Similar results were reported for neutralization of kenaf seed oil (Chew et al., 2017) in which excessively low or high levels of NaOH increased FFA. As a result, the FFA content in neutral oil was lower than that of CODEX standard (0.4%). In Figure 4.2 (C), higher temperatures (X_2) and extended time (X_3) reduced the efficiency of FFA removal.

4.5.4 Effect of neutralization parameters on gamma-oryzanol

In Table 4.6, the linear terms X_1, X_2 and X_3, the interaction terms X_1X_2, X_1X_3, and the quadratic terms X_1^2, X_3^2 significantly impacted the gamma-oryzanol content ($p<0.05$). Specifically, X_1 and X_3 significantly lowered gamma-oryzanol content. This is consistent with Engelmann et al. (2017); Gopala Krishna et al. (2001), who reported that the decrease in gamma-oryzanol was accelerated by increased NaOH concentrations and process time.

Figure 4.3 (A) illustrates the interaction between X_1 and X_2 on the gamma-oryzanol content in NRBO, in which the bioactive decreased significantly ($p<0.05$) with increase in X_1 (from 15 to 30°Be) and X_2 (from 80 to 90°C). The maximum gamma-oryzanol content was achieved under 19.86°Be (X_1) and 78.36°C (X_2) condition. In Figure 4.3 (B), gamma-oryzanol decreased as X_2 and X_3 increased from 75.34 to 90°C and 10.62 to 15min, given X_1 of 20°Be. Specifically, under higher temperatures and extended time, gamma-oryzanol in NRBO was reduced. In Figure 4.3 (C), given X_2 of 75ºC, the elliptical response surface plot indicated that the interaction between X_1 and X_3 was significant ($p<0.05$). The maximum gamma-oryzanol content was achieved under 19.51°Be (X_1) and 11.18min (X_3) condition.

4.5.5 Optimization of the neutralization parameters and model validation

Based on the CCF design, the optimal neutralization condition that achieved high oil recovery and quality was that of 19.24°Be for NaOH concentration (X_1), 74.79°C for temperature (X_2), and 11.18min for process time (X_3). Under the optimal condition, the predicted oil recovery, FFA, and gamma-oryzanol were 80.00%, 0.31%, and 1.00%, respectively. Meanwhile, the experimental results for the oil recovery, FFA, and gamma-oryzanol were 80.12%, 0.29%, and 1.02%. The

predicted and experimental data were in good agreement, suggesting that the response models could be applied to optimizing the RBO neutralization parameters for enhanced oil yield and quality.

4.5.6 Physiochemical properties and fatty acid profile

Table 4.7 compares the fatty acid profile and physicochemical properties of CRBO, DRBO, and NRBO under the optimal neutralization condition (19.24°Be, 74.79°C, and 11.18min). In this study, CRBO was obtained by thermal cooking followed by cold press extraction. Since CRBO contains impurities, particularly phospholipids which have unfavorable effects on the flavor and shelf life of the oil, the compounds were thus removed by degumming (Zufarov et al., 2008).

Fatty acid profile is an important indicator of the nutritive value and quality of the oil. According to Oluremi et al. (2013); Pena Muniz et al. (2015), high-quality RBO contains high percentages of unsaturated fatty acids, mainly oleic and linoleic. In this study, oleic acid (C18:1) and linoleic acid (C18:2) were dominant fatty acids, followed by palmitic acid (C16:0). In addition, the degumming and neutralization had minimal impact on the quality of oil in terms of fatty acid composition. This is consistent with Chew et al., (2017); Karabulut et al., (2005).

The peroxide value, iodine value, free fatty acid (FFA), saponification value, gamma-oryzanol value, and relative density of DRBO were not significantly different from those of CRBO ($p>0.05$). However, Ortega-García et al. (2006) documented that peroxide value of degummed safflower oil was higher than that of its crude oil, probably due to the presence of moisture and high temperature (70°C) during degumming, promoting the primary oxidation compounds. In this study, the phosphorus value of DRBO (33.59 mg/kg) was drastically decreased, compared with

CRBO (318.52 mg/kg) ($p<0.05$). The finding suggested that citric acid degumming process at 70°C effectively removed phospholipids from CRBO, consistent with Smiles et al. (1988); Zufarov et al. (2008). Mei et al. (2013) also reported that the phosphorus value of *Silybum marianum* seed oil was reduced by 53.1% after citric acid degumming. In addition, the melting point of DRBO was lower than CRBO's. The presence of impurities (phospholipids, wax, and peroxide) in CRBO contributed to higher melting point (Taghvaei et al., 2013) .

The peroxide value, FFA, saponification value, gamma-oryzanol content, phosphorus value, and melting point of NRBO were significantly lower than those of CRBO and DRBO ($p<0.05$). In comparison with CRBO, the phosphorus value, FFA, and gamma-oryzanol of NRBO were reduced by 98.03%, 91.12%, and 44.75%, respectively. The reduction in phosphorus value, FFA, and gamma-oryzanol was attributable to the formation of soapstock (De at al., 2010; Van Hoed et al., 2006). The lower saponification value and melting point were probably due to the absence of impurities in NRBO (Taghvaei at al., 2013). Nevertheless, the neutralization had no effect on the iodine value and relative density of RBO, consistent with Oluremi et al. (2013).

Total phenolic contents (TPC) and α-tocopherol were slightly reduced in degumming and a significant reduction in neutralization of the rice bran oils. The TPC and α-tocopherol of CRBO and DRBO were 4.39 mg GAE/g oil, 200ppm and 4.35 mg GAE/g oil, 197ppm, respectively whereas NRBO was 3.18 mg GAE/g oil and 130ppm. This reduction was due to the formation of soapstock. Similar results were reported by for palm oil (Prasanth Kumar and Gopala Krishna, 2014; Czerniak and Łaszewska, 2015), and olive oil (Nergiz, 1993). Nergiz. (1993) reported that some

bioactive compounds of oil (i.e., phenolic compounds and α-tocopherol) were almost destroyed during neutralization refining (95.01 mg/L to 64.03 mg/L). According to Prasanth Kumar and Gopala Krishna. (2014); Čmolík et al. (2000) the decline of α-tocopherol may be due to the fact that α-tocopherol is unstable in the presence of longer contact time with air and alkali.

During the degumming and neutralization process, chlorophyll, waxes, and phospholipids decomposes and is absorbed through soapstock which increase the bright oil colored (Sabah, 2007). The NRBO had the high lightness (L*) values (23.12±0.15) (p<0.05), wheares CRBO and DRBO had a low L^* value (22.59±0.05 and 22.79±0.11). The L* parameter color value obtained indicates that CRBO is darker than NRBO. The NRBO possess the highest yellow (b^*) value (4.91±0.16) (p<0.05), while there was non-significant changes in a^* value, which related to tonality of color (from red to greenness) of DRBO and NRBO in comparison with CRBO. The results obtained are quite similar with previous research in other type of oil ((Lamas et al., 2016; Lamas et al., 2016).

4.6 Conclusions

Nowadays, market competition is forcing the oil producers to optimize the refining process for reducing oil loss and quality. Thus, this research investigated the effect of neutralization parameters on the oil recovery (Y_1), FFA level (Y_2), and gamma-oryzanol content (Y_3) of neutralized rice bran oil, using response surface methodology (RSM) with central composite face-centered design. The quadratic RSM models were generated and statistical analysis performed to give an insight the effects of processing parameters. The ANOVA results showed that regression models were statistically good with a significance level of p<0.0001 and the model had no

significant lack of fit ($p>0.05$). Therefore, the well-fitting model was successfully generated with a good predictor of all experimental results. The optimal condition for chemical neutralization was 19.24°Be, 74.79°C, and 11.18min. The predicted and experimental results were agreeable in which the predicted oil recovery, FFA level, and gamma-oryzanol were 80.00%, 0.31%, and 1.00% under the optimal condition, while the corresponding experimental results were 80.12%, 0.29%, and 1.02%. The findings verified high predictive ability of the response models and thus could be deployed for optimization of rice bran oil neutralization parameters to enhance the oil yield and quality.

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Table 4.7 Fatty acid profile and physicochemical properties of crude rice bran oil (CRBO), degummed rice bran oil (DRBO), and neutralized rice bran oil (NRBO).

Quality of oil	**CRBO [1]**	**DRBO [2]**	**NRBO [3]**
1. Fatty acid profile (%)			
Myristic (C14:0)	3.59	3.57	3.43
Palmitic (C16:0)	16.75	16.51	17.11
Stearic (C18:0)	3.45	3.58	3.44
Oleic acid (C18:1)	41.91	41.59	42.04
Linoleic (C18:2)	24.19	24.49	24.21
Dihomo γ-linolenic (C20:3)	4.59	4.46	4.43
Heneicosylic (C21:0)	4.52	4.62	4.34
Nevonis (C24:0)	1.00	1.10	1.00
Saturated fatty acid	29.31	29.38	29.32
Unsaturated fatty acid	70.69	70.62	70.68
2. Physicochemical properties			
Peroxide Value ($meqO_2/kg$)	5.27^{a}±0.03	5.29^{a}±0.05	5.31^{b}±0.05
Iodine value (g I_2/100g)	99.98±1.22	99.72±0.50	99.96±0.25
Free fatty acid (%oleic acid)	4.44^{b}±0.05	4.37^{b}±0.25	0.31^{a}±0.15
Saponification value (mgKOH/g oil)	176.98^{b}±0.98	176.68^{b}±0.50	173.12^{a}±0.50
Gamma-oryzanol value (%)	1.81^{a}±0.02	1.80^{a}±0.03	1.00^{b}±0.25
Phosphorus value (mg/kg)	318.52^{c}±1.04	33.59^{b}±0.15	6.25^{a}±1.15
Relative density	0.92±0.01	0.92±0.02	0.91±0.25
Melting point (°C)	11.25^{c}±0.02	10.05^{b}±0.01	9.90^{a}±0.50
Total phenolic (mg GAE/g oil)	4.39^{a}±0.04	4.35^{a}±0.04	3.82^{b}±0.03
α-tocopherol (mg/L)	95.01±1.54	88.03±1.04	64.43±0.89

Table 4.7 Fatty acid profile and physicochemical properties of crude rice bran oil (CRBO), degummed rice bran oil (DRBO), and neutralized rice bran oil (NRBO) (Continue).

Quality of oil	**CRBO [1]**	**DRBO [2]**	**NRBO [3]**
3. Color of rice bran oil			
L^*	22.59^{c}±0.05	22.78^{b}±0.11	23.12^{a}±0.15
a^*	4.23^{b}±0.11	4.13^{a}±0.09	4.15^{a}±0.10
b^*	3.53^{c}±0.10	4.86^{b}±0.12	4.91^{a}±0.16

[1,2,3] Different letters in each row of physicochemical properties denote statistically significant differences between treatments ($p<0.05$). The values are mean of three replications ± standard deviation.

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(A)

(B)

(C)

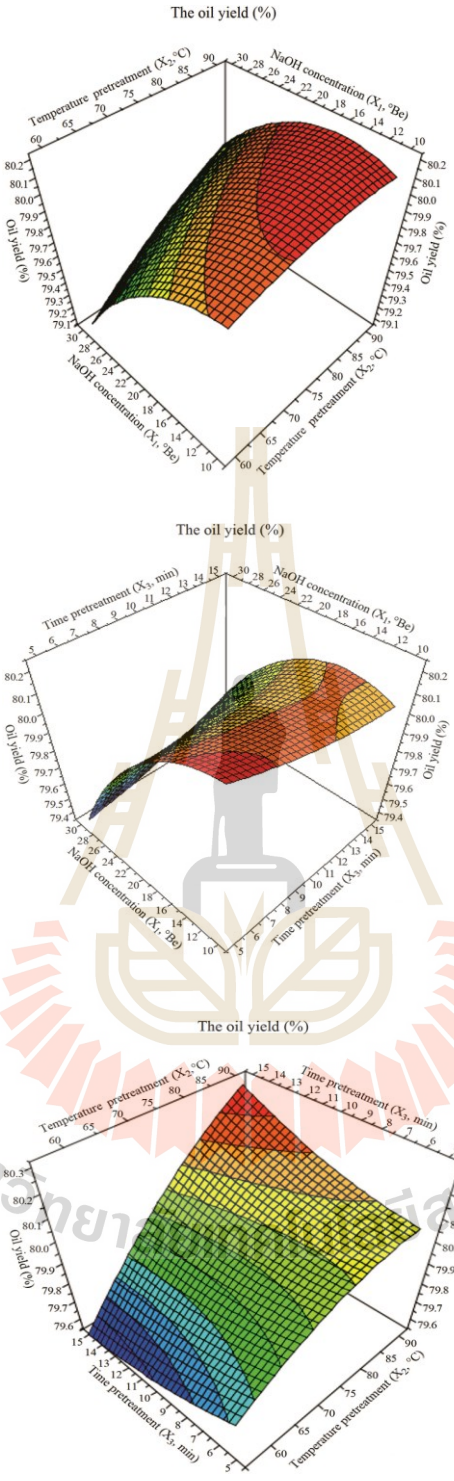

Figure 4.1 Response surface plots of interaction between: (A) NaOH concentration (X_1) and temperature (X_2); (B) NaOH concentration (X_1) and time (X_3); (C) temperature (X_2) and time (X_3) on the neutralized rice bran oil yield.

(A)

(B)

(C)

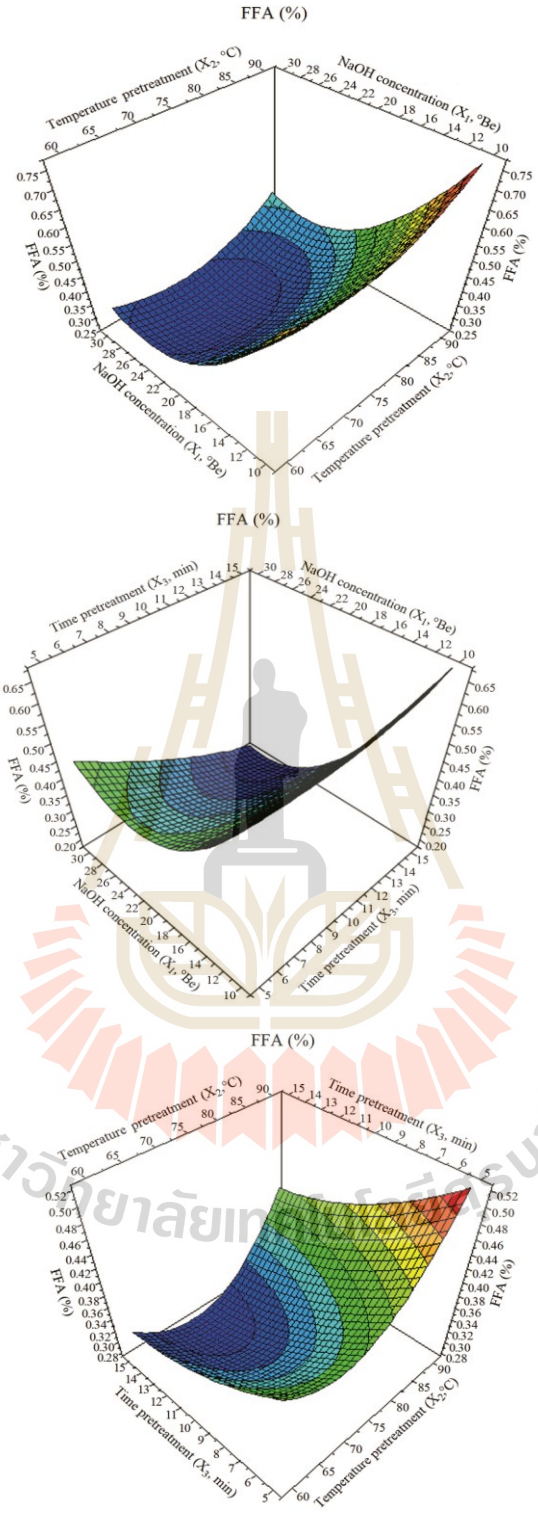

Figure 4.2 Response surface plots of interaction between: (A) NaOH concentration (X_1) and temperature (X_2); (B) NaOH concentration (X_1) and time (X_3); (C) temperature (X_2) and time (X_3) on the FFA of neutralized rice bran oil.

