การสกัดแกมมา-ออริซานอลและโปรตีนจากรำข้าวและกากรำข้าวด้วยของไหลภาวะกึ่งวิกฤตและเหนือวิกฤต

นางสาวศศิธร สรรพ่อค้า

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเคมีเทคนิค ภาควิชาเคมีเทคนิค
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

EXTRACTION OF γ-ORYZANOL AND PROTEINS FROM RAW AND DE-OILED RICE BRAN USING SUBCRITICAL AND SUPERCRITICAL FLUIDS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Chemical Technology
Department of Chemical Technology
Faculty of Science
Chulalongkorn University
Academic Year 2011

Thesis Title	EXTRACTION OF γ-ORYZANOL AND PROTEINS FROM RAW AND DE-OILED RICE BRAN USING SUBCRITICAL AND SUPERCRITICAL FLUIDS
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ศศิธร สรรพคำ : การสกัดแกมมา-ออริซานอลและโปรตีนจากรำข้าวและกากรำข้าวด้วยของไหลภาวะกึ่งวิกฤตและเหนือวิกฤต. (EXTRACTION OF γ-ORYZANOL AND PROTEINS FROM RAW AND DE-OILED RICE BRAN USING SUBCRITICAL AND SUPERCRITICAL FLUIDS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. สมเกียรติ งามประเสริฐสิทธิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : Prof. Yoshito Oshima, Ph.D., ผศ. ดร. วรินทร ชวศิริ, 132 หน้า.

ในปัจจุบันมีการศึกษาการสกัดสารต่างๆ จากชีวมวลด้วยของไหลภาวะกึ่งวิกฤตและเหนือวิกฤตในวงกว้าง สำหรับงานวิจัยนี้ มุ่งเน้นการสกัดแกมมา-ออริซานอลและโปรตีนจากรำข้าวและกากรำข้าว เพื่อเพิ่มมูลค่าของรำข้าวและกากรำข้าวนอกเหนือจากการนำไปใช้เป็นอาหารสัตว์ ผลการทดลองชี้ให้เห็นว่า การใช้คาร์บอนไดออกไซด์ภาวะเหนือวิกฤตสามารถสกัดน้ำมันที่มีแกมมา-ออริซานอลความเข้มข้นสูงจากรำข้าวและกากรำข้าวได้ โดยผลิตภัณฑ์ที่ได้มีความเข้มข้นสูงถึง 15,000 – 19,000 ppm ในขณะที่การใช้น้ำภาวะกึ่งวิกฤตสามารถสกัดโปรตีนจากรำข้าวได้ถึงร้อยละ 100 อย่างไรก็ตาม จากการศึกษาจลนพลศาสตร์ของการสลายตัวของโปรตีนจากรำข้าวภายใต้น้ำภาวะกึ่งวิกฤต พบว่าโปรตีนจะสลายเป็นกรดอะมิโนได้ดีที่อุณหภูมิสูงกว่า 200 องศาเซลเซียส และเวลาในการทำปฏิกิริยามากกว่า 30 นาที การสกัดโปรตีนจึงควรเลือกภาวะที่เหมาะสม หรือมุ่งเน้นไปที่การผลิตกรดอะมิโนแทนการสกัดโปรตีน นอกจากศึกษาการสกัดสารแต่ละชนิดด้วยของไหลต่างชนิดแบบแยกส่วนกันแล้ว งานวิจัยนี้ได้ทดลองสกัดแกมมา-ออริซานอลด้วยคาร์บอนไดออกไซด์ภาวะเหนือวิกฤต และการสกัดโปรตีนด้วยน้ำภาวะกึ่งวิกฤตแบบต่อเนื่องกัน โดยใช้ภาวะที่เหมาะสมที่ได้จากการทดลองขั้นต้น จากการทดลองพบว่าการสกัดด้วยของไหลภาวะกึ่งวิกฤตและเหนือวิกฤตแบบต่อเนื่องกันนั้น สามารถทำได้โดยให้ผลไม่ต่างจากการสกัดแบบแยกส่วนกัน ผลิตภัณฑ์ที่ได้ นอกจากน้ำมันรำข้าวที่มีแกมมา-ออริซานอลความเข้มข้นสูงและโปรตีนแล้ว ยังได้น้ำตาล กรดอะมิโน และสารมูลค่าเพิ่มอื่นๆ อีกด้วย

ภาควิชา เคมีเทคนิค	ลายมือชื่อนิสิต .....
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5173887923 : MAJOR CHEMICAL TECHNOLOGY
KEYWORDS : SUBCRITICAL WATER / SUPERCRITICAL CARBON-DIOXIDE / PROTEINS / γ-ORYZANOL / RICE BRAN

SASITHORN SUNPHORKA : EXTRACTION OF γ-ORYZANOL AND PROTEINS FROM RAW AND DE-OILED RICE BRAN USING SUBCRITICAL AND SUPERCRITICAL FLUIDS. ADVISOR : ASSOC. PROF. SOMKIAT NGAMPRASERTSITH, Ph.D., CO-ADVISORS: PROF. YOSHITO OSHIMA, Ph.D., ASST. PROF. WARINTORN CHAVASIRI, Ph.D., 132 pp.

Subcritical and supercritical fluid extractions have been developed in order to recover valuable compounds from lignocellulosic biomass. In this work, the authors focused on γ-oryzanol and proteins extraction from rice bran and de-oiled rice bran which are generally used as an animal feed. The experimental results showed that supercritical carbon dioxide (SC-CO_2) could be used to extract rice bran oil with relatively high γ-oryzanol concentration whilst subcritical water (SW) could recover 100% of protein. The kinetic information of rice bran protein hydrolysis under SW atmosphere indicated that proteins/polypeptides hydrolyze to amino acids within two pathways. Moreover, the increase in temperature and reaction time (over 200 °C and 30 min) favored the proteins/polypeptides degradation rate. Thus, the extraction condition for SW extraction should be optimized or the amino acid production would be focused instead. Finally, the multi-fluid processing or consecutive extraction process using SC-CO_2 followed by SW was investigated. The suitable condition obtained from each single-fluid extraction was used. The results showed that SC-CO_2 extraction and SW extraction could be operated sequentially without any interaction. The obtained products were not only rice bran oil with high concentrated γ-oryzanol and proteins, but also sugars, amino acids and other value-added products.

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude my supervisors, Assoc. Prof. Somkiat Ngamprasertsith, Prof. Yoshito Oshima and Asst. Prof. Warintorn Chavasiri who reviewed this thesis during its preparation and offered many helpful suggestions, supervision and much encouragement throughout pass four years of my research.

I would like to acknowledge Assoc. Prof. Kejvalee Pruksathorn, Prof. Patarapan Prasassarakich, Asst. Prof. Prapan Kuchontara and Dr. Vorakan Burapatana for their participation on the dissertation chairman and members of thesis committee, respectively. I am also deeply grateful to Dr. Kunakorn Poochinda who gave helpful advices.

Sincerest appreciation also extends to The Royal Golden Jubilee Scholarship (Thailand Research Fund) for financial support of this research and Mr. Pravit Santiwattana, from the Thai Edible Oil Co., Ltd., for supplying the rice bran and de-oiled rice bran and also for the NIR analysis of both raw materials.