(A)

(B)

(C)

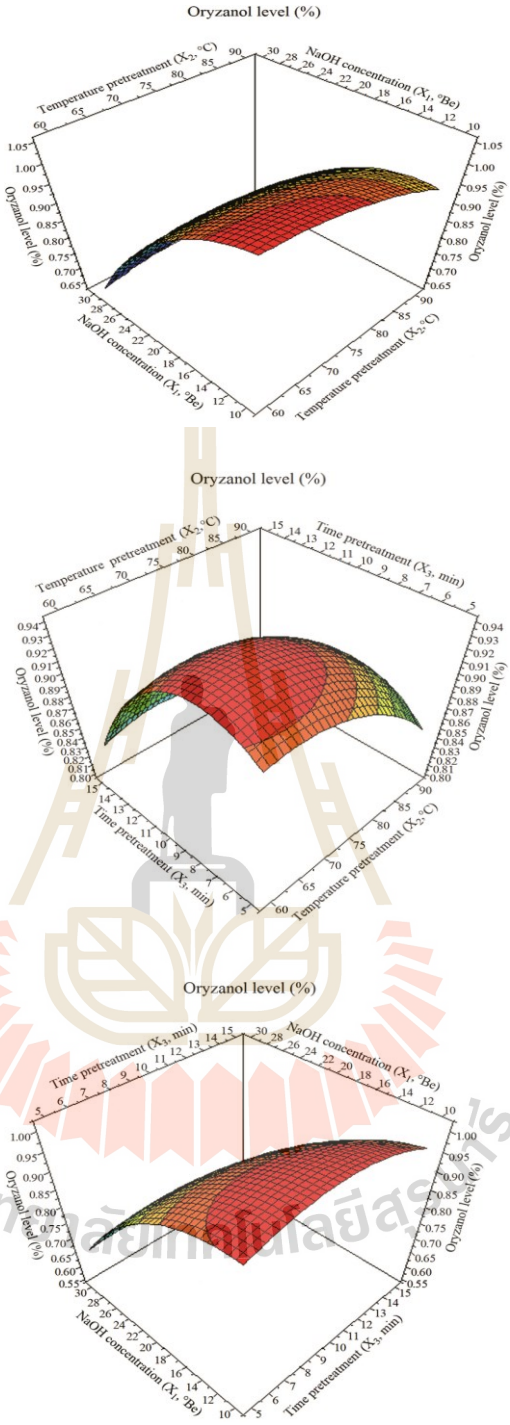

Figure 4.3 Response surface plots of interaction between: (A) NaOH concentration (X_1) and temperature (X_2); (B) temperature (X_2) and time (X_3); (C) NaOH concentration (X_1) and time (X_3) on the gamma-oryzanol content of neutralized rice bran oil.

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CHAPTER V

OPTIMIZATION OF PROCESS PARAMETERS AND KINETIC MODEL OF ULTRASOUND ASSISTED SOLVENT EXTRACTION GAMMA-ORYZANOL FROM DRIED RICE BRAN SOAPSTOCK

5.1 Abstract

Gamma-oryzanol extraction from dried rice bran soapstock (DRBS) was performed using the ultrasound assisted solvent extraction (UASE) technique with a mixture solvent of ethyl acetate and ethanol. Response surface methodology (RSM) with a face-centered composite design (FCCD) was used to investigate the optimal extraction conditions including ultrasound power (X_1) (0.5-4.5 W/g), temperature (X_2) (35-55ºC), and extraction time (X_3) (4-26min). The quadratic response model was generated and statistical analysis performed to validate the model. The maximum gamma-oryzanol recovery was found to be 98.03% at the optimal extraction conditions of 4.0 W/g, 50.0ºC, and 21.5min. The predicted value of gamma-oryzanol recovery was 98.15%, suggesting in good agreement with the experimental data. In addition, the extraction experiments were performed for describing the kinetics behavior of UASE of gamma-oryzanol from the DRBS under selected conditions and results were well correlated using a second-order kinetic model. The theoretical gamma-oryzanol concentration at saturation (C_s), extraction rate constant (k), and

initial rate of extraction (*h*) at various ultrasound powers and temperatures ranging from 0.5-4.5 W/g and 35-55ºC, respectively, and extraction time of 21.5 min were determined. The obtained results from the predicted models were consistent with experimental value (R^2>0.97) and suggesting that the high ultrasound power and moderate temperature provided a best performance on gamma-oryzanol extraction. In addition, sonication time of 21.5min was sufficient to obtain the maximum gamma-oryzanol recovery.

Keywords: Optimization, gamma-oryzanol, rice bran soapstock, response surface methodology, kinetic model.

5.2 Introduction

Rice bran oil is an excellent source of essential nutrients, including fatty acids, vitamin E (tocopherols and tocotrienols), vitamin K, and gamma-oryzanol (Lerma-García et al., 2009; Patel and Naik, 2004; Pestana-Baueret al., 2012; Gunstone, 2011). In particular, gamma-oryzanol helps reduce plasma cholesterol (Wilsonet et al., 2007), platelet aggregation, and cholesterol absorption (Sharma and Rukmini, 1986). Gamma-oryzanol is also used in cosmetic products for its anti-itching and anti-dandruff properties (Seetharamaiah and Prabhakar, 1986).

However, rice bran oil in crude form is high in free fatty acids (FFA) and wax (Gunstone, 2011), requiring chemical refining (i.e., degumming, neutralization, bleaching, and deodorization) to remove the undesirable compounds. Specifically, chemical neutralization removes FFA by using aqueous sodium hydroxide (NaOH) to form soapstock, trapping neutralized rice bran oil and gamma-oryzanol. According Gopala Krishna et al. (2001), nearly 90% of gamma-oryzanol loss occurred through wet soapstock.

At present, soapstock is mostly converted into toiletry and detergent products (Narayan et al., 2006), feedstock (Ju and Vali, 2005), and lecithin products (Thurman, 1961). In fact, gamma-oryzanol present in rice bran soapstock holds promising potential for commercial-scale production (Narayan et al., 2006). Thus, several authors focused their attention on gamma-oryzanol extraction from rice bran soapstock. In 2009, Kumar et al. investigated the extraction of gamma-oryzanol from dried rice bran soapstock using various solvents (ethyl acetate, ethyl methyl ketone, hexane, acetone, and isopropanol) assisted by conventional extraction. The authors recognized ethyl acetate was most suited for gamma-oryzanol extraction (97-99% yield under 6 hour extraction) probably due to low polarity and viscosity of this solvent. In 2010, Kaewboonnum et al. also extracted gamma-oryzanol from rice bran soapstock using ethyl acetate because of the most suitable extraction solvent. Even though ethyl acetate is accepted to use in food and pharmaceutical due to its low toxicity, ethanol is used as a nontoxic and an ecologically-friendlier replacement to extract vegetable oil and antioxidant compounds (Franco et al., 2009; Moreau and Hicks, 2005). Interestingly, there is no study on gamma-oryzanol extraction from rice bran soapstock using a mixture of ethyl acetate and ethanol as solvent extraction. Therefore, in this study the possibility of replacing ethyl acetate with ethanol to be a mixture solvent for gamma-oryzanol extraction from the DRBS was investigated.

Conventional extraction technique is time-consuming. Thus, it is necessary to find a novel technique for extracting gamma-oryzanol economically. Ultrasound assisted extraction is one of the most promising alternative extraction method because of the advantages of lower process temperatures and extraction time, ease of operation, and high efficiency (Zhang, Yang, and Wang, 2011; Gayas and Kaur, 2017). The difference between ultrasound assisted extraction and conventional solvent extraction

method is here mechanical cavitation and thermal effects. Specifically, mechanical cavitation can produce physical effects (liquid turbulence and liquid circulation) which accelerated diffusion of solvent in particles, disrupted of cell wall, and enhanced the mass transfer efficiency of target compounds in the solvent, and thus increase extraction yield. (Vetal, Lade, and Rathod, 2013). Although the previous studies have sucessfully extracted gamma-oryzanol from rice bran soapstock using conventional solvent extration (Venkatadri and Sreesaila, 2005; Kumar et al., 2009; Seetharamaiah and Prabhakar, 1986; Kaewboonnum et al., 2010), the advanced technique such as ultrasound assisted extraction has not been applied.

The extraction efficiency of the gamma-oryzanol is closely related to the UASE operating parameters, these factors having as well a high influence on its kinetics. Kinetic studies help in understanding various factors that affected to the extraction process. Nowadays, many kinetic models of solid-liquid extraction for oil and polyphenols from natural sources were carried out such as the second-order kinetic model (Lazar et al., 2016; Rakotondramasy-Rabesiakaet al., 2009), Fick's second law (Petrović et al., 2012), film theory (Bora et al., 2008), or Peleg's model (Jesus et al., 2010). However, the second-order kinetic model of ultrasound assisted gamma-oryzanol extraction using a mixture solvent is still not well evaluated.

Thus, this research aims to investigate the effect of ultrasound assisted solvent extraction, using a mixture of ethanol and ethyl acetate, on gamma-oryzanol recovery from dried rice bran soapstock. Response surface methodology (RSM), specifically face-centered composite design (FCCD), was used for optimize the extraction conditions considering the effective variables of ultrasound power, temperature, and time on the maximum yield of gamma-oryzanol. In this study, ultrasound power was varied between 0.5, 2.5, and 4.5 W/g, extraction temperature between 35, 45, and

55ºC, and extraction time between 4, 15, and 26min. In addition, a second-order kinetic model was used to study the kinetics of the UASE of gamma-oryzanol under selected conditions.

5.3 Objectives

This study was designed to investigate the effect of ultrasound assisted solvent extraction on the gamma-oryzanol recovery.

5.4 Materials and Methods

5.4.1 Materials

Rice bran oil wet soapstock was first pretreated following Venkatadri and Sreesaila (2005), whereby 1.25%wt NaOH solution was added and reacted at 80°C for 15-30min with constant stirring until the moisture content was 55.0%. The soapstock was then centrifuged and decanted prior to dehydration in vacuum evaporator (100ºC, 550mmHg) for 2-3 hours to obtain dried rice bran soapstock (DRBS) with 4.0-4.5% moisture content and used as the starting material for extraction.

Methanol (Mallinckrodt), *n*-hexane, ethanol (<98.5%), and ethyl acetate absolute value (99.5%) were from Sigma-Aldrich (Darmstadt, Germany), and NaOH anhydrous pellets (98%) from Carlo Erba reagent (Burkina Faso, France).

5.4.2 Experimental methods

5.4.2.1 Soxhlet extraction (SE)

A 20 g DRBS in thimbles was extracted with ethyl acetate at 90ºC for 4 hours (Kaewboonnum et al., 2010) with a solvent to solid ratio of 10:1 (Kumar et al., 2009). Gamma-oryzanol rich fraction was then centrifuged at 10,000

rpm and 10ºC for 10min to remove solids, and the solvent vacuum-evaporated (55°C, 500 mmHg) to dryness. The resulting gamma-oryzanol content was 4.41±0.21 g/100 g DRBS (control 1).

5.4.2.2 Gamma-oryzanol extraction by mixed solvent extraction

A 20 g DRBS was extracted by a mixture of ethanol in ethyl acetate (MEE), where ethanol content was varied between 5%, 10%, 15%, 20%, and 25%. The solvent to solid ratio was 10:1 (Kumar et al., 2009), and the extraction was carried out at 90ºC for 4 hours. Gamma-oryzanol rich fraction was then centrifuged and the solvent evaporated. The optimal ethanol content in MEE provided a maximum gamma-oryzanol recovery was used as a control 2.

5.4.2.3 Ultrasound assisted solvent extraction

An ultrasonic generator with 40 kHz operating frequency and 750 W ultrasonic power (VCX750 Vibracell; Sonic and Materials, Inc., Newtown, CT, USA) was used in ultrasound assisted solvent extraction (UASE). In extraction, 20 g DRBS in 500 mL flask was mixed with 200 mL MEE at optimal ethanol concentration. The mixtures were ultrasound-treated, given the ultrasound power (X_1) of 0.5-4.5 W/g, extraction temperature (X_2) of 35-55ºC, and sonication time (X_3) of 4-26min, with 5 s pulse duration. Gamma-oryzanol rich fraction was then centrifuged at 10,000 rpm for 10 min, and vacuum-evaporated (55°C, 500 mmHg) to dryness. The gamma-oryzanol recovery was determined in relation to control 2, as expressed in Eq. 1:

$$\text{Gamma-oryzanol recovery (\%)} = \frac{\text{Weight of recovered gamma-oryzanol}}{\text{Control 2}} \times 100 \qquad (5.1)$$

5.4.2.4 Experimental design for gamma-oryzanol extraction from dried rice bran soapstock

A face-centered composite (FCCD) design was used in the implementation of response surface methodology (RSM) to optimize the extraction parameters, using MODDE version 5.0. The extraction parameters (independent variables) included ultrasound power (X_1), extraction temperature (X_2), and sonication time (X_3), and each parameter was varied between low, moderate, and high, coded -1, 0, and +1, respectively (Table 5.1). In Table 5.2, the design of experiment consisted of 17 experimental runs in 3 replicates, and the response (*Y*) were averages.

Table 5.1 Extraction parameters (independent variables) of the face-centered central composite design (FCCD).

Symbol	Variables	Levels		
		-1	**0**	**+1**
X_1	Ultrasound power (W/g)	0.5	2.5	4.5
X_2	Extraction temperature (°C)	4	15	26
X_3	Sonication time (min)	35	45	55

A second order polynomial model was applied to predict the gamma-oryzanol recovery: (*Y*)

$$f(Y) = \beta_0 + \sum_{n=1}^{k} \beta_i X_i + \sum_{n=1}^{k} \beta_{ii} X_i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j \quad (5.2)$$

where Y is the predicted response; β_0 is a constant; X_i and X_j are independent variables, β_i and β_{ij} are the linear coefficients, and cross coefficients; β_{ii} is the

quadratic coefficients. The model adequacy was evaluated by lack of fit, F-test, and coefficient of determination (R^2) by analysis of variance (ANOVA) that was generated by MODDE version 5.0. Model validation was subsequently carried out. The statistical difference was at 5% significance level ($p<0.05$).

5.4.2.5 Kinetic model of ultrasound assisted solvent extraction

A deeper analysis of ultrasound powers and extraction temperatures of gamma-oryzanol yield were investigated by the study of kinetic of extraction. The second-order kinetic model was applied to determine the kinetic parameters following Lazar et al. (2016) method. The experimental tests included: (i) ultrasound powers (0.5, 2.5, and 4.5 W/g), carrying on the extraction temperature up to 45ºC; (ii) temperatures (35°C, 45°C, and 55°C), employing the ultrasound at 2.5 W/g. Mixture of ethyl acetate and ethanol at 85:15 (v/v) was used as an extraction solvent. The experiments were performed at sonication time from 0-21.5min.

According to Lazar et al. (2016), the rate of dissolution of gamma-oryzanol extraction from the DRBS can be described by the second-order model as shown in Eq. 3.

$$\frac{dC_t}{dt} = k(C_s - C_t)^2 \quad (5.3)$$

Table 5.2 Experimental and predicted gamma-oryzanol recovery under variable ultrasound powers (X_1), extraction temperatures (X_2), and sonication times (X_3).