Many thanks go to Dr. Ruengwit Sawangkeaw and other colleagues and friends at the Department of Chemical Technology, Dr. Saipin Boonkert at the Department of Chemistry, Chulalongkorn University and also Oshima & Otomo laboratory's members, Asst. Prof. Rumiko Hayashi and Dr. Osamu Sawai at The University of Tokyo for their helpful suggestions, supports, encouragement and friendships during the period of this research.

Finally, I wish to acknowledge the support, encouragement and love of my family throughout my Ph.D. program.

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NOMENCLATURES

C_{aa}	amino concentration (g/L)
C_{agg}	aggregated protein concentration (g/L)
C_p	protein concentration (g/L)
C_{po}	Disaggregated proteins/ polypeptides concentration (g/L)
C_{p0}	Initial protein concentration (g/L)
E_a	activation energy (kJ/mol)
$k_{agg,aa}$	rate constant (amino acid production from aggregated protein) (g/L•min)
$k_{agg,po}$	rate constant (disaggregated proteins/polypeptides production from aggregated protein) (L/g•min)
$k_{i,j}$	rate constant (species *i* converts to species *j*)
$k_{p,agg}$	rate constant (protein aggregation) (g/L•min)
$k_{po,aa}$	rate constant (amino acid production from disaggregated proteins/polypeptides) (g/L•min)
l	reaction order for amino acid production from disaggregated proteins/polypeptides
m	reaction order for protein aggregation
n	reaction order for disaggregated proteins/polypeptides production from aggregated protein
o	reaction order for amino acid production from aggregated protein
R	universal gas constant (8.3143 J/mol•K)
t	time (min)
T	temperature (K)
τ	time for the consecutive reaction (min^{-1})

CHAPTER I

INTRODUCTION

1.1 Motivation

Rice (*Oriza Sativa* L.) is the most important food source for human, especially in Asia. Thailand is the top ten rice producing country and top three rice exporter of the world. Thai rice production is increasing continuously to meet rising global demand. However, since rice is sold as white rice, white rice production process generates several wastes such as rice husk, rice straw and rice bran. Nowadays, rice is produced in Thailand about 30 million tones/year and results in the production of 3 million tones/year of rice bran [1]. The rice bran contains high amount of proteins, oil and nutrients. γ-oryzanol in rice bran acts as antioxidant which prevents Alzheimer's disease and can be used to reduce cholesterol level and treat nerve imbalance. Moreover, rice bran oil has other nutrients such as β-carotene, omega 3, omega 6 and protein such as lipase and α-1 antitrypsin [2]. However, small amount of them is extracted to recover rice bran oil. Apart of rice bran and the de-oiled rice bran are used as animal feed. Only small amount of them was mixed with food or recover some vitamin and wax for cosmetics [3]. Therefore, rice bran and de-oiled rice bran containing high level of protein have not been utilized to their full potential. In recent years, many studies investigate the nutrients and other chemicals extraction from rice bran and de-oiled rice bran for recovery the valuable chemicals.

In case of γ-oryzanol and oil extraction, the conventional process of rice bran oil extraction is chemical consumption processes. Hexane is generally used as a solvent. The extraction process can be carried out at 50 ^{o}C and atmospheric pressure. However, it must have several processes for product purification and solvent recovery [4 – 6]. The major problems of this process are hexane leakage into the environment and hexane separation resulting in high operating cost. The obtained γ-oryzanol concentration is only 2,000 – 4,000 ppm. Recently, many researches have been interested in using supercritical carbon dioxide (SC-CO_2) in stead of hexane [2, 5, 11,

12]. The SC-CO_2 is normally used as medium to extract non-polar compounds from solid matrix due to its relatively low critical temperature and pressure, non-toxic, non-flammable, noncorrosive, inert and inexpensive. It can be separated easily from rice bran oil by reducing pressure. The extraction process is normally carried out in semi-continuous system. The obtained γ-oryzanol concentration is over than 10,000 ppm. Previous works on SC-CO_2 extraction were carried out at high operating pressure about 50 – 70 MPa and low CO_2 flow rate (0.5 – 1 g/min) or at pressure about 30 – 50 MPa and higher CO_2 flow rate (10 – 40 g/min). Such high operating pressure and CO_2 flow rate require high energy consumption, resulting in high operating cost. However, there is still a lack of study on SC-CO_2 extraction at relatively lower pressure, especially with low CO_2 flow rate, to achieve high oil yield and γ-oryzanol concentration.

In case of protein extraction, the conventional extraction process employs chemicals such as sodium hydroxide and hydrochloric acid. The process typically consists of several sub-processes and operating steps, leading to complication and time consumption during operation. Recently, subcritical water (SW) was employed for the production of protein and amino acids from both rice bran and de-oiled rice bran [7 – 10, 13]. The extraction process using SW can be served as a one-step process for extracting protein and can achieve 100% extraction of original protein in forms of protein hydrolysates. In order to utilize full potential of rice bran as rice bran oil with high γ-oryzanol concentration and protein, the rice bran would be extracted consecutively by integrating the SW process with the SC-CO_2 process. Moreover, the SC-CO_2 extraction and SW extraction should be performed in the same type of reactor, making the process less complicated. However, it was found that all of previous works on protein extraction using SW were carried in a batch-type reactor while most works on SC-CO_2 extraction were done in semi-continuous reactor.

Therefore, the goal of this work is to develop the consecutive process for γ-oryzanol and protein extraction using SC-CO_2 and SW in a semi-continuous reactor. Furthermore, this work tried to explore the γ-oryzanol extraction using SC-CO_2 at relatively low operating pressure and CO_2 flow rate. An experimental design was also

performed using analysis of variance (ANOVA) and response surface methodology to determine optimum conditions for each step.

In addition, this work suggested a new kinetic model of protein aggregation, disaggregation and decomposition reaction pathway. Since the SW extraction is carried out at severe condition, some of extracted protein might be degraded to other polypeptides and amino acids. However, previous works were mentioned only one-step reaction pathway of amino acid production from proteins. The previous kinetic model cannot describe protein aggregation and disaggregation under SW. The new kinetic model was proposed and the reaction orders and rate constants were also determined.

1.2 Objectives

1. Study the extraction of γ-oryzanol using SC-CO_2 with relatively low operating pressure.
2. Study the extraction of protein using SW in semi-continuous reactor.
3. Propose a new kinetic model that emphasizes the rice bran protein aggregation, disaggregation and decomposition in SW atmosphere.
4. Investigate a new integrated process of consecutive subcritical and supercritical fluids extraction process for extracting γ-oryzanol and protein from rice bran in one experimental set-up.

1.3 Scope of This Work

This work was separated into 4 parts. In part 1, the oil and γ-oryzanol was extracted from rice bran and de-oiled rice bran using SC-CO_2. The experiments were carried out in a semi-continuous reactor at temperature range of 40 – 100 ^{o}C, pressure of 10 – 30 MPa and 5 – 60 min of operation. These chosen operating conditions are relatively low compared to literature reviews. In part 2, the protein was extracted from rice bran and de-oiled rice bran using SW. The experiments were also carried out in semi-continuous reactor at temperature range of 150 - 250 ^{o}C, pressure of 4 - 10 MPa and 5 – 60 min of operation. In both two parts, an experimental design, ANOVA and

response surface methodology were used to analyze the experimental results and identify the optimum conditions. Finally, results of this work were compared to those of literature reviews.