Run	Coded variable						Experimental value	Predicted value
	X_1	X_2	X_3	X_1	X_2	X_3	(%)	(%)
1	-1	-1	-1	0.5	35	4	73.21±1.21	72.92±1.01
2	1	-1	-1	4.5	35	4	82.89±0.95	82.37±0.93
3	-1	1	-1	0.5	55	4	86.12±1.04	85.90±0.64
4	1	1	-1	4.5	55	4	91.99±1.45	91.89±1.02
5	-1	-1	1	0.5	35	26	86.37±1.00	86.04±0.89
6	1	-1	1	4.5	35	26	90.82±1.34	90.61±1.01
7	-1	1	1	0.5	55	26	95.56±1.61	95.31±1.45
8	1	1	1	4.5	55	26	96.46±1.07	96.42±0.84
9	-1	0	0	0.5	45	15	90.20±1.41	90.96±1.31
10	1	0	0	4.5	45	15	95.27±0.94	96.24±1.08
11	0	-1	0	2.5	35	15	85.18±1.91	86.54±1.75
12	0	1	0	2.5	55	15	95.19±1.15	95.94±1.01
13	0	0	-1	2.5	45	4	86.50±1.21	87.63±1.04
14	0	0	1	2.5	45	26	95.84±1.59	96.45±1.45
15	0	0	0	2.5	45	15	95.27±1.81	94.59±1.41
16	0	0	0	2.5	45	15	95.27±1.40	94.59±1.03
17	0	0	0	2.5	45	15	96.71±1.24	94.58±1.05

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where C_t is the concentration of the gamma-oryzanol (g/L) at a given extraction time t (min). k is the second-order extraction rate constant (L/g.min), and C_s is the concentration of gamma-oryzanol at saturation in the liquid extraction (g/L). To determine the kinetic parameters, the integrated rate law of a second-order extraction under the boundary conditions $C_t = 0$ to C_t and $t = 0$ to t, can be written as Eq. 4 or a linearized Eq. 5:

$$C_t = \frac{C_s^2.k.t}{1+C_s.k.t} \tag{5.4}$$

$$\frac{t}{C_t} = \frac{1}{k.C_s^2} + \frac{t}{C_s} = \frac{1}{h} + \frac{t}{C_s} \tag{5.5}$$

where h is initial extraction rate (g/L.min) when t and C_t approach 0 and can be defined as (Pan et al., 2011):

$$h = k.C_s^2 \tag{5.6}$$

The C_s and k were determined from experimentally from the slope and intercept by plotting t/C_t against t.

5.4.3 Analytical method

5.4.3.1 Quantitative analysis of gamma-oryzanol content

ORF content was analyzed by UV-spectrophotometry/NIR (Shimazu, UV-2600, Japan) (Joshi et al., 2016), whereby ORF was dissolved in hexane and absorptions at multi wavelength between 200-400 nm were measured. Gamma-oryzanol was determined by high-performance liquid chromatography (HPLC)/UV following Sakunpaket et al. (2014). The HPLC/UV composed a pump

(LPG 3X00), auto-sampler (ACC-3000), diode-array UV/VIS detector (DAD), and a Poroshell 120 EC-C 18 column (3.0 mm×150 mm, 2.7µm). The UV detector and the column temperature were 325 nm and 25°C, respectively. The mobile phase of 100:0 (v/v), 50:50 (v/v), and 40:60 (v/v) methanol and acetonitrile was carried out sequentially for 5, 10, and 15 min each, with gradient elution at a flow rate of 1.0 mL/min.

5.4.3.2 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM JEOL, JSM 6010 LV, Technology Development Ltd., Japan) was used to characterize the effect of ultrasound on the morphology of dried rice bran soapstock. In the analysis, untreated and treated dried rice bran soapstock were placed on a metal stub and gold-sputtered. The shape and surface characteristics of untreated and treated DRBS were determined. The most representative images were selected at 600x magnification.

5.4.3.3 Data statistical analysis

The entire analysis was performed in triplicate and the averages and standard deviations were computated in Stagraphic centrution XV (Statsoft Inc., USA) and Modde 5.0 (Umea, Sweeden). A one-way analysis of variance (ANOVA) was carried out to assess the effects of extraction process on the ORF recovery. Tukey-HSD test was used to compare the means, given the 5% significance level ($p<0.05$).

5.5 Results and discussions

5.5.1 Mixed solvent extraction

In this study, ethanol was chosen as a replacing ethyl acetate solvent for gamma-oryzanol extraction from the DRBS, taking into account its recommendation as an eco-friendly and safe solvent for use in food. Therefore, mixtures of ethyl acetate with ethanol at various concentrations (0-25% v/v) were studied to obtain an optimal gamma-oryzanol extraction. Table 5.3 presented the effect of introduced ethanol into ethyl acetate as a mixture of ethanol in ethyl acetate (MEE) on the gamma-oryzanol recovery from the dried rice bran soapstock (DRBS) using conventional technique (Soxhlet extraction, SE). Replacing ethyl acetate with ethanol from 0 to 15% (v/v) did not affect the efficiency of gamma-oryzanol extraction yields (4.39 to 4.41 g/100 g DRBS) ($p>0.05$). However, higher concentrations of ethanol (>15% v/v) in the MEE significantly reduced the yield of gamma-oryzanol ($p<0.05$). This was probably because high polarity and viscosity of ethanol affecting the efficiency of gamma-oryzanol extraction. The varying ratios of the MEE modify the solvent polarity influencing the solubility of gamma-oryzanol, as the MEE could not penetrate the DRBS where hydrophobicity is prevalent. In addition, a high viscosity of solvent resist mass transfer, hence it could not effectively extract gamma-oryzanol (Kumar et al., 2009; Lebovka et al., 2012). According to previous statements, some authors indicated the lower selectivity of alcohol towards gamma-oryzanol due to less solubility of gamma-oryzanol in the solvent mixture (Narayan et al., 2006; Antonio, et al., 2011), which was in agreement with our results. For further experiment, the replacement of ethyl acetate with ethanol by 15% in the MEE was considered suitable for study the optimizing the parameters of ultrasound assisted solvent extraction

(UASE) of gamma-oryzanol extraction from the DRBS. Under this concentration is suitable in view of possible application of the gamma-oryzanol extractions in food industries because it is not only a safe solvent, but it is also cheaper when compared with the ethyl acetate.

Table 5.3 Effect of ethanol concentration in the MEE on gamma-oryzanol extraction from dried rice bran soapstock.

Ethyl acetate/ethanol ratio (v/v)	**100:0**	**95:5**	**90:10**	**85:15**	**80:20**	**75:25**
Gamma-oryzanol (g/100**g** DRBS)	$4.41^{a}\pm$ 0.26	$4.40^{a}\pm$ 0.15	$4.41^{a}\pm$ 0.29	$4.39^{a}\pm$ 0.15	$4.05^{b}\pm$ 0.31	$4.05^{b}\pm$ 0.37

Different letters in each row denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications ± standard deviation.

5.5.2 Evaluation of regression for gamma-oryzanol extraction

In this study, the response surface methodology (RSM) with face-centered central composite design (FCCD) was used to determine the optimum conditions for the ultrasound assisted solvent extraction (UASE) of gamma-oryzanol from the dried rice bran soapstock. The variables used were ultrasound power (W/g, X_1), extraction temperature (X_2) and sonication time (min, X_3). The relationships between these three variables on gamma-oryzanol yield also were evaluated. The experimental and predicted values were given in Table 5.2. The recovery of gamma-oryzanol obtained from the DRBS was 73.21-96.71%.

The statistical significance of each coefficient was determined by using the F-test, p-value, and lack of fit (LOF) obtained from the analysis of variance (ANOVA). Table 5.4 showed the ANOVA results of the fitted quadratic models on the response of gamma-oryzanol recovery. The high F-values for gamma-oryzanol recovery (45.38) and very low p-value ($p<0.05$) indicated the model terms were significant. Quanhong and Caili (2005); Yolmeh et al. (2014) who documented the larger F-value and smaller p-value in optimization model would be a greater significant effect on the corresponding response variable. Moreover, the p-value of the LOF in model was 0.2830, indicating non-significant ($p>0.05$), which illustrated the model satisfactorily fitted to the experimental data.

The robustness and predictive ability of the model developed were evaluated based on the correlation coefficient (R^2), adjusted R^2, and model predictive ability (Q^2). The high R^2 value (0.983) and adjusted R^2 (0.961) of the gamma-oryzanol recovery showed a good agreement between the experimental data and predicted data. While Q^2 value of the gamma-oryzanol recovery was 0.933. The result indicated that the variability was explained well by the models and has a small prediction error on new predictive models. Additionally, the difference between adjusted R^2 and Q^2 value in our results was less than 0.3, which presented models were in reasonable agreement (Analytics Solutions, 2013).

The quadratic equations were used to establish the relationship between the independent variables (X_1 = ultrasound power, X_2 = temperature treatment and X_3 = sonication time) of UASE and responses (Y = gamma-oryzanol recovery) were as followed:

$$Y=94.596+2.641X_1+4.698X_2+4.410X_3-1.220X_1*X_3-3.560X^2_2-2.559X^2_3 \quad (5.7)$$

Table 5.4 ANOVA results of the response surface quadratic model on gamma-oryzanol recovery.

	Degree of freedom	Sum of squares	Mean square	F-value	p-value
Total Corrected	16	659.750	41.234		
Regression	9	648.634	72.071	45.3854	0.0000
Residual	7	11.116	1.5879		
Lack of Fit	5	9.733	1.947	2.81634	0.2830
Pure Error	2	0.691	0.691		

R^2 = 0.983; Adjusted R^2 = 0.961; Q^2=0.933; p-value<0.05 indicates statistical significance.

Table 5.5 Regression coefficients and p-values for gamma-oryzanol recovery after backward elimination.

Source [a]	Regression coefficients	p-value [b]
Constant	94.595	0.0000
X_1	2.641	0.0002
X_2	4.698	0.0000
X_3	4.410	0.0000
X_1* X_3	-1.212	0.0499
X_2^2	-3.360	0.0029
X_3^2	-2.559	0.0111

[a] X_1, X_2, and X_3 denote ultrasound power, extraction temperature, and sonication time, respectively. [b] p<0.05 indicates statistical significance.

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5.5.3 Effect of process variables on gamma-oryzanol recovery

Table 5.5 presents the effect of the UASE parameters on the gamma-oryzanol recovery. The result showed that the linear term of X_1, X_2 and X_3, cross-product term of X_1X_3 and quadratic of X_2^2, X_3^2 had a positive effect on the gamma-oryzanol extraction from rice bran soapstock. The interaction term of X_1X_2, and X_2X_3, and quadratic term of X_1^2 on the model significantly did not significantly affected ($p>0.05$) the gamma-oryzanol recovery, thus, they were eliminated.

Figure 5.1A illustrated the interaction between ultrasonic power (X_1) and extraction temperature (X_2) on the gamma-oryzanol recovery, given sonication time at 15min. The recovery increased by increasing X_1 and X_2, and maximum value (96.98%) was achieved under 4.5 W/g and 49ºC. Increase extraction temperature to 55ºC under 4.5 W/g slightly reduced the gamma-oryzanol recovery.

Figure 5.1B showed that ultrasound power (X_1) and sonication time (X_3) had a significant influence on the gamma-oryzanol recovery. The maximum gamma-oryzanol recovery was achieved (97.15%) under the ultrasound power (X_1) of 4.5 W/g and sonication time (X_3) of 19.40min, given a fixed extraction temperature of 45ºC. The positive influence to gamma-oryzanol recovery was also indicated after prolonging the time treatment from 19.40 to 26min at 4.5 W/g. The results could be due to high ultrasonic power and an extended sonication time generates high temperatures and violent pressure, resulting in severe turbulence in the medium and the subsequent disintegration of the microstructures (Balachandran et al., 2006; Zhang et al., 2008).

In Figure 5.1C, the gamma-oryzanol recovery was maximum value of 97.59%, when X_2 and X_3 were 50.30ºC, and 21.54min, respectively, given X_1 of 2.5 W/g. Although Sharma and Gupta, (2006); Zhang et al. (2008), who reported that the high extraction temperature and extended sonication duration enhances the mass

liquid transfer by the cavitation forces resulting in increased the extraction yield. In this study, the high extraction temperature (51-55ºC) and extended sonication duration (22-26 min) resulted in slightly reduction of gamma-oryzanol recovery.

5.5.4 Optimization of the extraction process

Table 5.6 indicates the optimum UASE condition for the maximum recovery of gamma-oryzanol from DRBS. Based on the FCCD, the ultrasound power of 4.00 W/g, extraction temperature of 50ºC, and sonication time of 21.50min resulted in the optimal conditions of the UASE for gamma-oryzanol recovery of 98.15% from DRBS. The predicted gamma-oryzanol recovery was 98.03% under the optimal condition. These experimental and predicted values validated the response model with a good correlation, suggesting that the response model could be applied to optimizing the UASE parameters for enhanced gamma-oryzanol recovery.

To characterize the gamma-oryzanol composition obtained from the DRBS using UASE under optimal parameters, HPLC was employed in order to compare with gamma-oryzanol standard. The chromatograms of gamma-oryzanol presented in Figure 5.2A and 5.2B. It obviously showed similar profiles with four peaks of gamma-oryzanol standard and obtained in this study including campesteryl ferulate (RT = 7.753min and 7.765min), 24-methylene cycloartanyl ferulate (RT = 7.050min and 7.065min), β-sitosteryl ferulate (RT = 9.006min and 8.989min) and cycloartenyl ferulate (RT = 6.235min and 6.243min), respectively. Specifically, cycloartanyl ferulate and 24-methylene cycloartanyl ferulate exhibited the maximum proportions in gamma-oryzanol, while the minimum proportion of campesteryl ferulate and β-sitosteryl ferulate in gamma-oryzanol were observed. Thus, the HPLC results confirmed that the gamma-oryzanol components obtained from DRBS were comparable with gamma-

oryzanol standard. Moreover, similar profiles with other study were obtained by Kumar et al. (2009); Sakunpak et al. (2014).

Table 5.6 Model validation for maximum gamma-oryzanol recovery given the optimal UASE condition.

Ultrasound power (X_1,W/g)	Temperature treatment (X_2,°C)	Sonication time (X_3, min)	Experimental value (%)	Predicted value (%)
4.00±0.65	50±0.85	21.50±0.41	98.03±0.98	98.15±0.75

5.5.5 Rice bran soapstock microstructure

Figure 5.3 presents the SEM images of the DRBS and the residue after the optimal UASE condition. The morphology of the DRBS (Figure 5.3A) was irregularly spherical in shape and the heterogeneity size. It can be explained due to the stickiness of soapstock. Similar result was observed with Lamas et al., (2016), who reported that no uniformity of rice bran soapstock because the material has asticky characteristic. The formation of dented surfaces of soapstock particles after sonication indicated that the efficiency of extraction was increased (Figure 5.3B). In addition, it suggested that the ultrasound treatment leads to destroy the soapstock structure and increase a porous characteristic enhancing the extraction efficiency. The finding confirmed the extraction efficiency when using the UASE technique. While the DRBS (untreated) shows a completely dense structure. The results were consistent with; Kurian et al. (2015); Yi et al. (2016), who reported that the ultrasonic treatments induced the structural changes, fissures, and cavities in the sample.

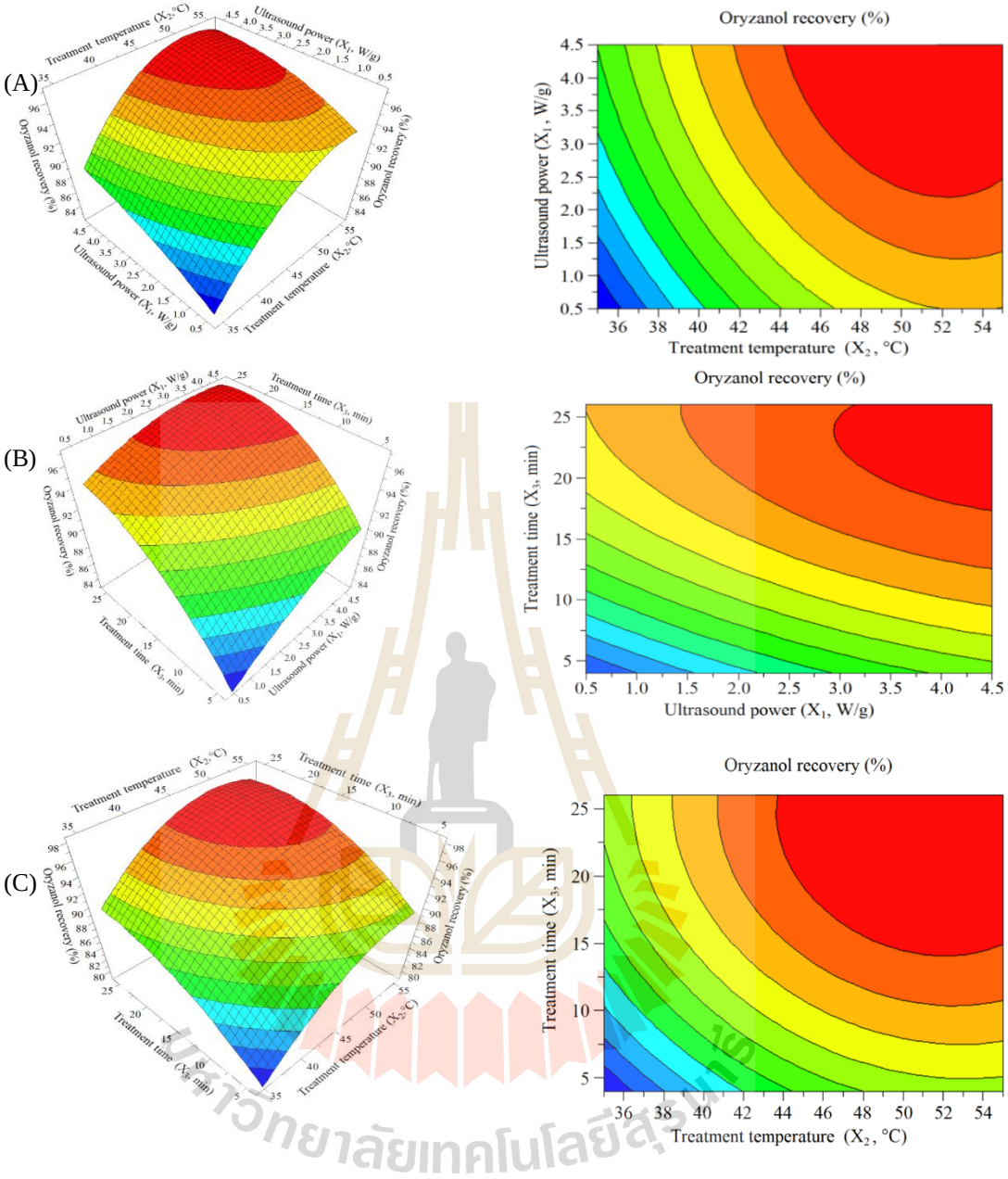


Figure 5.1 The response surface plots of the effects of ultrasound power, extraction temperature, and sonication time on gamma-oryzanol recovery: (A) fixed sonication time at 15min; (B) (extraction temperature at 45°C; and (C) fixed ultrasound power at 2.5 W/g.

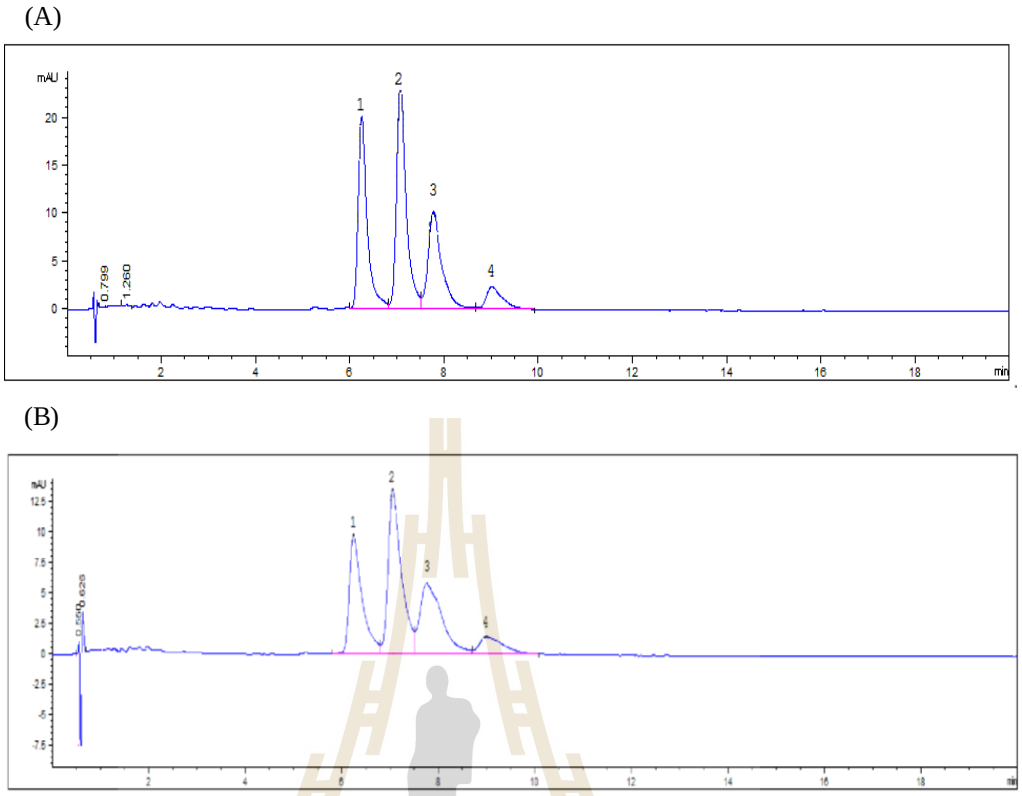
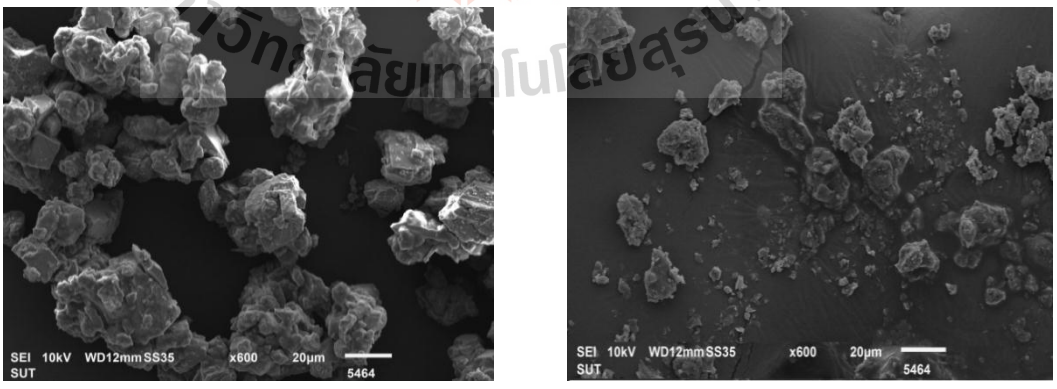


Figure 5.2 HPLC chromatograms of gamma-oryzanol (1) cycloartenylferulate, (2) 24-methylenecy cloartanyl ferulate, (3) campesterylferulate, and (4) *β*-sitosteryl ferulate): (A) standard and (B) recovered gamma-oryzanol from DRBS.



(A) (B)

Figure 5.3 SEM images of: (A) untreated and (B) UASE treated DRBS.

5.5.6 Kinetic of UASE process

The extraction kinetics were studies in order to describe the effect of UASE process parameters on the gamma-oryzanol extraction. Experimental data on the concentration of gamma-oryzanol of UASE from the DRBS using the MEE composition ethyl acetate/ethanol 85:15 (v/v) for the three investigated ultrasound powers and extraction temperatures as function of extraction time were reported in Figure 5.4. Because based on the optimal parameters of the UASE obtained from the RSM, the extraction time between 0-21.5min was used for the kinetics studies. The calculated values from the second-order kinetic model were compared with the experimental data for the gamma-oryzanol extractions with UASE under different conditions. In Figure 5.4A and 5.4B, the solid lines are the model prediction and the points are the experimental data. The calculated gamma-oryzanol concentrations over time obtained were fitted with the experimental data, showing the validity relationship of the second-order kinetic models. These results indicated that the two-third of gamma-oryzanol extractions (more than 3.0 g/L) occurred during the first stage extraction time (0-8min). Extended extraction time from 8 to 21.50min resulted slightly increase in gamma-oryzanol concentrations (Figure 5.4A and 5.4B). However, at high temperature of 55ºC (Figure 5.4B), small reduction of the concentration was observed agreeing with the RSM results (Figure 5.1C). According to Lebovka et al. (2012), the longer extraction time lead to greater the chance of solvent dissolving other solutes from the solid, or desired solute (target compound) to be degraded by temperature or expose to the atmosphere. According to Pan et al. (2011), the extraction of total phenolics from pomegranate peel using three different extraction methods (continuous and pulsed ultrasound assisted extraction (CUAE and PUAE), and conventional extraction (CE)) presented two stage extractions. First stage is

characterized by a rapid extraction rate involving the dissolution of soluble substances near particle surfaces into the solvent. Second stage is characterized by a slow extraction rate involving mass transfer of soluble substances from the internal material into the solvent by diffusion process (Pan et al., 2011; Lebovka et al., 2012).

The gamma-oryzanol concentration significantly increased with increase intensity level from 0.5 W/g to 2.5 W/g ($p<0.05$) over the extraction time, however, from 2.5 W/g to 4.5 W/g only slightly increase in concentration was observed ($p>0.05$) (Figure 5.4 A). According to Goula (2013), ultrasound intensity level influenced on the extraction process, a larger ultrasound power increasing the mass transfer process and resulting in a moderate rise in the recovered yield. In addition, gamma-oryzanol concentration increased with elevated extraction temperature from 35ºC to 45ºC ($p<0.05$). As Lazar et al. (2016) reported that higher extraction temperature using ultrasound assisted extraction increase on the extraction rates of polyphenolic compounds from different biomass due to under higher temperature, the solubility and diffusivity of the phenolic compounds are increased, enhancing the mass transfer and accelerating the extraction process. However, elevated temperature from 45ºC to 55ºC at 21.5min extraction time small decrease in concentration were observed ($p>0.05$). High temperature could lead to thermal degradation of phenolic compounds and cause a faster evaporation of the solvent, resulting in the efficiency of the whole process (Lazar et al., 2016; Goula, 2013).

Figure 5.5 showed the linearized forms of the second-order model for the UASE process (Eq. 5). The gamma-oryzanol concentration at saturation (C_s), and extraction rate constant (k) were calculated from the slope and intercept by plotting t/C_t against t. The initial extraction rate (h) was calculated following the Eq. 6. Table 5.7 presents the kinetic parameters for the second-order kinetic models of the gamma-

oryzanol extraction. The C_s, k, *h* values trended to increase with increasing ultrasounds from 0.5 to 4.5 W/g, and with increasing temperature from 35 to 45°C. While, the C_s, and *h* values slightly decreased when increased temperature from 45 to 55°C. These results suggested that more efficiency of the UASE for increasing gamma-oryzanol extraction rate were obtained when the conditions of high ultrasound power (4.5 W/g) and moderate temperature of 45°C. Based on the coefficients of determination (R^2 = 0.9722-0.9971) and the RSME (0.0907-0.2134) (Table 5.7), the second-order kinetic model resulted the fitting models for all the investigated ultrasound powers and temperatures, and could be used for describing the effect of UASE parameters for the gamma-oryzanol extraction form the DRBS.

Table 5.7 Kinetic parameters of gamma-oryzanol recovery from the DRBS for different ultrasound powers and extraction temperatures.

Kinetic parameters	**Ultrasound power (W/g)**			**Extraction temperature (°C)**		
	0.5	**2.5**	**4.5**	**35**	**45**	**55**
C_s experimental (g/L)	3.910	4.290	4.310	3.890	4.317	4.219
C_s calculated (g/L)	3.841	4.251	4.330	3.724	4.195	3.986
k (L/g.min)	0.207	0.215	0.215	0.193	0.216	0.217
h (g/L.min)	3.165	3.975	3.994	2.920	4.025	3.863
R^2	0.9766	0.9971	0.9962	0.9722	0.9964	0.9955
RMSE	0.0907	0.0912	0.0989	0.1136	0.1736	0.2134

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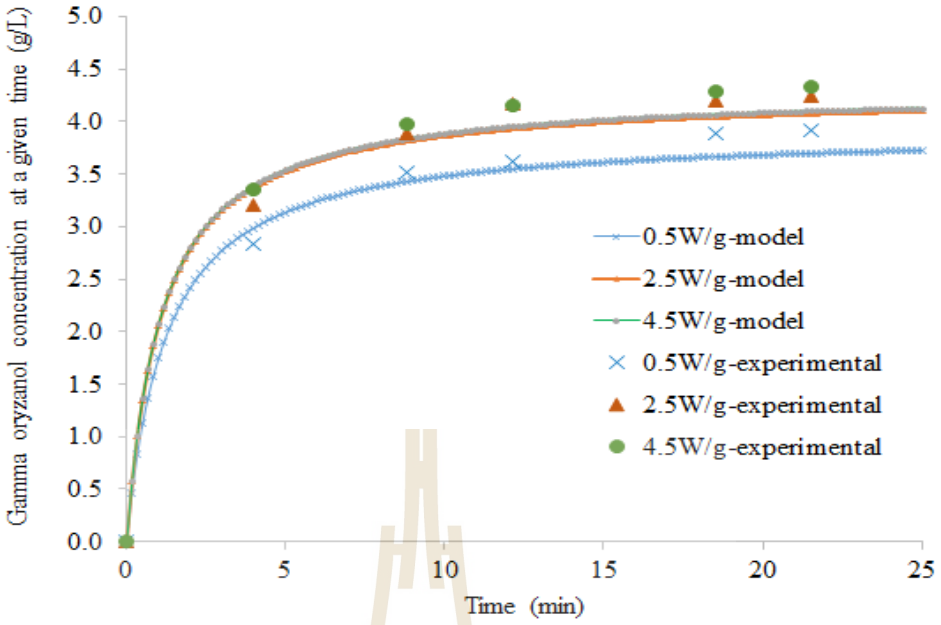
(A)



(B)

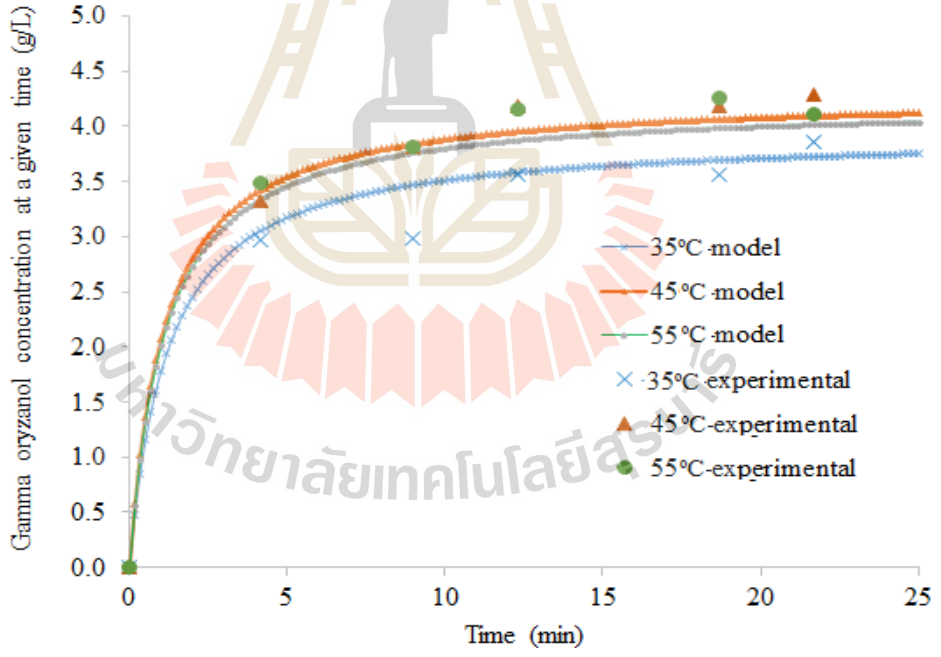


Figure 5.4 Gamma-oryzanol concentration at a given sonication time (C_t): (A) extraction temperature at 45°C, (B) ultrasound power at 2.5 W/g.