In part 3, kinetic models of rice bran protein aggregation, disaggregation and decomposition were presented. The simplified model was proposed. The differential equations of each reaction step were generated and fitted to experimental results. It can be used to describe the phenomena of protein change under SW atmosphere. In part 4, the integrated process of part 1 and part 2 was investigated. SC-CO_2 extraction was performed followed by SW extraction in order to extract oil with high γ-oryzanol concentration and protein in one process. The optimum conditions obtained from statistical analysis of part 1 and 2 were used. The composition of products and residue were analyzed.

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Rice Bran and Its Antioxidant Contents

2.1.1 Composition of rice bran and de-oiled rice bran

Rice is grown in many countries around the world including China, India, Indonesia, Bangladesh, Burma, Vietnam, Japan, the Philippines and Thailand. The rice production is increasing since the demand is constantly rising in many countries. It is considered as a staple food for more than half of humanity [14]. After harvesting, the harvested rice or so-called paddy is milled to remove its outer layer, hull, to produce brown rice and then this brown rice sent to further process to produce white rice. The removed brown layer is called rice bran [15]. The rice bran and its composition is shown in Fig. 2-1. It is a good source of nutrients since it has relatively high level of antioxidants, vitamins and protein as shown in Table 2-1 [2, 16, 17].

Recently, rice bran is used in many food application including crackers, beverage and medical foods.

Figure 2-1. Rice and its composition [17]

Table 2-1. Nutrients found in commercial stabilized rice bran

Composition		Value/100 g rice bran	Composition		Value/100 g rice bran
Moisture	(g)	6.00	Vitamin B Complex		
Protein*	(g)	14.50	- Thiamin	(mg)	2.65
Ash	(g)	8.50	- Niacin	(mg)	46.87
Total Carbohydrates	(g)	51.00	- Riboflavin	(mg)	0.28
- Dietary Fiber**	(g)	29.00	Pantothenic Acid	(mg)	3.98
Total Fat	(g)	20.50	Vitamin B6	(mg)	3.17
- Saturated Fat	(%)	3.70	γ-Ozyzanol	(mg)	245.15
Tocols	(mg)	25.61	Phytosterols	(mg)	302.00

* Protein includes albumin, globulin, glutelin and prolamin, the most interesting proteins are lipases and α-1 antitrypsin

** The indigestible carbohydrates

There are many products those are made from rice bran such as rice bran wax and other rice bran extract. They have been used in wide variety of cosmetics and foods. The most well-known rice bran product is rice bran oil which contains relatively high level of vitamins, protein and commercially-important phytonutrients such as tocopherol and γ-oryzanol; most of them interest pharmacy and cosmetics [12, 18]. In order to produce rice bran oil, rice bran is extracted and purified by various chemicals and processes. Then, the de-oiled rice bran which contains small amount of oil, but high value of protein and other vitamins is disposed as a waste. The photonutrients analysis of de-oiled rice bran presents in Renuka Davi and Arumughan's work [19]. The report indicates that de-oiled rice bran contains of γ-oryzanol, ferulic acid, tocopherol, fat, protein and other minerals those can be extracted by alcohol, acetone and other organic solvents. However, there are some attempts to use whole de-oiled rice bran for an animal feed as reported in many researches [20, 21]. Garg and coworkers [20] investigated the replacing of maize grain with de-oiled rice bran on intake of adult ewes and concluded that the grain can be replaced up to 50% concentrate mixture without any effect on ewe's performance.

2.1.2 γ-Oryzanol and rice bran oil

Rice bran oil is superior to traditional cooking oils and quickly becomes a favorite in commercial frying. This is due to its components those have high health benefits. Since the rice bran contains high levels of antioxidants including tocopherol, γ-oryzanol and other polyphenol compounds in different quantities and qualities depending on the variety [22], there has much attraction to recover those nutrients, especially γ-oryzanol, for human consumption. A γ-oryzanol is mainly composed of esters of *trans*-ferulic acid (*trans*-hydroxycinnamic acid) and phytosterols (sterols and triterpenic alcohols) [12]. The molecular structures of four phytosterols: cycloartenol, b-sitosterol, 24-methylenecycloartenol and campesterol predominate, is shown in Fig. 2-2. A γ-oryzanol in rice bran has been reported to reduce cholesterol levels in serum and have antioxidant functions due to ferulic acid, a strong antioxidant, containing in its structure. Moreover, it is found that a higher quantity of γ-oryzanol than vitamin E in rice bran cause the higher in antioxidant activities and maybe more important antioxidant in rice bran to reduce cholesterol oxidation compared to vitamin E [23].

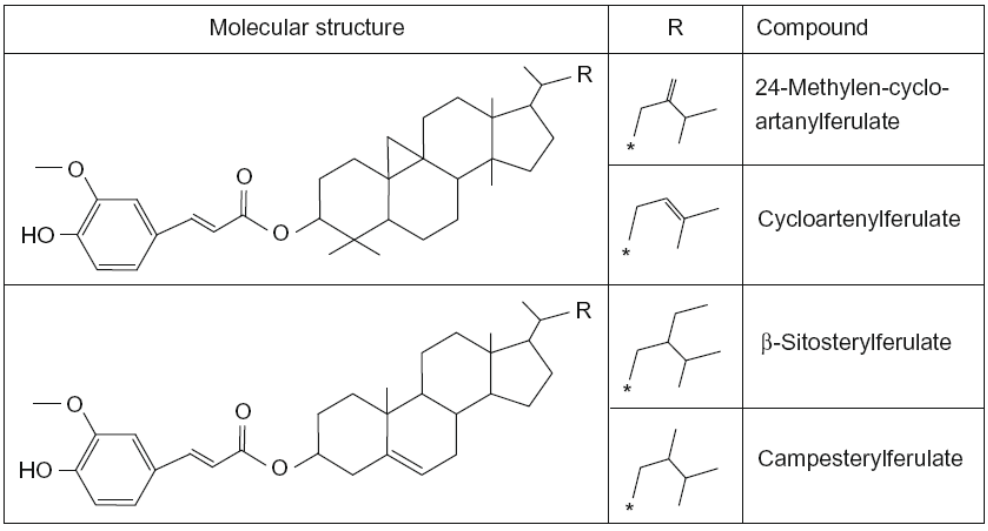


Figure 2-2. Chemical structures of 4 phytosterols found in rice bran [12]

Due to a largely lipophilic of γ-oryzanol, it is extracted from rice bran with rice bran oil (around 20%wt content in rice bran). The rice bran oil is well-known as heart oil because of its lowering cholesterol properties. Many extraction processes such as chemical extraction, enzymatic processes, subcritical and supercritical fluids extraction have been developed to obtain phytochemical-rich fraction. The concentration of γ-oryzanol in rice bran oil is varied with extraction method as reported in Lerma-Gacia and coworkers' and Patel and coworkers' works [12, 16]

However, the various concentrations and grades of rice bran oil are produced due to their applications. The γ-oryzanol concentration of commercial cooking rice bran oil is about 2000 – 3000 ppm and price of this edible oil is taken to be around 3 USD/kg [24, 25]. Especially for health food, high concentrated γ-oryzanol and other phytonutrients in rice bran oil was sold in form of soft-capsule or tablet [26]. The rice bran oil that contains γ-oryzanol more than 1% is normally packed in soft-capsule (about 500 mg/capsule) and it costs around 0.5 USD/capsule [27, 28]. Nowadays, many people around the world concern about their health and they are interested in this kind of natural product. Thus many processes and experiments have been investigated and developed for many years. The researches were summarized and described in further down.