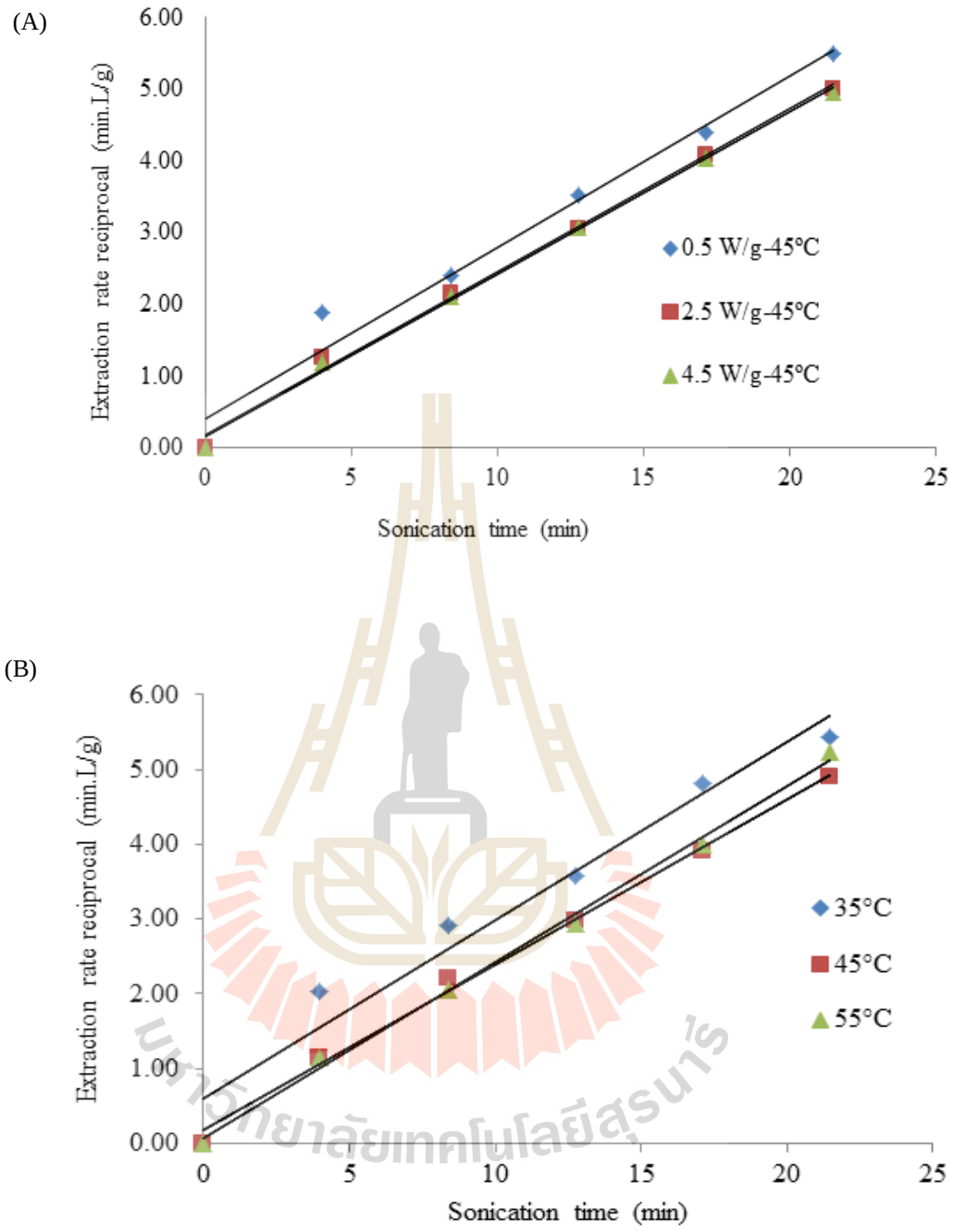


Figure 5.5 Extraction rate reciprocal (t/C_t) of gamma-oryzanol recovery from the DRBS at different sonication time (t): (A) extraction temperature at 45°C, (B) ultrasound power at 2.5 W/g.

5.6 Conclusion

In this study, UASE of gamma-oryzanol recovery from DRBS were investigated and compared. RSM and FCC were applied to give an insight into the effects and interactions of all parameters during extraction. The results illustrated that the well-fitting model was generated with a good predictor of all experimental results. The optimum conditions for extraction gamma-oryzanol extraction were an ultrasonic power of 4.0 W/g, temperature process of 50ºC, and 21.50 min for the ultrasonication process, which achieved ORF of 98.03%, respectively. HPLC profile showed four chromatographic peaks in ORF (campesteryl ferulate, 24-methylene cycloartanyl ferulate, β-sitosteryl ferulate and cycloartenyl ferulate). The experimental data fits well with the second-order model and kinetics parameters included the initial extraction rate (h), the extraction rate constant (k), the concentration at saturation (C_e) and the activation energy (E_a). These data were calculated using the obtained model. The activation energy obtained 39.05 kJ mol^{-1} indicated the endothermic nature of the UASE process. Hence in this study, the optimization UASE parameters of gamma-oryzanol from rice bran soapstock are reliable, stable and available in practice. In addition, the fitting equation of kinetic model fitting equation can apply to describe the mechanism of ultrasound-assisted extraction.

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CHAPTER VI

ISOLATION AND CHARACTERIZATION OF GAMMA-ORYZANOL ISOLATED FROM RICE BRAN SOAPSTOCK

6.1 Abstract

This research investigates the effect of ethanol concentrations in ethyl acetate/ethanol mixture and crystallization conditions on the recovery and purity of gamma oryzanol isolated from dried rice bran soapstock, using two-step crystallization. The ethanol concentrations were varied between 0, 10, 20, 30, and 40% v/v, and the crystallization parameters included solvent to crude gamma oryzanol rich fraction solution (ORF) ratio, crystallization temperature, and crystallization time. In the first crystallization, the solvent to ORF ratio was varied between 2.5:1, 5.0:1, 7.5:1 and 10:1 (v/v), crystallization temperature between -60, -45, -30, -15 and 0°C, and crystallization time between 5, 7.5, 10, 12.5, and 15 h. The liquid phase was subsequently subjected to second crystallization for gamma oryzanol crystal, where the crystallization temperatures and time were varied between 0, 5, 10, 15 and 20ºC; and 1, 6, 11 and 15 h. The optimal ethanol concentration in the solvent mixture was 30% v/v. The optimal first-crystallization solvent to ORF ratio, crystallization temperature and time were 7.5:1 (v/v), -15°C, and 7.5 h. The optimal second-crystallization temperature and time were 5ºC and 6 h, with gamma oryzanol recovery and purity of 58.03-61.17% and 87.10-90.29%, respectively. The physiochemical properties of gamma oryzanol crystal also indicated good agreement between isolated

gamma oryzanol and gamma oryzanol standard.

Keywords: Optimization, soapstock, gamma-oryzanol purity, crystallization.

6.2 Introduction

Rice bran, a by-product of rice milling, contains 12-23% oil (Wongwaiwech et al., 2018). Rice bran is also rich in nutraceuticals, including gamma oryzanol, tocopherols, and tocotrienols, making crude rice bran oil highly nutritious. However, high contents of free fatty acids (FFA), waxes, gums, and pigments in crude rice bran oil complicate the refining process to improve the odor, appearance, and shelf life to meet the standards of edible oil (Gunstone, 2011; De et al., 2011).

In rice bran oil refining, FFA and mucilaginous impurities are removed by alkaline neutralization (Engelmann et al., 2017). The alkaline neutralization process nevertheless causes nearly 90% gamma oryzanol loss through wet soapstock (Gopala Krishna et al., 2001). At present, rice bran soapstock is mostly converted into detergent soaps (Narayan et al., 2006), feedstock (Ju and Vali, 2005), and lecithin products (Thurman, 1961). In fact, residual gamma oryzanol contained in rice bran soapstock presents a promising opportunity for gamma oryzanol production on a commercial scale (Narayan et al., 2006). Research have shown that gamma oryzanol helps reduce plasma cholesterol (Wilson et al., 2007; Kumar et al., 2009), platelet aggregation, and cholesterol absorption (Sharma et al., 1986; Seetharamaiah et al., 1986). It is also used in cosmetic for anti-itching and anti-dandruff properties (Gopala Krishna et al., 2001; Patel and Naik, 2004) and in food products (Patel and Naik, 2004). Therefore, many extraction methods have been proposed for isolating gamma oryzanol from alkaline oil cake (soapstock).

Masao and Yashizane (1968) isolated gamma oryzanol from alcoholic solution (ethanolic and methanolic) of rice bran oil soapstock using CO_2 as anti-solvent. They successfully derived white-crystal gamma oryzanol with 85% purity; however, gums and waxes were mostly insoluble in gaseous solvent mixture. Das et al. (1998) isolated gamma oryzanol from rice bran oil soapstock using calcium ion-induced precipitation. Although the recovery and purity were high (76% and 96%), the technique was highly complex and required a toxic solvent (chloroform).

Narayan et al. (2004) and Zullaikah et al. (2009) experimentally isolated gamma oryzanol from rice bran oil and rice bran soapstock by using preparative high performance liquid chromatography (HPLC); and reported 90% gamma oryzanol recovery with 90-98% purity. Nonetheless, the method was ideal for laboratory-scale production due to low productivity. Zullaikah et al. (2009) isolated gamma oryzanol from rice bran oil at a very low temperature (-60ºC) by a mixture of ethyl acetate and methanol as crystallization solvent using two-step crystallization; and reported 57-59% gamma oryzanol crystal recovery with 93-95% purity. However, the sub-zero temperature (-60ºC) during the first crystallization necessitated a special cooling device. Kaewboonnum et al. (2010) isolated gamma oryzanol from rice bran oil soapstock with 20:80 (v/v) ethyl acetate/methanol mixture using two-step crystallization (3°C and 1 h for the first crystallization, followed by 5°C and 24 h for the subsequent step). However, the recovery and purity of gamma oryzanol were relatively low (55.17% and 74.60%).

In this research, ethanol was used in combination with ethyl acetate. Ethanol is commonly used in food and pharmaceuticals because of its low toxicity and cost-effectiveness. Besides, ethanol, when combined with ethyl acetate, moderates the

solvent polarity, thus enhancing gamma oryzanol crystallization. However, there exists no study on gamma oryzanol isolation using ethyl acetate/ethanol mixture.

Thus, this research investigates the effect of ethanol concentrations (0, 10, 20, 30, and 40% v/v) in ethyl acetate/ethanol mixture and crystallization conditions (i.e., solvent to ORF ratio, crystallization temperature and time) on gamma oryzanol isolated from dried rice bran soapstock. The isolation was carried out using two-step crystallization. In the first crystallization, the solvent to ORF ratio was varied between 2.5:1, 5.0:1, 7.5:1 and 10:1 (v/v), the crystallization temperature between -60, -45, -30, -15 and 0°C, and the crystallization time between 5, 7.5, 10, 12.5, and 15 h. The liquid phase from the first crystallization was subsequently subjected to second crystallization where the crystallization temperatures and time were varied between 0, 5, 10, 15 and 20ºC; and 1, 6, 11 and 15 h, respectively, for gamma oryzanol crystal. The gamma oryzanol recovery and purity were determined and the physicochemical properties characterized.

6.3 Objectives

This study was to investigate the effect of crystallization conditions on gamma-oryzanol recovery and purity.

6.4 Materials and Methods

6.4.1 Materials

Wet rice bran oil soapstock, following Venkatadri and Sreesaila (2005), was heated to 80ºC in a water bath and saponified by adding 1.25% (w/v) NaOH solution with constant stirring for 30 min before centrifugation at 10,000 rpm and 10ºC for 15 min to remove liquid. The solid was then vacuum-evaporated (100ºC,

550 mmHg) for 2 h for dried rice bran soapstock (DRBS) with 4-4.5% moisture content, measured by a moisture meter (A&D, AD-4714A, Japan). The DRBS was used as the starting material for gamma oryzanol extraction.

In extraction, 50 g DRBS was extracted in 500 mL ethanol/ethyl acetate mixture (15% ethanol: 85% ethyl acetate) by using ultrasound assisted extraction (4.0 W/g, 50.0ºC, and 21.5min). The crude gamma oryzanol rich fraction solution (ORF) was then centrifuged to remove solid before vacuum evaporation (Evaporator, Cole Parmer EW-28615-01, Cole Parmer, USA). The ORF contained 4.41 g gamma oryzanol/100 g DRBS.

Gamma oryzanol standard (Oryza Oil and Fat Chemical Co., Ltd, Japan) was used for quantification. Meanwhile, ethyl acetate (99.5%), acetonitrile (99.8%), and ethanol (98.5%) were from Sigma-Aldrich (Darmstadt, Germany).

6.4.2 Experimental methods

In the first crystallization, the mucilaginous impurities (waxes and gums) in the crude ORF solution were removed by a mixture of ethyl acetate and ethanol and stirred for 15 min at room temperature. The ethyl acetate to ethanol ratio was varied between 100:0, 90:10, 80:20, 70:30, and 60:40% (v/v); solvent to crude ORF solution ratio between 2.5:1.0, 5.0:1.0, 7.5:1.0, and 10.0:1.0 (v/v); crystallization temperature between 0, -15, -30, -45, and -60ºC; and crystallization time between 5, 7.5, 10, 12.5, and 15 h. After crystallization, liquid phase (LP) and solid phase (SP) were separated by filtering with Whatman filter paper no.1 under vacuum condition (350 mmHg). Gamma oryzanol recovery in LP was determined by spectrophotometry and HPLC.

In the second crystallization, the LP was subjected to different crystallization temperatures (0, 5, 10, 15, and 20ºC) and times (1, 6, 11, and 15 h). Gamma oryzanol crystal was precipitated and separated by vacuum filtering. The recovery and purity of gamma oryzanol were calculated by Eqs. (1) and (2).

$$\text{Gamma-oryzanol recovery (\%)} = \frac{\text{Weight of gamma-oryzanol in LP/isolated}}{\text{Weight of gamma-oryzanol in ORF}} \times 100 \quad (6.1)$$

$$\text{Gamma-oryzanol purity (\%)} = \frac{\text{Weight of gamma-oryzanol in sample}}{\text{Weight of sample}} \times 100 \quad (6.2)$$

6.4.3 Analytical methods

6.4.3.1 Quantitative analysis of gamma-oryzanol content

Gamma oryzanol contents were analyzed by UV-spectrophotometer/NIR (Shimazu, UV-2600, Japan) and HPLC, following Joshi et al. (2016) and Sakunpaket et al. (2014). HPLC consisted of a pump (LPG 3X00), auto-sampler (ACC-3000), diode-array UV/VIS detector (DAD), and Poroshell 120 EC-C18 column (3.0 mm x 150 mm, 2.7µm). The column temperature and UV detector were set at 25°C and 325 nm. The gradient mobile phase consisted of 100:0, 50:50, and 40:60 (v/v) methanol:acetonitrile for 5, 10, and 30 min. The flow rate was 1.0 ml/min throughout the HPLC analysis, and the sample injection volume was 20 µl.