2.1.3 Rice bran proteins

Besides γ-oryzanol, rice bran protein is one of valuable by-products of rice bran. It is an alternative to soy protein and can be an ingredient for wide range of food products. As shown in Table 2-1, protein is typically contented in rice bran around 14 – 17 %wt which includes 37% albumin (water-soluble), 36% globulin (salt-soluble), 22% glutelin (alkali-soluble), and 5% prolamin (alcohol-soluble) [29, 30]. However, albumin is reported to be insoluble in distilled water due to its molecule can form head-to-tail aggregates together by electrical force, resulting in a strong aggregation. Low salt concentrations neutralize this charge and allow it soluble but higher salt concentrations will precipitate it again [31 – 39].

In food application, rice bran protein concentrated is used as ingredient in skin conditioner or sold in form of powder which will be mixed with bread, beverages, pasta, confections, and weaning foods. Rice bran proteins are generally extracted by NaOH solution, then precipitated by HCl solution and finally freeze-dried to obtain concentrated proteins. Its price depends on its concentration that varies around 70% or more [40, 41]. In addition, rice bran proteins are interesting in terms of a container of essential amino acids. The essential amino acids contained in concentrated rice bran consists of isoleucine (second-limiting amino acid), leucine, lysine, methionine, cystine, phenylalanine, tyrosine, threonine (first- limiting amino acid), tryptophan and valine [33].

2.2 Conventional Extraction and Subcritical/Supercritical Fluids Extraction

2.2.1 Conventional extraction process for rice bran oil motivation

The production of rice bran oil includes three main processes; stabilization/preparation, crude rice bran oil production and rice bran oil refinery. Preparation process is firstly conducted to decrease moisture content and treat rice bran particles for enhancing extractability. Rice bran is stabilized by heating with steam at 90 – 100 ^{o}C to inactivate enzyme-lipase. The bran is then dried to decrease its moisture content. It expands as it exits the stabilizer. After that, the pretreated rice bran is transferred to extraction process to produce crude rice bran oil. The flowchart of preparation process, crude rice bran oil process and solvent recovery process of Bran Oil Co., Ltd., Surin province of Thailand, is shown in Fig. 2-3. In the extraction process, hexane is normally used as a solvent using batch or continuous reactor. The process is operated at 50 ^{o}C to remove oil from rice bran particles. The oil/hexane mixture or so-called miscella is then separated by series of physical processes such as cyclone separation and evaporation. As can be seen in this flowchart, the wastes include steam, dust, hexane, wastewater and bran fines. The crude bran oil can be used for manufacture of soap, glycerine, cosmetics and etc [6, 42].

The major problem of solvent extraction process is hexane leakage into the environment, since hexane is volatile, flammable and explosive. Moreover, some report indicated that the largest operating cost of using hexane to extract edible oil from plant is hexane separation from extracted oil. The traces amount of chemicals those remain in the product has negative effect on both quality of products and human health [43]. To avoid this severe problem, the other methods has been modified and one of them is supercritical fluid extraction which has several advantages compared to the conventional process as described in another section.

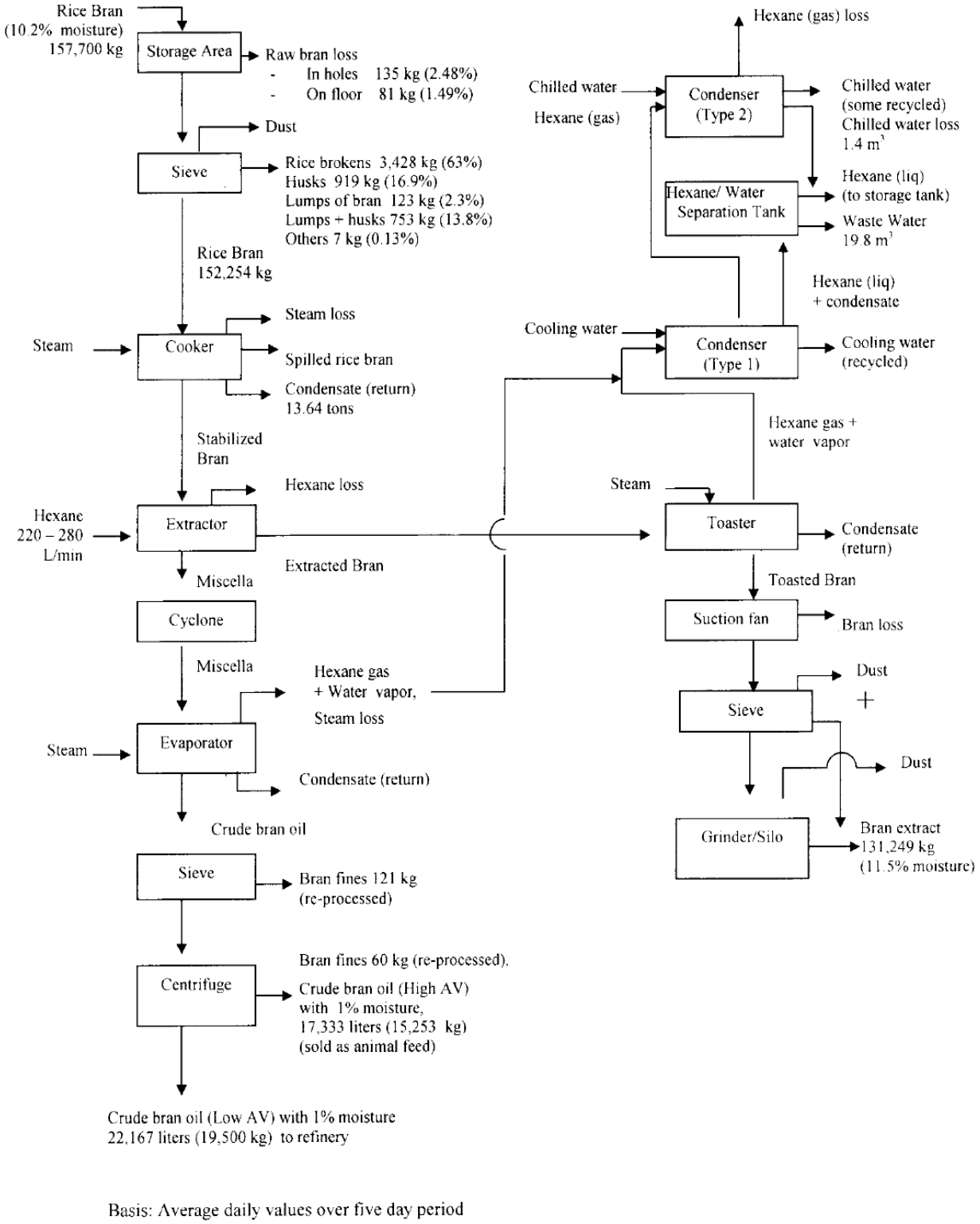


Figure 2-3. Crude rice bran oil production; Surin, Bran Oil Co., Ltd. [6]

Table 2-2 and 2-3 shows the composition of crude rice bran oil and commercial rice bran oil, respectively. It can be found that crude rice bran oil has high level of free fatty acids compared to rice bran oil. The further processes are thus preformed in order to upgrade the crude rice bran oil to commercially food grade rice bran oil. Food grade rice bran oil was produced by both physical refinery and chemical refinery process involving bran fines removal, degumming, dewaxing, deacidification, bleaching and deodorization [16, 44, 45]. However, some refinery processes cause the strong effecting on refining losses which is lowering the refinery yield. The main process causing the highest refining losses of rice bran oil is neutralization [46]. In this process, free fatty acid is partially neutralized by caustic and then washed by water to remove soap. In case of high content free fatty acid, higher amount of caustic and water is added which rise to great loss of soluble rice bran oil in wastewater. The extraction process that has high potential to produce less free fatty acid and wax content rice bran oil is thus an attractive way to avoid the complicated refinery process and prevent the refining losses.