6.4.3.2 Thermal behavior analysis

The melting point of gamma oryzanol was determined by differential scanning calorimetry (DSC 7, Perkin Elmer Corp., Norwalk, USA). The baseline was established using two empty sealed aluminum pans. Then, 3-5 mg gamma oryzanol crystal was weighed in aluminum pan and sealed. The DSC chamber was flushed with nitrogen (0.5 mL/min) and the samples were heated from 25°C to

200°C at 10°C/min and maintained at 200°C for 10 min and the melting curve plotted. The samples were then cooled down from 200°C to 25°C at 10°C/min.

6.4.3.3 Liquid chromatography–mass spectrometry (LC-MS)

The components of gamma oryzanol were characterized by Dionex Ultimate 3000 HPLC coupled with Bruker Amazon SL mass spectrometer (Thermo Fisher Scientific, USA), following Sakunpak et al. (2014). The samples were separated at 25ºC on Poroshell 120EC-C18 column (3.0 mm x 150 mm, 2.7µm) using gradient mobile phase consisting of 95:5, 40:60, and 5:95 (v/v) methanol:acetonitrile containing 0.1% formic acid for 2, 20, and 8 min, with a flow rate of 1.0 ml/min. The injection volume was 20 µL and the UV detector wavelength was 325 nm. The mass spectrometer was equipped with electrospray ionization (ESI) and quadrupole-ion trap. The mass spectra were observed in negative ion mode and recorded on a mass to charge ratio (m/z) range of 100-800. The capillary voltage was 4500 V, the drying gas temperature was 200°C with a flow rate of 4.5 L/min, and the nebulizer pressure was 200 kPa. The gamma oryzanol components were individually identified using multiple reaction monitoring (MRM) mode and data analyzed by LTQ Orbitrap mass spectrometer software (Thermo Fischer Scientific, U.S.A.).

6.4.3.4 Field emission scanning electron microscope (FESEM)

The particle size and morphology of gamma oryzanol were determined by FESEM (HITACHI JEOL JSM 7800F, Technology Development Ltd., Japan). In the analysis, standard and isolated gamma oryzanol were placed on the metal stub and gold-sputtered; and 25,000x images of the shape and surface characteristics of gamma oryzanol crystal were taken.

6.4.4 Other analytical methods

Color values were measured by Minolta CR-300 Chroma Meter (Osaka, Japan) and expressed in accordance with the Commission Internationale de l'Eclairage (CIE) L*a*b* system (lightness, redness, and yellowness). The particle size distribution of gamma oryzanol crystal was determined by a laser scattering particle size distribution analyzer (Horiba, LA 920, Kyoto, Japan), following Hong, Nakayama, & Park, (2002). The particle size curve and mean particle size were analyzed by LA-920 software analyzer (Horiba, LA 920, Kyoto, Japan).

6.4.5 Statistical analysis

The entire analysis was performed in triplicate and the averages and standard deviations were computed in Stagraphic centrution XV (Statsoft Inc., USA). A one-way analysis of variance (ANOVA) was carried out to assess the effects of isolation processes on the gamma-oryzanol recovery and physiochemical properties of gamma-oryzanol isolated. The Tukey-HSD comparison was used to compare the mean values given that the significant differences at $p<0.05$ were observed.

6.5 Results and discussions

6.5.1 First step crystallization

6.5.1.1 Effect of ethanol content in solvent mixture composition

Solvent mixture polarity plays a crucial role in isolation of gamma oryzanol from crude ORF solution. In the first crystallization, crude ORF solution was mixed, on a trial basis, with ethyl acetate/ethanol mixture (i.e., 0, 10, 20, 30, and 40% v/v ethanol concentrations), given 5.0:1.0 v/v solvent to ORF ratio (Kaewboonnum et al., 2010) under constant stirring. The mixture was then retained at

-60°C for 15 h (Zullaikah et al., 2009) until liquid (LP) and solid phases (SP) were formed. The LP was filtered through Whatman filter paper no.1 for enriched gamma oryzanol.

Table 6.1 tabulates the effect of ethanol concentrations in ethyl acetate/ethanol mixture on gamma oryzanol recovery. Gamma oryzanol contents in LP were insignificantly different ($p>0.05$) under 0, 10, 20, and 30% (v/v) ethanol conditions. With 40% (v/v) ethanol concentration, gamma oryzanol recovery was reduced significantly ($p<0.05$), which could be attributed to low solubility of gamma oryzanol in alcohol (Narayan et al., 2006; Antonio, et al., 2011). In other words, gamma oryzanol was trapped in the residual impurities.

The LP was then subjected to second crystallization for further isolation of gamma oryzanol, given the crystallization temperature and time of 5°C and 15 h (Kaewboonnum et al., 2010). The recovery and purity of gamma oryzanol were analyzed by spectrophotometry and HPLC. Higher ethanol concentration (40% v/v) signficantly lowered gamma oryzanol recovery (47.35% and 44.87% for spectrophotometry and HPLC) ($p<0.05$). However, variations in ethanol concentrations had no significant effect on gamma oryzanol purity (89.93-90.38% and 87.38-87.40% for spectrophotometry and HPLC) ($p>0.05$). As a result, a 30% ethanol concentration (70:30% v/v) was adopted to isolate gamma oryzanol from crude ORF solution.

6.5.1.2 Effect of solvent to ORF ratio on the first crystallization

The ethyl acetate/ethanol mixture solvent to crude ORF ratio was varied between 2.5:1.0, 5.0:1.0, 7.5:1.0, and 10.0:1.0 (v/v), given 70:30% (v/v) ethyl acetate/ethanol mixture, and retained at -60°C for 15 h (Zullaikah et al., 2009).

In Table 2, gamma oryzanol recovery in LP significantly increased as the ratio increased from 2.5:1.0 to 7.5:1.0 (v/v). This is because higher solvent to ORF ratio increased concentration gradients between solute and solvent, thereby enhancing gamma oryzanol solubility in LP. However, under 10.0:1.0 (v/v), gamma oryzanol recovery was insignificantly different from that of 7.5:1.0 (v/v) ratio.

As shown in Table 6.2, high gamma oryzanol contents in LP in turn increased the recovery and purity of gamma oryzanol in second crystallization. By comparison, the recovery and purity of gamma oryzanol under 2.5:1.0 v/v were lowest due to large quantities of residual mucilage in LP, which subsequently co-precipitated in the second crystallization. On the other hand, higher solvent to ORF ratios (7.5:1.0 and 10.0:1.0 v/v) significantly improved gamma oryzanol recovery and purity ($p<0.05$). However, use of large quantities of solvent translates into high production costs. As a result, the solvent to ORF ratio of 7.5:1.0 (v/v) was employed, given the comparably high gamma oryzanol recovery and purity.

6.5.1.3 Effect of temperature on the first crystallization

The first-crystallization temperatures were varied between 0, -15, -30, -45, and -60°C, given 15 h crystallization time. In Table 6.3, the recovery of gamma oryzanol in LP was insignificantly different as temperatures rose from -15°C to -60°C ($p>0.05$). Gamma oryzanol contents in LP under subzero conditions were higher than at 0°C. This is consistent with Zullaikah et al. (2009), who documented that the crystallization temperature of 0°C achieved low gamma oryzanol recovery and subsequently low gamma oryzanol crystal.

First-crystallization LP was further subjected to second crystallization, given the crystallization temperature and time of 5°C for 15 h

(Kaewboonnum et al., 2010). The second-crystallization results showed no significant difference in the recovery and purity, given subzero first-crystallization temperatures (-15, -30, -45, and -60°C). On the other hand, at 0°C, the recovery and purity were comparatively low. The low recovery could be attributed to crystallization of gamma oryzanol with impurities during the first crystallization. The mucilaginous impurities in LP after the first crystallization also co-precipitated in the second crystallization under 0°C condition, resulting in low purity. As a result, the first-crystallization temperature of -15°C was adopted and used in subsequent experiments.

6.5.1.4 Effect of crystallization time on the first crystallization

The first-crystallization time was varied between 5, 7.5, 10, 12.5, 15 h, given -15°C crystallization temperature. In Table 6.4, gamma oryzanol recovery belonging to 5 h crystallization time was significantly lower than that of 7.5 h condition ($p<0.05$). However, the differences were insignificant between 7.5, 10.0, 12.5, and 15.0h crystallization time ($p>0.05$).

Additionally, after the second crystallization at 5°C for 15 h (Kaewboonnum et al., 2010), the recovery and purity of gamma oryzanol improved, given the first-crystallization time between 5.0-7.5 h. However, the recovery and purity of gamma oryzanol were insignificantly different for the first-crystallization time beyond 7.5 h. As a result, the first-crystallization time of 7.5 h was adopted as it could optimally remove impurities.

6.5.2 Second crystallization

6.5.2.1 Effect of temperature on the second crystallization

The second crystallization isolates gamma oryzanol with higher purity from LP derived from the first crystallization. The second-crystallization

temperatures were varied between 0, 5, 10, 15, and 20°C, given 15 h crystallization time. In Table 6.5, gamma oryzanol recovery at 0 and 5°C were insignificantly indifferent ($p>0.05$). Higher temperatures (10-20°C) lowered gamma oryzanol recovery as dissolution of gamma oryzanol was accelerated, adversely affecting the recovery. According to Zullaikah et al. (2009), the recovery and purity of gamma oryzanol at high process (crystallization) temperatures could be improved by prolonging the crystallization time from 24 to 72 h. In this research, the purity of gamma oryzanol was insignificantly different between different second-crystallization temperatures ($p>0.05$), consistent with Kaewboonnum et al. (2010), who reported that 5°C was optimal for gamma oryzanol crystallization. As a result, the second-crystallization temperature of 5°C was adopted.

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Table 6.1 Effect of solvent mixture composition on gamma-oryzanol recovery.

Ethyl acetate:ethanol (v/v)	First crystallization[1]		Second crystallization[2]			
	Gamma oryzanol recovery (%)[3]		Gamma oryzanol recovery (%)[3]		Purity (%)[3]	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
100:0	80.03[a]±0.15	75.90[a]±1.10	60.10[a]±1.51	ND[4]	90.38[a]±1.57	87.40[a]±2.00
90:10	79.80[a]±0.93	75.98[a]±0.93	59.92 [a]±1.23	57.93[a]±1.54	90.31[a]±1.23	87.38[a]±2.01
80:20	79.79[a]±1.01	75.90[a]±1.01	60.13 [a]±1.59	58.03[a]±2.01	90.29[a]±1.35	87.39[a]±1.95
70:30	79.78[a]±1.05	75.92[a]±1.05	59.97[a]±1.45	57.99[a]±1.58	90.31[a]±1.56	87.40[a]±1.69
60:40	74.94[b]±1.10	71.94[b]±1.10	47.35[b]±1.20	44.87[b]±2.11	89.93[a]±1.61	87.38[a]±1.75

[1] Experimental condition was solvent:ORF = 5.0:1.0; crystallization temperature = -60°C; crystallization time = 15 h.

[2] Experimental condition was crystallization temperature = 5°C; crystallization time = 15 h.

[3] The values are the mean of three replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$).

[4] ND = not determined.

Table 6.2 Effect of solvent to ORF on gamma-oryzanol recovery on the first crystallization.

Solvent to crude ORF Ratio (v/v)	First crystallization[1]		Second crystallization[2]			
	Gamma oryzanol recovery (%)[3]		Gamma oryzanol recovery (%)[3]		Purity (%)[3]	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
2.5:1.0	73.92^{c}±1.17	69.17^{c}±1.65	49.02^{c}±2.05	ND[4]	76.31^{c}±1.13	71.01^{c}±1.81
5.0:1.0	79.27^{b}±1.35	76.44^{b}±1.87	57.61^{b}±1.98	55.01^{b}±1.21	89.96^{ab}±1.21	87.35^{ab}±2.15
7.5:1.0	80.72^{a}±1.89	77.55^{a}±2.19	60.79^{a}±1.82	57.95^{a}±1.04	89.99^{a}±1.43	87.41^{a}±1.69
10.0:1.0	80.75^{a}±2.17	77.56^{a}±2.62	59.99^{a}±2.04	57.93^{a}±0.89	90.01^{a}±2.01	87.38^{a}±1.75

[1] Experimental condition was ethyl acetate:ethanol = 70:30 (v/v); crystallization temperature = -60°C; crystallization time = 15h.

[2] Experimental condition was crystallization temperature = 5°C; crystallization time = 15 h.

[3] The values are the mean of three replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$).

[4] ND = not determined.

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Table 6.3 Effect of temperature on gamma-oryzanol recovery on the first crystallization.

Temperature (ºC)	First crystallization[1]		Second crystallization[2]			
	Gamma oryzanol recovery (%)[3]		Gamma oryzanol recovery (%)[3]		Purity (%)[3]	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
0	44.96^{b}±0.87	42.34^{b}±1.98	35.21^{b}±1.83	ND[4]	89.90^{ab}±1.89	84.61^{ab}±1.11
-15	80.49^{a}±1.29	75. 02^{a}±1.78	59.98^{a}±1.08	57.82^{a}±0.78	90.19^{a}±1.59	87.69^{a}±1.65
-30	80.57^{a}±0.94	74.98^{a}±2.12	60.17^{a}±1.32	57.93^{a}±1.25	90.15^{a}±1.65	87.60^{a}±1.69
-45	80.55^{a}±1.12	75.11^{a}±2.45	59.89^{a}±1.05	57.85^{a}±1.16	90.21^{a}±1.54	87.59^{a}±1.55
-60	80.59^{a}±1.15	75.09^{a}±2.38	59.89^{a}±1.19	57.90^{a}±1.42	90.18^{a}±1.72	87.60^{a}±1.69

[1] Experimental condition was ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1.0 (v/v); crystallization time = 15 h.

[2] Experimental condition was crystallization temperature = 5°C; crystallization time = 15 h.

[3] The values are the mean of three replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$).

[4] ND = not determined.

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Table 6.4 Effect of crystallization time on gamma-oryzanol recovery in the first crystallization.

Time (hour)	First crystallization[1]		Second crystallization[2]			
	Gamma oryzanol recovery (%)[3]		Gamma oryzanol recovery (%)[3]		Purity (%)[3]	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC	Spectrophotometry	HPLC
5	44.84^{b}±0.87	39.29^{b}±1.05	45.21^{b}±1.83	42.01^{b}±1.05	89.91^{ab}±2.09	87.51^{ab}±1.11
7.5	80.06^{a}±0.99	75.00^{a}±1.31	59.98^{a}±1.08	57.98^{a}±1.68	90.09^{a}±2.10	87.59^{a}±1.65
10	80.05^{a}±1.50	74.98^{a}±1.10	60.10^{a}±1.32	57.88^{a}±1.55	90.12^{a}±1.84	87.62^{a}±1. 69
12.5	80.05^{a}±1.20	74.99^{a}±0.99	59.95^{a}±1.05	57.91^{a}±1.41	90.11^{a}±1.79	87.59^{a}±1.55
15	80.03^{a}±1.02	75.01^{a}±1.01	59.93^{a}±1.19	57.86^{a}±1.49	90.13^{a}±1.83	87.60^{a}±1.69

[1] Experimental condition was ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1.0 (v/v); crystallization temperature = -15°C.

[2] Experimental condition was crystallization temperature = 5°C; crystallization time = 15 h.

[3] The values are the mean of three replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$).

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Figures 6.1 A-E illustrate the FESEM images and particle size histograms of gamma oryzanol crystal under different second-crystallization temperatures (0, 5, 10, 15, and 20°C). Large particle sizes were present at low temperatures (0 and 5°C), with 75% of the particles larger than 150 µm. At higher crystallization temperatures (10, 15, and 20°C), 74% of gamma oryzanol crystals were smaller than 150 µm. The results could be attributed to the fact that low crystallization temperatures induced crystal growth and aggregation of crystals, resulting in larger particle sizes.