Table 2-2. Lipid composition of crude rice bran oil [47]

Component	**%wt**
Saponifiable lipids	**90 – 96**
Neutral lipids	88 – 89
Triacylglycerols	83 – 86
Diacylglycerols	3 – 4
Monoacylglycerols	6 – 7
Free fatty acids	2 – 4
Waxes	3 – 4
Glycolipids	6 – 7
Phospholipids	4 – 5
Unsaponifiable lipids	**4.2**
Phytosterols	43[a]
4-Methyl sterols	10
4-Dimethyl sterols (trierpene alcohol)**	28
Hydrocarbons *	18
Tocopherols and tocotrienols	3

*Squalene 16 – 40%, i.e. 0.12 – 0.3% in oil

**Mainly oryzanol

[a]These Figure are % of total unsaponifiable lipids

Table 2-3. Physical and chemical characteristics of rice bran oil [47]

Parameter	Typical	Range
Specific gravity (20^{o}C)	0.916	0.916 – 0.922
Refractive index (20^{o}C)	1.470	1.470 – 1.474
Free fatty acid (as %oleic)	0.05	0.05 – 0.12
Iodine value	95	90 – 110
Saponification value	193	180 – 195
Smoke point (^{o}C)	213	-
Colour Lovibond (5.25 inch)	2.5R, 27Y	2.5 – 3.5R, 25 – 35Y
Fatty acid composition (%wt)		
14:0	0.4	0.2 – 0.7
16:0	19.8	12 – 28
16:1	0.2	0.1 – 0.5
18:0	1.9	2 – 4
18:1	42.3	35 – 50
18:2	31.9	29 – 45
18:3	1.2	0.5 – 1.8
20:0	0.9	0.5 – 1.2
20:1	0.5	0.3 – 1.0
22:0	0.3	0.1 – 1.0
Others	0.6	1.0 max

2.2.2 General subcritical and supercritical fluids extraction

1) Subcritical and supercritical fluids properties

The subcritical and supercritical regimes are shown in Fig. 2-4. When substances come beyond their critical points, their properties will be liquid-like densities, gas-like viscosities and diffusion coefficients located in the range between gas and liquid states as shown in Table 2-4. At the critical point, it can be observed that the interface between vapor and liquid phases of the substance disappears, so, transition from gas-phase boundary to the supercritical phase is smooth. Moreover, the most attractive property of supercritical fluids is the ability to tune their

solubilizing power by adjusting pressure and additionally temperature. The possibility of using supercritical fluids to extract solute from various characteristic of raw material such as polarity and molecular size is thus rising [48].

Due to these unique properties, supercritical fluids have been chosen for several engineering applications such as solvent extraction, adsorption, desorption, and also supercritical fluid extraction processes [49].

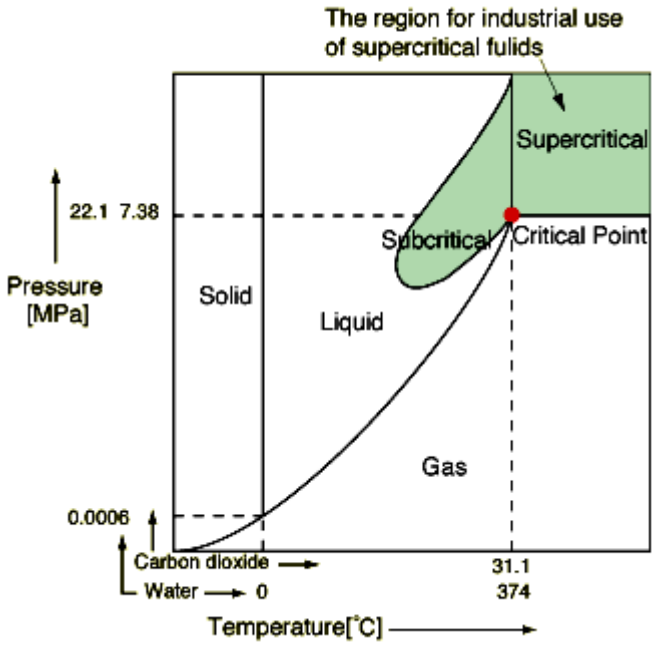


Figure 2-4. P-T diagram of a water and carbon dioxide [50]

Supercritical water: $T>T_c$ (374 ^oC); $p>p_c$ (22.05 MPa)

Subcritical water: $T<T_c$; $p>p_{Saturation}$ (p may also be above p_c)

Steam: $T<T_c$ (T may also be above T_c); $p<p_{Saturation}$ [51]

Table 2-4. Physical properties of supercritical fluids [49]

Fluid	**P/T (MPa/K)**	**Density ρ (kg/m^3)**	**Diffusion coefficient D$_{ij}$ (cm^2/s)**	**Viscosity μ (g/cm·s)**
Gas	0.1/298	0.6 – 2.0	0.1 – 0.4	(1 – 3) x 10^{-4}
Supercritical fluid	P_c/T_c	200 – 500	0.7 x 10^{-3}	(1 – 3) x 10^{-4}
Liquid	0.1/298	600 – 1600	2 x 10^{-6} – 2 x 10^{-5}	(0.2 – 3) x 10^{-2}

There are several fluids those are chosen to use at above its critical point. Each substance has its own advantages and disadvantages and can be used in different applications. Table 2-5 presents several substances which may be used as supercritical fluids [49]. Especial attention is given to carbon dioxide (CO_2), the most commonly used supercritical fluid worldwide. In comparison to other substances, CO_2 presents a critical temperature (T_c) close to room temperature and a relative low critical pressure (P_c), what is interesting when considering the energy requirements for the solvent delivery at a determined operational pressure. Besides, it is abundant, relatively inexpensive, inert, can be used in high purity, non-flammable, atoxic and non-explosive, following the environmental and health organizations´ restrictions.

Table 2-5. Critical data of some pure components [49]

Component	**T_c (^oC)**	**P_c (MPa)**
Ethylene	9.4	5.04
Carbon dioxide	31.1	7.38
Ethane	32.3	4.87
Nitrous oxide	36.6	7.26
Propane	96.8	4.25
n-Hexane	234.5	3.01
Acetone	235.1	4.70
Methanol	239.6	8.09
Ethanol	240.9	6.14
Ethyl acetate	250.2	3.83
Water	374.1	22.06

2) General extraction process

Although the Soxhlet extraction is widely used for liquid-solid extraction, this technique sometimes takes long time to complete and requires solvent separation and disposal process. Supercritical fluid extraction is a relatively new technique for recovery an interested compounds from solid matrix. It can be improved a short sample preparation time, short operation time and less requirement of product

separation/purification process [52]. Two main mechanisms those would be focused in this work include:

(1) In case of extracting lipid from raw material, it has interaction between only solvent and solute in solid phase. The process is thus the solution of extractable matter such as oil and oil-soluble substrate, γ-oryzanol, but the solvent does not dissolve the solid matrix.

(2) In case of extracting some component from lignocellulosic material to obtain agricultural-derived product, the solvent reacts with normally insoluble solute and then the products can be dissolved into solvent. In this case, the extraction is followed by further separation process to recover the desired products from solvent. The subcritical fluid, especially subcritical water, can be used in this purpose.

Fig. 2-5 shows the schematic diagram of the supercritical fluid extraction process. Four main units are involved: extractor, pressure reducer, separator and high-pressure pump [53]. Solid is charged in the reactor and then fluid is fed into the reactor through high-pressure pump. The extract and fluid medium are sent to and separated at separator, after they reach the desired condition. However, supercritical fluid extraction process for solid feed is a semi-continuous process as shown in Fig. 2-5. In the supercritical CO_2 extraction process, a co-solvent is often pumped and mixed with the high-pressure CO_2 for enhancing the solvent power or selectivity.

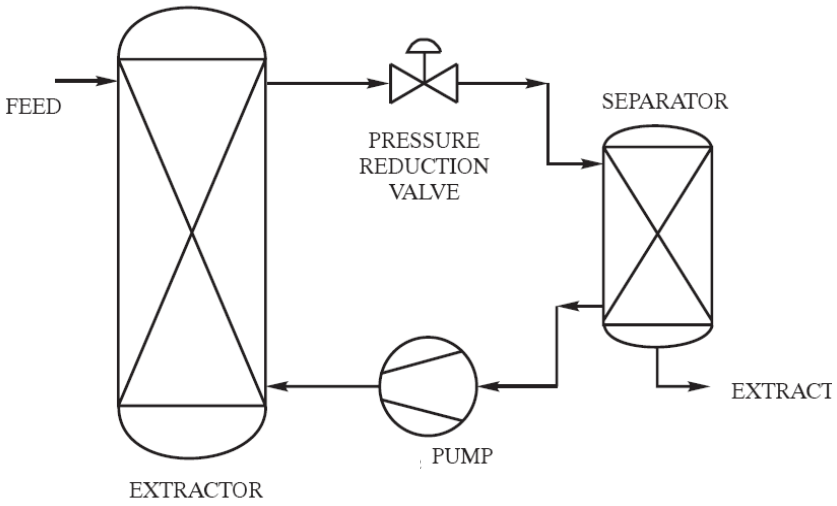


Figure 2-5. The schematic diagram of supercritical fluid extraction process [53]

Major advantages of supercritical fluids extraction are shown as follows [54, 55]:

(1) By using supercritical fluids which have relatively low viscosity but high diffusivity than normal liquid, they can penetrate into solid matrix and leads the faster and more complete extraction.

(2) The solvent power can be adjusted easily by changing pressure and temperature. The tunable property makes it achieving high selectivity and useful for extraction of complex compounds.

(3) In case of CO_2, the extraction process can be operated at low temperature. The mild condition prevents the desired products from undesired reaction and the products are thus not degraded under operating condition. This advantage is useful for extracting volatile and thermal labile compounds. In addition, the solvent and solute can be separated easily by depressurization resulting in no residue solvent in the solute. The separated solvent can be recycled or reused in the large scale process.

(4) Many common used fluids such as CO_2 and water has less environmental or health effects.

(5) Supercritical fluids extraction technique can be applied to different scales; from laboratory scale to large industrial scale.

Anyway, some disadvantages appear for this process such as high initial investment and maintenance cost, large number of operating parameters which are described as further down and difficulty in scale up due to limitation of commercial equipment design and performance [55, 56]. However, many industries, especially for food industries such as decaffeination of coffee and tea, flavors extraction from herbs and edible oil extraction from plant seed, have been operated by using supercritical CO_2 with some modifiers [57]. These processes reach commercially successful and have been developed for achieving more specified products

In case of rice bran oil extraction using supercritical carbon dioxide (SC-CO_2), the properties of carbon dioxide, process detail and expected process variables will be discuss in next section.

2.3 Supercritical Carbon Dioxide Extraction of γ-Oryzanol

2.3.1 Supercritical carbon dioxide (SC-CO_2)

Among supercritical fluids, supercritical carbon dioxide (SC-CO_2) has more attention for extraction process. The carbon dioxide (CO_2) presents a relatively low critical temperature (Tc) which is close to room temperature (31.1 °C) and a relatively low critical pressure (Pc = 7.38 MPa). This critical property involves the relatively low extraction condition which provides less energy requirement compared to other supercritical fluids processes. Moreover, CO_2 is inert, can be used in high purity, relatively inexpensive, non-flammable, nontoxic following the environmental and health organizations´ restrictions [49].

As described above, the solubility of substance in SC-CO_2 can be tuned by adjusting the operating temperature and pressure. The density of CO_2 is increased with higher pressure but lower temperature as shown in Fig. 2-6 (a). Its diffusivity is about 1-2 orders of magnitude higher than that of normal liquids as shown in Fig. 2-6 (b) [58]. Due to this advantage feature, a solute-solvent interaction during the mixing and penetrating small pores in a solid matrix is more effectively. The selectivity can be modified, dissolving power can be adjusted easily to the

approaching liquid phase and the extraction process can be fast performed compared to conventional extraction [2].