6.5.2.2 Effect of crystallization time on the second crystallization

The second-crystallization time was varied between 1, 6, 11, and 15 h, given 5°C crystallization temperature. In Table 6, gamma oryzanol recovery was significantly positively correlated with crystallization time ($p<0.05$). At 1 h crystallization time, the resulting gamma oryzanol crystal was of small particles (Figure 6.2A). By comparison, extension of crystallization time increased the size of gamma oryzanol crystal (Figures 6.2B-D), but the crystal sizes (100-300 nm) under prolonged crystallization time were insignificantly different and the crystal shape was similar. In addition, the crystalline structure and shape were similar to gamma oryzanol standard (Figure 2E). Besides, variation in the crystallization time had no effect on gamma oryzanol purity (Table 6.6). Thus, the crystallization time of 6 h was adopted as the second-crystallization duration.

In short, the optimal ethanol concentration in the solvent mixture was 30% v/v. The optimal first-crystallization solvent to ORF ratio, crystallization temperature and time were 7.5:1 (v/v), -15°C, and 7.5 h. The optimal second-crystallization temperature and time were 5°C and 6 h, with gamma oryzanol recovery and purity of 58.03-61.17% and 87.10-90.29%, respectively.

Table 6.5 Effect of temperature process on gamma-oryzanol recovery on the second crystallization.

Temperature (°C)	Gamma oryzanol recovery (%)[1]		Purity (%)[1]	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC
0	59.91[a]±0.78	ND[2]	90.21[a]±0.95	87.10[a]±0.95
5	59.98[a]±0.95	57.68[a]±1.14	90.24[a]±1.01	87.09[a]±1.01
10	58.15[b]±0.98	55.61[b]±0.89	90.19[a]±1.01	87.15[a]±0.95
15	55.94[c]±0.90	51.15[c]±0.93	90.23[a]±1.10	87.10[a]±1.01
20	51.00[d]±0.90	46.00[d]±1.06	90.21[a]±1.10	87.08[a]±1.05

[1] Second crystallization time was 15 h after the first crystallization (ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1.0 (v/v); crystallization temperature = -15°C, and crystallization time = 7.5 h). The values are the mean of three replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$).

[2] ND = not determined.

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Table 6.6 Effect of second-crystallization time on gamma oryzanol recovery.

Time (hour)	Gamma oryzanol recovery (%)[1]		Purity (%)[1]	
	Spectrophotometry	HPLC	Spectrophotometry	HPLC
1	55.60[b]±1.18	51.16[b]±1.28	90.01[a]±2.55	87.09[a]±0.99
6	61.17[a]±1.05	58.03[a]±1.35	90.29[a]±2.12	87.10[a]±1.46
11	61.12[a]±2.08	57.98[a]±1.43	89.99[a]±1.31	87.11[a]±1.15
15	61.16[a]±1.99	58.05[a]±1.32	90.21[a]±2.10	87.09[a]±1.21

[1] Second crystallization temperature was 5°C after the first crystallization (ethyl acetate:ethanol = 70:30 (v/v); solvent:ORF = 7.5:1.0 (v/v); crystallization temperature = -15°C, and crystallization time = 7.5 h). The values are the mean of three replications ± standard deviation. Different letters in each column denote statistically significant differences between treatments ($p<0.05$).

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6.5.3 The physiochemical properties of gamma-oryzanol

The physicochemical properties of gamma oryzanol crystal under the optimal two-stage crystallization condition were characterized using HPLC, LC-MS, and DSC; and results compared against gamma oryzanol standard.

Figure 6.3 compares the HPLC chromatograms of gamma oryzanol standard and isolated gamma oryzanol (i.e., gamma oryzanol crystal) with four similar peaks: cycloartenyl ferulate (retention time (RT) = 6.235 and 6.243 min), 24-methylene cycloartanyl ferulate (RT = 7.050 and 7.065 min), campesteryl ferulate (RT = 7.763 and 7.765 min), and β-sitosteryl ferulate (RT = 9.006 and 8.089 min). Cycloartanyl ferulate and 24-methylene cycloartanyl ferulate were dominant in gamma oryzanol crystal, consistent with (Patel & Naik, 2004; Kumar et al., 2015; Inamuddin, 2012; Gopala Krishna et al., 2001; and Khuwijitjaru et al., 2009). Interestingly, in this study, 24-methylene cycloartanyl ferulate and β-sitosteryl ferulate in the standard and isolated gamma oryzanol were significantly different ($p<0.05$) (Table 6.7). This could be attributed to different solvent extraction methods and starting material effect (Kurma et al., 2009).

The four characteristic peaks of isolated gamma oryzanol were identified using LC-MS and compared with those of gamma oryzanol standard and previous studies. In Figures 4A-D, the isolated gamma oryzanol showed fragmentation patterns, including cycloartenyl ferulate (RT = 12.41 min, deprotonated molecular ion $[M-H]^-$ at m/z 601.9 and 587), 24-methylcycloartenyl ferulate (RT = 13.75 min, $[M-H]^-$ at m/z 615.9 and 601.0), campesteryl ferulate (RT = 15.11 min, $[M-H]^-$ at m/z 575.9 and 561.0), and β-sitosteryl ferulate (RT = 17.51 min, $[M-H]^-$ at m/z 589.9 and 575.0). The results were consistent with those of gamma oryzanol standard and in

cold-pressed rice bran oil (Kumar et al., 2009; Das, Chaudhuri, Kaimal, & Bhalerao, 1998) and soapstock (Sakunpak et al., 2014). The melting points of isolated gamma oryzanol and gamma oryzanol standard were between 125-130°C, which were slightly different from that of isolated oryzanol from rice bran oil soapstock of Rao et al. (2002) (120-125°C) and of Das et al. (1998) (132-135°C).

The HPLC, LC-MS, and DSC results of isolated gamma oryzanol confirmed that gamma oryzanol derived from crude ORF of this research was comparable to gamma oryzanol standard and those of previous studies. However, gamma oryzanol standard had a higher lightness (L^*) (94.25±0.50) than isolated gamma oryzanol (83.33±0.98) ($p<0.05$). On the other hand, redness (a^*) and yellowness (b^*) of gamma oryzanol standard were lower than isolated gamma oryzanol. The high redness (0.57±0.02) and yellowness (4.69±0.03) of isolated gamma oryzanol could be attributed to the impurities, e.g., triglyceride and mucilage.

6.6 Conclusion

This research investigated the effect of ethanol concentrations (0, 10, 20, 30, and 40% v/v) in ethyl acetate/ethanol mixture and crystallization conditions (solvent to ORF ratio, crystallization temperature, and crystallization time) on the recovery and purity of gamma oryzanol isolated from dried rice bran soapstock. Two-step crystallization was performed to isolate gamma oryzanol. The results showed that the optimal ethanol concentration in the solvent mixture was 30% v/v. Given 70:30 (v/v) ethyl acetate/ethanol mixture, the optimal first-crystallization solvent to ORF ratio, crystallization temperature and time were 7.5:1 (v/v), -15°C, and 7.5 h. The liquid phase derived from the first crystallization was subsequently subjected to second crystallization for gamma oryzanol crystal. The optimal second-crystallization

temperature and time were 5ºC and 6 h, with gamma oryzanol recovery and purity of 58.03-61.17% and 87.10-90.29%, respectively. Furthermore, the physicochemical properties of gamma oryzanol crystal were characterized and results indicated good agreement between isolated gamma oryzanol and gamma oryzanol standard. Essentially, the findings revealed that ethyl acetate/ethanol mixture, together with two-step crystallization, is operationally viable for gamma oryzanol extraction from rice bran soapstock.

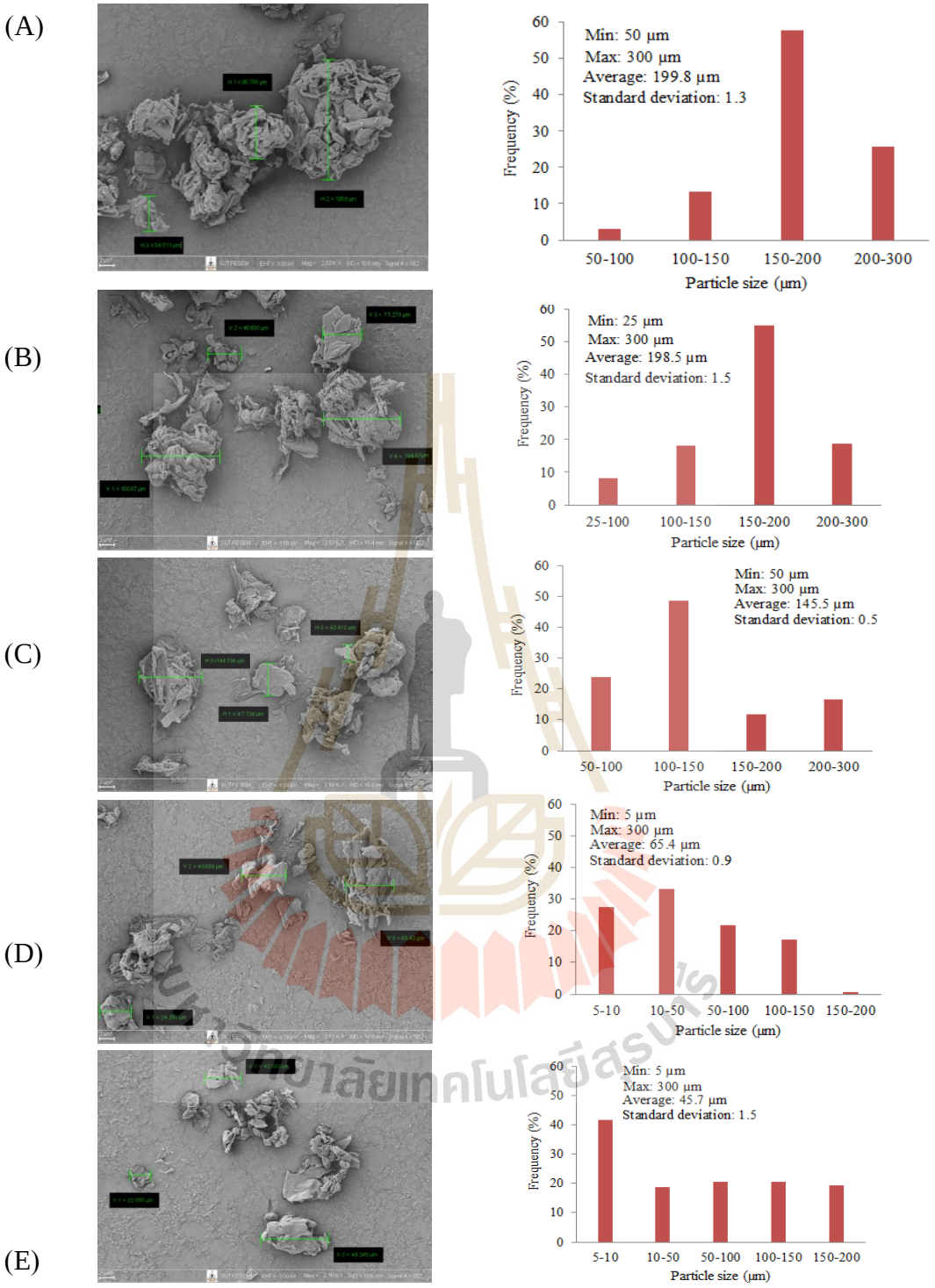


Figure 6.1 FESEM images and particle size histogram of gamma-oryzanol isolated from ORF: (A) 0°C, (B) 5°C, (C) 10°C, (D) 15°C, and (E) 20°C.

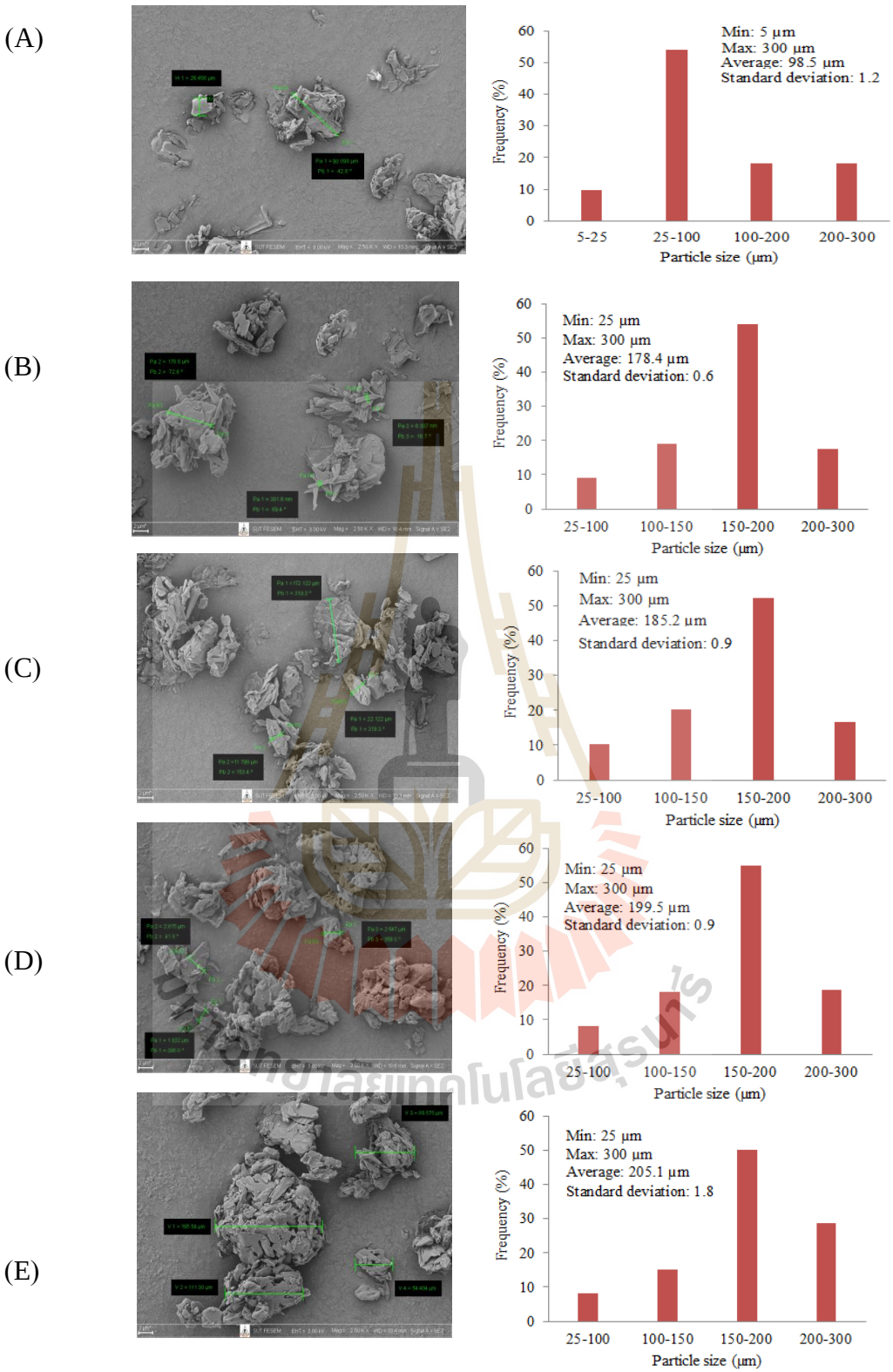
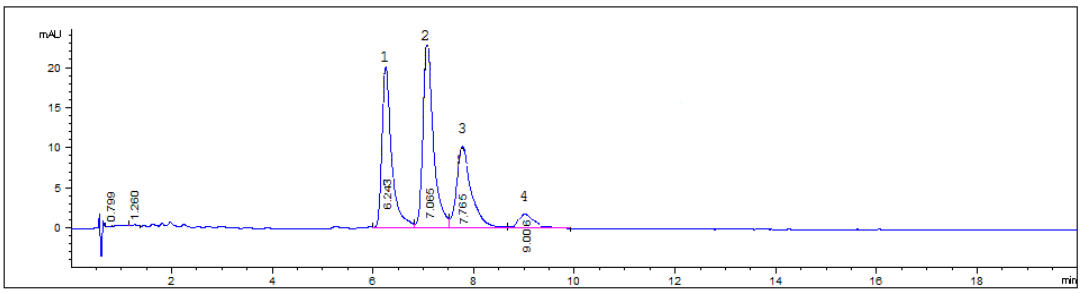


Figure 6.2 FESEM images and particle size histogram of gamma-oryzanol isolated from ORF: (A) 1 hour, (B) 6 hours, (C) 10 hours, (D) 15 hours, and (E) standard.