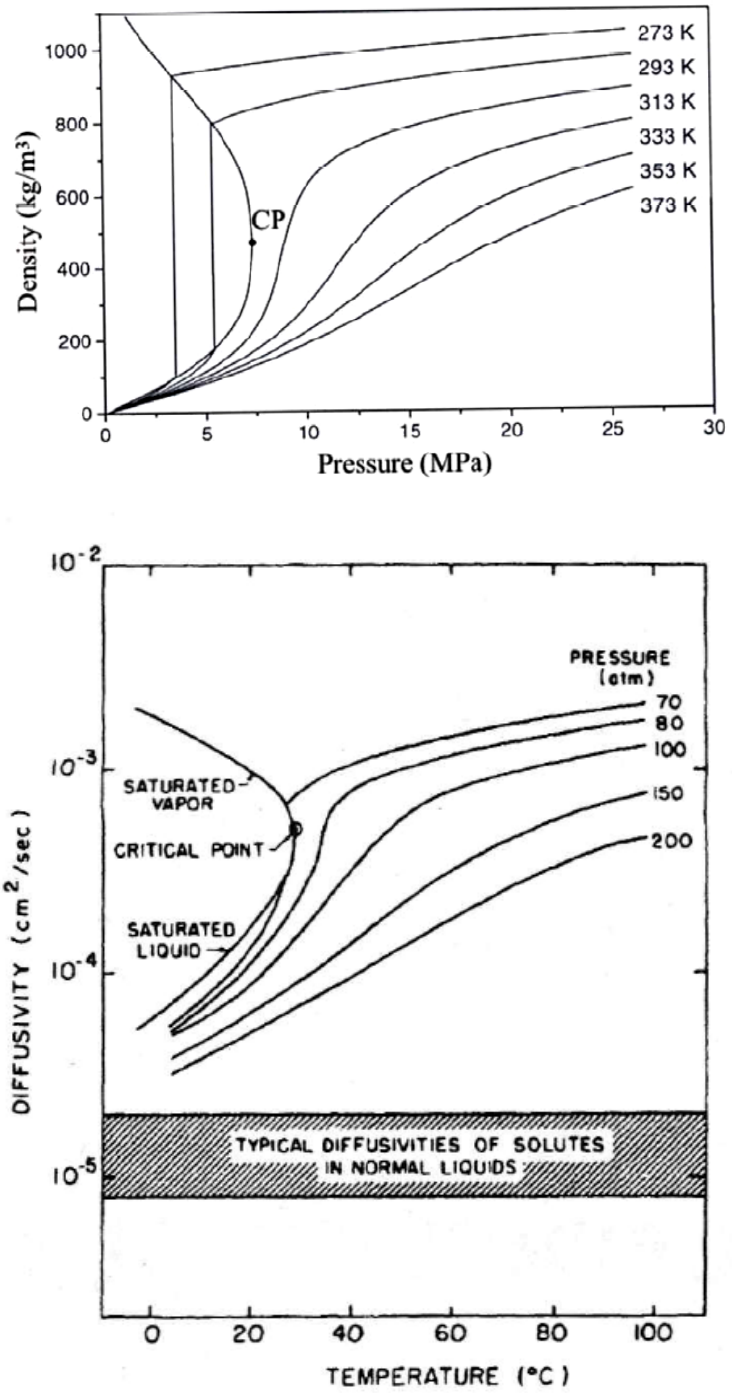


Figure 2-6. Density (a) and diffusivity (b) of pure CO_2 at difference conditions [49, 58].

Especial attention for recovery of relatively high purity non-polar substrates is given to using SC-CO_2. SC-CO_2 usually yields good recovery many sensitive bioactive components of natural oils sterols, tocols, carotenoids, and polyunsaturated fatty acids [59]. The usage of SC-CO_2 for extracting many kinds of lipids from different nuts for food products is also reported in a review paper of Sehana and coworkers [54].

2.3.2 Process parameters for SC-CO_2 extraction of oil and γ-oryzanol/ phytonutrients from solid material

Since the process parameters play important roles for process operation and the properties of supercritical fluids is sensitive with some variables, the process parameters have been studied for achieving maximum yield. The involving parameters are summarized and shown in table 2-6.

Table 2-6. Process parameters for supercritical fluids-solid extraction [11, 55, 56, 59, 60]

Operating step	Main parameters
Raw material preparation	Particle size
	Water or moisture content
Extraction process	Temperature
	Pressure
	Extraction time
	Solvent flow rate and solvent/feed ratio
	Packing material
	Type of supercritical fluid/co-solvent
Product/solvent separation	Temperature
	Pressure

In preparation step, the sample is modified in order to increase mass transfer and extraction rate during extraction process. In generally, the raw material is ground to increase the surface area, increase solvent accessibility and decrease path length which solvent can penetrate into pores and solute thus reaches the bulk phase easily. The different particle sizes will suit for different types of raw material and extraction process. Besides raw material size, moisture content in raw material acts as mass transfer barrier has negative effect on oil extraction and causes breakage of seed. Moreover, the oil content in extracted also has negative effect on product stability. To avoid these obstacles, moisture content has to be controlled. The influence of moisture content in a range of 3% to 10% generally has no effect on mass transfer of seed oil [56, 59].

In extraction process, each parameter and its effects will be described and shown in many literature reviews as follow:

1) Pressure and temperature

As mentioned above, the solubility of product into solvent depends on mostly pressure and temperature. In generally, at constant temperature, oil solubility increases with pressure. At constant pressure which is near critical point, the oil solubility is increased by decreasing temperature. This phenomenon is caused from increment of solvent density at higher pressure, but lower temperature. Anyway, the effects of temperature with various pressures may have some differences depending on solubility isotherm [61]. At near critical pressure, an increasing in temperature causes the solubility decrease as described above. In contrast, at higher pressure, an increasing in temperature causes the increasing in vapor pressure resulting in solubility increase.

The different optimal temperatures and pressures depend on various raw materials, processes and desired products as presented in Sehana and coworkers' report [54]. In case of oil and nutrients extraction from rice bran, many researchers have been reported the suitable condition and quality of oil products. Perretti and coworkers [62] indicated that an extraction under 10,000 psi (68 MPa) and 80 ^{o}C gave the highest yield of extracted oil, using 50 mL semi-batch reactor. The highest yield

of γ-oryzanol was also obtained at this condition. Increasing pressure at constant temperature provided higher oil yield, amount of extracted γ-oryzanol and α-tocopherol, which comprises vitamin E. Imsanguan and coworkers [2] also studied the extraction of γ-oryzanol and α-tocopherol from rice bran under the effect of temperature (45 – 65^{o}C) and pressure (38 and 48 MPa). The CO_2 flow rate was 0.45mL/min and 0.5 g of rice bran was packed in the semi-continuous reactor. The results showed that amount of extracted γ-oryzanol and α-tocopherol was increased with temperature and pressure and reached the maximum yield at 65^{o}C and 48 MPa. This work corresponds with Balachandran and coworkers [11] who studied the effects of several variables such as pressure (350, 425 and 500 bar), temperature (50, 60 and 70^{o}C) and time (0.5, 1 and 1.5 hr) on the extraction yields. At optimal condition, the phytonutrients content in the extract were found to be a range of tocols about 1500–1800 ppm, sterols about 15,350–19,120 ppm and oryzanol about 5800–11,110 ppm.

Lerma-Garcia and coworkers [12] summarized several literature reviews and reported that the triglycerides and phytosterol losses could be minimized, at low pressure and high temperature. The authors used a response surface methodology to determine the optimum condition and the result indicated that concentration of γ-oryzanol was increased and reached the highest value at 40^{o}C and 30 MPa. Jesus and coworkers [63] studied the recovery of γ-oryzanol from rice bran oil by-product, the residue of fatty acids distillation from rice bran oil soapstock, using SC-CO_2 in continuous reactor. The global yield which is defined as a ratio between an extract mass and mass of sample fed into the reactor was increased with high pressure but low temperature. The optimal condition was found to be 30 MPa and 30 ^{o}C that provide the highest concentration of γ-oryzanol around 32,000 ppm.

For pilot scale, Shen and coworkers carried out the rice bran oil extraction in 300 g of sample-size semi-continuous reactor using SC-CO_2 flow rate of 2.5 – 3.5 kg/h [64, 65]. The oil yield reached 98% of hexane-extractable oil at 30 MPa and 40 ^{o}C. The results indicated that free oil can be extracted more quickly at higher CO_2 density conditions; higher pressure but lower temperature. After extraction process, the oil product was passed continuously to second-stage column to separate oil from SC-CO_2 by various controlled temperatures and pressures. Although

oryzanol and α-tocopherol could not be reduced, all of water was removed and free fatty acid concentration could be reduced by this fractionated separation.

2) Extraction time

Due to the structure of raw material, the diffusion and accessibility of solvent might be limited by complex structure of solid matrix. The extraction time is thus important to hold as long as enough for allowing solvent-solute diffuse from pore through solid surface. Many works operated a rice bran oil extraction process at 4 – 6 hrs which almost extractable products could be obtained [2, 63, 64]. The extraction time depends on many process parameters such as temperature and pressure and also particle size. Higher pressure and smaller particle size can force the solvent to penetrate into solid matrix faster providing shorter retention time. The operating time can be reduced to 1.5 hrs with increasing pressure to 50 MPa [11]. Anyway, too much extraction time decreased the concentration of desired compounds such as tocopherols and tocotrienols, since more of other components in rice bran were extracted.