(A)



(B)

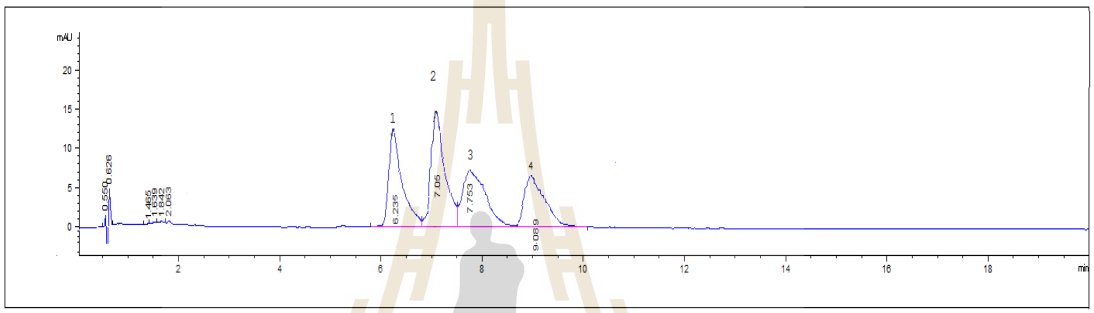


Figure 6.3 HPLC chromatogram showing retention times of gamma-oryzanol analogues at 325 nm (A:gamma-oryzanol standard; and B: gamma-oryzanol isolated from ORF): (a) cycloartenyl ferulate; (b) 24 methylene cycloartanyl ferulate; (c) campesteryl ferulate; (d) *β*-sitosteryl ferulate. Numbers on HPLC peaks represent elution times (min).

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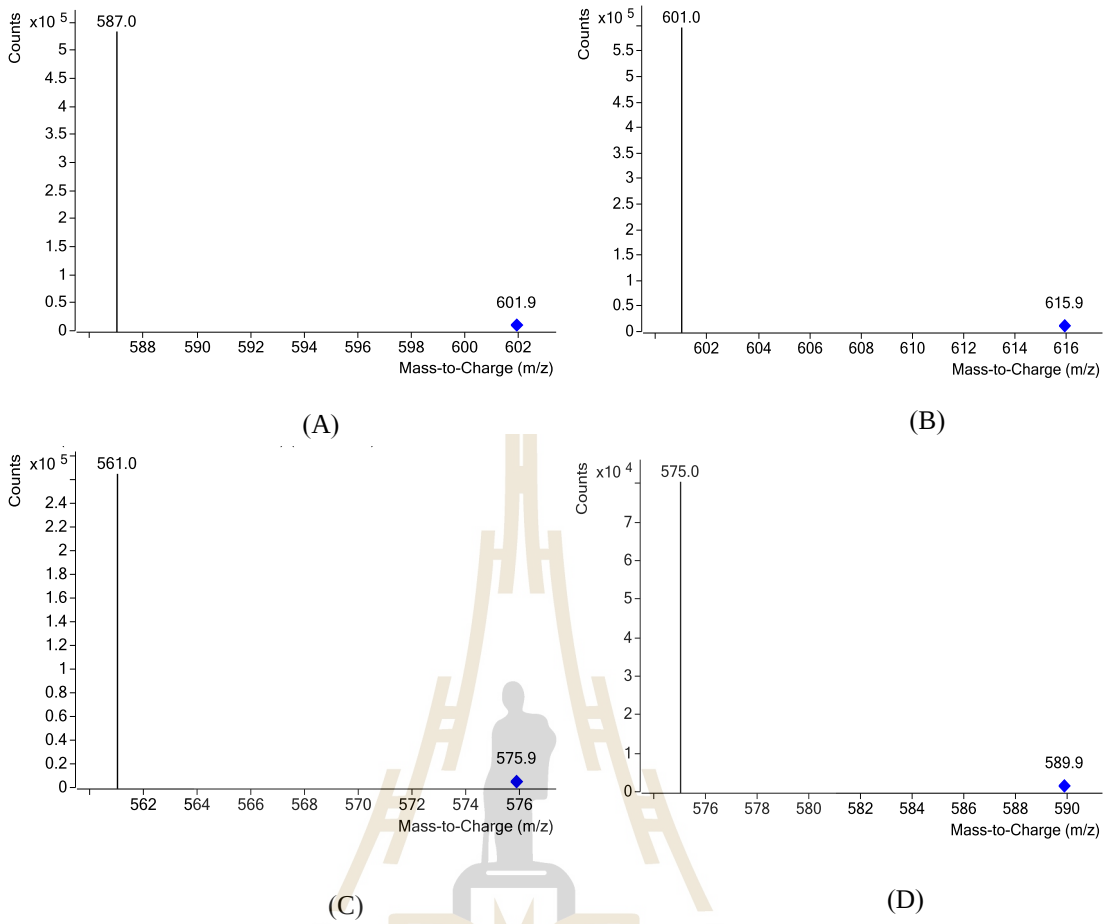

Figure 6.4 Mass spectra of isolated gamma oryzanol: (A) cycloartenyl ferulate, (B) 24-methylene cycloartanyl ferulate, (C) campesteryl ferulate, (D) β-sitosteryl ferulate.

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Table 6.7 The properties of gamma-oryzanol standard and gamma-oryzanol isolated from rice bran soapstock.

	Gamma oryzanol standard[1]	**Isolated gamma oryzanol[1]**
Components (%)		
Cycloartenyl ferulate	26.12±0.94	26.68±0.43
24-Methylene cycloartanyl ferulate	35.27^{a}±1.05	30.70^{b}±0.76
Campesteryl ferulate	23.20±0.55	23.21±0.23
β-Sitosteryl ferulate	15.41^{b}±1.04	19.41^{a}±0.55
Color value		
L^{*}	94.25^{a}±0.16	83.33^{b}±0.95
a^{*}	-0.01^{a}±0.0	-0.57^{b}±0.01
b^{*}	0.76^{b}±0.05	4.69^{a}±0.25

[1] The values are the mean of three replications ± standard deviation. Different letters in each row denote statistically significant differences between treatments ($p<0.05$). Different letters in each column denote statistically significant differences between treatments ($p<0.05$). The values are the mean of three replications±standard deviation.

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CHAPTER VII

OVERALL CONCLUSION

The main purposes of this work were to determine the efficiency of the extraction of lipid and gamma-oryzanol from rice bran and evaluated the physiochemical properties of lipid and gamma-oryzanol obtained. For these purposes, 5 experiments were carried out. The first experiment focused on the effects of ultrasound and thermal pretreatments combined cold press extraction on the increase in the yield and the quality of rice bran oil (Chapter III). The next experiment was to evaluate the effects of chemical neutralization (i.e., NaOH concentration, time, and temperature process) on the oil recovery and physiochemical properties of the neutralized rice bran oil (Chapter IV). Response surface methodology (RSM) was used to find the optimal conditions for the chemical neutralization. The two last experiments were done to evaluate the effect of ultrasound assisted solvent extraction and kinetic model for the extraction of gamma-oryzanol from rice bran soapstock (Chapter V), and the isolation method used in collecting the gamma-oryzanol powder from gamma-oryzanol rich fraction (ORF) (Chapter VI). The summary of study was summarized as follows:

Regarding the extraction methods, the ultrasound pretreatment and the thermal pretreatment combined cold press extraction (CCE and UCE) were successful in extracting lipid from rice bran. Various criteria were used for evaluating the quality

and the quantity of rice bran oil extract (i.e., oil extractability, IV, PV, PPV, gamma-oryzanol content, color, and fatty acid profile). As our results, the ultrasonic power and thermal cooking time was positively correlated to the oil extractability. High ultrasound intensity (UCE 4.5- and UCE6.75-) and extended thermal cooking (CCE25 and CCE35) enhanced high oil extractability. However, high ultrasonic intensity and extended cooking time resulted in a low crude rice bran oil quality Meanwhile, short thermal cooking (CCE5) had less oil extractability than UCE (4.5- and 6.75-) and CCE (15, 25, and 35), but the high quality of crude rice bran oil, especially fatty acid was observed, which closely resembled those of the CE.

In the chemical refining, FFA, wax, and phospholipids were successfully removed by NaOH solution. The effect of variable chemical neutralization parameters on the oil recovery, free fatty acid) FFA (level, and gamma-oryzanol content of neutralized rice bran oil was evaluated by using response surface methodology .The response surface methodology (RSM) results indicated the positive correlation among NaOH concentration, temperature, and process time. These results also revealed that there were strong impacts of NaOH concentration and process temperature on FFA, gamma-oryzanol content and oil recovery and quality (Chapter IV). An increase in NaOH concentration (10-20°Be) and process temperature (60-75°C) accelerated the decrease of FFA and preserved gamma-oryzanol in oil. In addition, lower NaOH concentration (10-20°Be) and shorter time (5-12min) also yielded greater oil recovery and slightly reduced gamma-oryzanol. Whereas, an increase NaOH concentration from 20 to 30°Be and process time up to 15min led the oil yield loss and gamma-oryzanol loss. From our results, the optimum neutralization condition was found to be 19.24°Be, 74.79°C, and 11.18 min. Under the optimum condition, the quality of rice

bran was improved, with a low FFA (0.39% oleic acid), phosphorus content (6.25ppm) and peroxide value (5.31 meq O2/kg).

Gamma-oryzanol presented in dried rice bran soapstock was about 4.41 g/100 g dried soapstock. Our research aims to extract gamma-oryzanol from rice bran soapstock. A mixture of ethyl acetate and ethanol (MEE, 85:15 (v/v)) was found to be the highest yield of gamma-oryzanol rich fraction (ORF) using the ultrasound assisted solvent extraction (UASE) technique (Chapter V). In this chapter, response surface methodology (RSM) with a face-centered composite design) FCCD(was used to investigate optimal extraction conditions. The results showed that the optimum conditions for ORF extraction from rice bran soapstock were 4.0 W/g, 50ºC, and 21.50 min. ,optimal condition the Underthe gamma-oryzanol concentration was found to be 4.32 g gamma-oryzanol/100 g DRBS or 98.03% of yield compared with soxhlet extraction. From the extraction model, the ultrasound power and the temperature affected to the ORF recovery more significantly than that of extraction time. Furthermore, the kinetic model confirmed that ultrasound parameters have a strong affluence on the ORF recovery. Especially, ultrasound power and extraction temperature have a predominant influence in C_e, h, and k of the kinetic model.

The purification of gamma-oryzanol using low temperature was experimented by a two-step crystallization process (Chapter VI). In the first step crystallization, gamma-oryzanol rich fraction (ORF) was dissolved in a mixture of ethanol in ethyl acetate 30:70 (v/v) at 7.5:1 of solvent to solid ratio. The samples were then stored at -15°C for 7.5 hours. The solid phase (wax and the impurities) and liquid phase were separated by using high-speed centrifuge (10,000 rpm) and filtered using Whatman filter no1. The liquid phase (LP) was crystallized again at 5°C for 6 h (the second

crystallization). The yield and purity of gamma-oryzanol obtained from this step were 68.89 wt% and 87.10 wt% by HPLC measurement. The physical properties of the sample matrix such as color, melting, and individual composition were determined. The melting point of gamma-oryzanol isolated from ORF and standard was in a range of 125-130°C. In addition, gamma-oryzanol obtained was darker than gamma-oryzanol standard due to the remaining wax and impurities. The gamma-oryzanol standard and isolated from rice bran soapstock presented four important compounds including 24-methylene cycloartanyl ferulate, campesteryl ferulate, cycloartenyl ferulate or beta-sistosteryl ferulate. Specially, 24-methylene cycloartanyl ferulate and campesteryl ferulate are considered as a major fraction of gamma-oryzanol.

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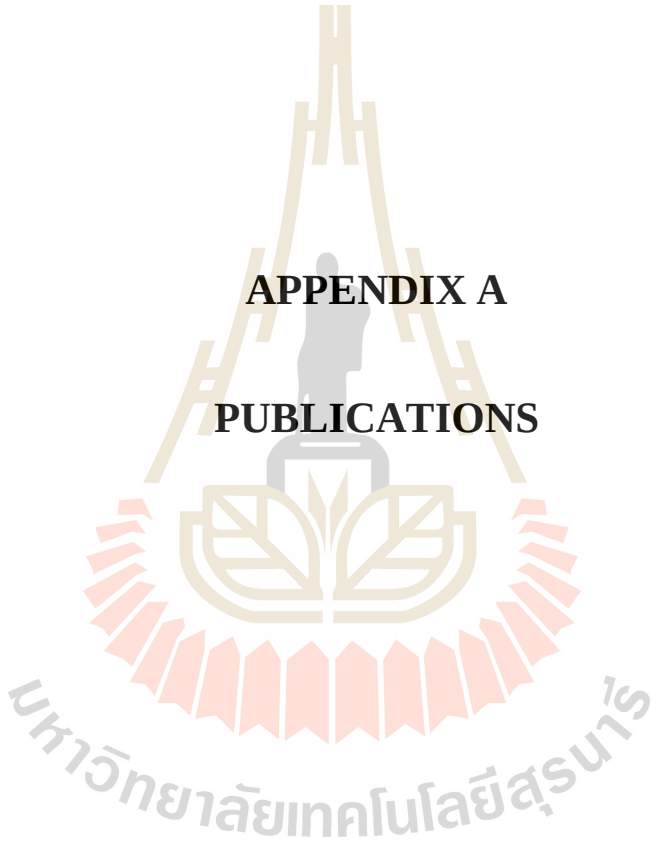
APPENDIX A

PUBLICATIONS



LIST OF PUBLICATIONS

Journals

Phan, V.M., Junyusen, T., Liplap, P., and Junyusen, P. (2018). Effects of ultrasonication and thermal cooking pretreatments on the extractability and quality of cold press extracted rice bran oil. **J. Food Process Eng.** 42(2): 1-8.

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Phan, V.M., Junyusen, T., Liplap, P., and Junyusen, P. (2019). Ultrasound assisted solvent extraction oil from dried rice bran soapstock. **The 12th TSAE International Conference,** 14-15 March 2019, Thailand.

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BIOGRAPHY

Mr. PHAN VAN MAN was born on the 5th of Sept 1986 in Quang Ngai province, Vietnam. He graduated Bachelor of Food Engineering from Faculty of Chemical and Food Engineering, HCM City University of Technology and Education in 2009. After graduation, he was studied master degree at Faculty of Chemistry, HCM City University of Technology, Vietnam. In 2016, he obtained the scholarship "*Provision of 2016 Postgraduate Scholarships for Foreign Students"* to presence a Doctor degree at school of Agricultural Engineering, Institute of Engineering, Suranaree University of Technology, under the supervision of Assist Prof. Dr. Tiraporn Junyusen and Assist Prof. Dr. Pansa Liplap. He conducted the research in the topic of extraction lipid and gamma-oryzanol from rice bran.

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