3) Solvent flow rate and solvent/feed ratio

High solvent flow rate causes an increasing in solvent/feed ratio which can increase productivity, but higher operating cost. In case of semi-continuous process, higher fresh solvent feed rate, at constant amount of raw material, affect an increasing of diffusion rate of product due to an increment of concentration in gradient throughout an operation. Anyway, Lerma-Garcia and coworkers [12] reported that increasing flow rate did not has any effect on product composition. An increased flow rate enhances degradation of the ligno-cellulosic substrate, resulting in the breakage of solid particle [66].

4) Packing materials

Balachandran and coworkers [11] reported the effects of packing material. An extraction process of rice bran without packing material yields only 50% of original oil content. It is probably due to the compacting of rice bran and consequent channeling. Using of structured stainless steel ring and glass bead as packing materials gave more extraction yield compared to using pebbles which reach almost 100% extraction. Badal [67] indicated that the compacting of rice bran decreases a solvent contact to the solutes and also proposed that designing of packing material is one of challenges to develop the supercritical fluids extraction.

For separation process, the temperature and pressure are reduced in order to decrease the solubility and recover only desired product. In one step separation, the temperature and pressure can be reduced to atmospheric condition to obtain all extractable products and recycle or reuse the solvent. In case of fractionation of product, the pressure is sequential reduced to suitable values for each desired product.

5) Co-solvent

Since CO_2 is a non-polar solvent, it can not dissolve polar substances. In order to improve the extraction of polar substance, the solvent's polarity of CO_2 has to be increased. Several researchers indicated that the small percentages of co-solvent such as methanol, ethanol, acetone or even water can be added to the system. The lipid and polyphenols solubility is increased by adding ethanol [54, 68]. In case of food industry, ethanol is permitted for using as a co-solvent. It can be separated from products by simple process as evaporating at room temperature. When ethanol is added to CO_2 as a co-solvent, the critical point of mixture is changed as shown in Table 2-7. The amount of ethanol have to be limited if the extraction is decided to occur in the supercritical regime at 40 – 100^{o}C. Anyway, subcritical fluids (CO_2 + ethanol) extraction process is investigated instead of extracting at supercritical regime and reported in several researches those will be described as follow down.

Table 2-7. Critical temperature and pressure of CO_2-ethanol mixture [69]

Ethanol concentration (%wt)	T_c (^oC)	P_c (MPa)
0	31.1	7.38
5	42.5	7.32
10	53.7	7.27
14	62.8	7.22
17	69.5	7.19
20	76.1	7.15
100	243.3	6.13

The applications of subcritical and supercritical CO_2/ethanol for recovery natural compounds from plants are presented in several researches. Vatai and coworkers [70] studied the extraction of phenolic compounds (mainly flavonols, 3-hydroxy-2-phenylchromen-4-one, and phenolic acids) from grape marc and elder berry using ethanol and SC-CO_2. The experiments were performed in semi-continuous reactor for investigating the effect of pressure (15 and 30 MPa) and type of solvents on the total phenol compounds. Compared with using ethanol alone, SC-CO_2 gave lower total phenols and anthocyanins content in the extract. When using SC-CO_2 with added ethanol for extraction, the extract contented two times more phenols and three times more anthocyanins compared with using pure SC-CO_2.

Hasbay Adil and coworkers used ethanol as a co-solvent for SC-CO_2 extraction of polyphenols from apple and peach pomaces [69]. The authors reported that optimum pressure and temperature were about 54.6 – 57 MPa and 55.7 – 58.4 ^oC for apple pomace and 50.6 – 51.0 MPa and 50.9 – 52.3 ^oC for peach pomace, respectively. The optimum ethanol concentration and time were 20% and 40 min, respectively. Moreover, this work concluded that more active polyphenols were selectively extracted. King [68] also reported about the selectivity increment of desired product during separation using SC-CO_2/ethanol mixture. In this work, the process steps for enrichment of phospholipids from vegetable oil or seeds using SC-CO_2 was presented. The soybean flakes were firstly de-fatting by SC-CO_2 and then the de-oiled flakes were extracted by SC-CO_2/ethanol mixture to remove

phospholipids. The phospholipids could not be removed by only SC-CO_2, but could be recovered by SC-CO_2/ethanol mixtures. These phospholipids were further enriched in an alumina preparative SFC column. The modified SC-CO_2 with a 5–30 vol.%, 9:1 ethanol: water was used to elute and fractionate.

Vaughn Katherine and coworkers [71] investigated the recovery of a lycopene, carotenoid, from watermelon. The results showed that extracted lycopene reached the maximum yield at 70 ^{o}C, 20.7 MPa and 15% ethanol in SC-CO_2. However, the interaction between process parameters had effects on the lycopene yield and presented the high yield even in other conditions such as at 70 ^{o}C, 41.4 MPa with 10% ethanol and 70 ^{o}C, 27.6 MPa with 15% ethanol.

2.3.3 Summarization of optimal conditions

The optimal conditions reported by many researchers who investigated the SC-CO_2 extraction of rice bran oil are summarized and shown in Table 2-8. It can be seen that all researches can recover ~100% of extractable rice bran oil which contents relatively high nutrient concentration. The extraction process was successfully operated at a pressure over 30 MPa and a temperature of 30 – 130 ^{o}C and operating time about 1.5 – 3 hrs.

Table 2-8. Optimal conditions for rice bran oil/byproduct extraction/purification using SC-CO_2

Researchers	Raw material	Reactor type	T (^oC)	P (MPa)	t (min)	flow rate	Oil yield/ nutrients content
Perretti and coworkers [62]	RB	50-mL semi-continuous	80	68	100	(1.082 g/min)	~100% yield α-tocopherols 1228 ppm γ-oryzanol 18,000 ppm
Imsanguan and coworkers [2]	RB	semi-continuous (0.5 g sample)	65	48	~180	0.45 mL/min	N/R α-tocopherols 127 mg/kgRB γ-oryzanol 11,371 mg/kgRB
Balachandran and coworkers [11]	RB	semi-continuous (500 g sample + 500g glass bead)	60	50	90	40 g/min	~100% yield α-tocopherols 1610 ppm γ-oryzanol 11,110 ppm
Jesus and coworkers [63]	The residue of fatty acids distillation from RB oil soapstock	semi-continuous (4.1 g sample)	30	30	-	10 g/min	high conc. γ-oryzanol 32,000 ppm
Shen and coworkers [64]	RB	semi-continuous (300 g sample)	40	30	180 - 240	2.5 kg/h (~42 g/min)	98% yield γ-oryzanol ~15,000 ppm

N/R – not reported, RB – rice bran

2.4 Subcritical Water Extraction of Proteins

2.4.1 Subcritical water (SW)

Subcritical water (SW; T_c = 374.20 ^oC, P_c = 22.05 MPa) is defined as hot water that maintains the liquid state in the temperature ranging between 100 – 374 ^oC and pressure over its saturated pressure. Its alternative terms include pressurized hot water (PHW) and hot compressed water (HCW) [8-10, 72, 73]. When the temperature of the water is increased from ambient to 250 ^oC, its relative dielectric constant is decreased from about 80 to nearly 27 as shown in Fig. 2-7. This value is almost equal to that of acetone at ambient temperature (20.7). As shown in Fig. 2-7, the ion product of the SW is significantly increased by increasing temperature from ambient to near critical point and the SW thus can catalyze some chemical reactions without using catalyst.

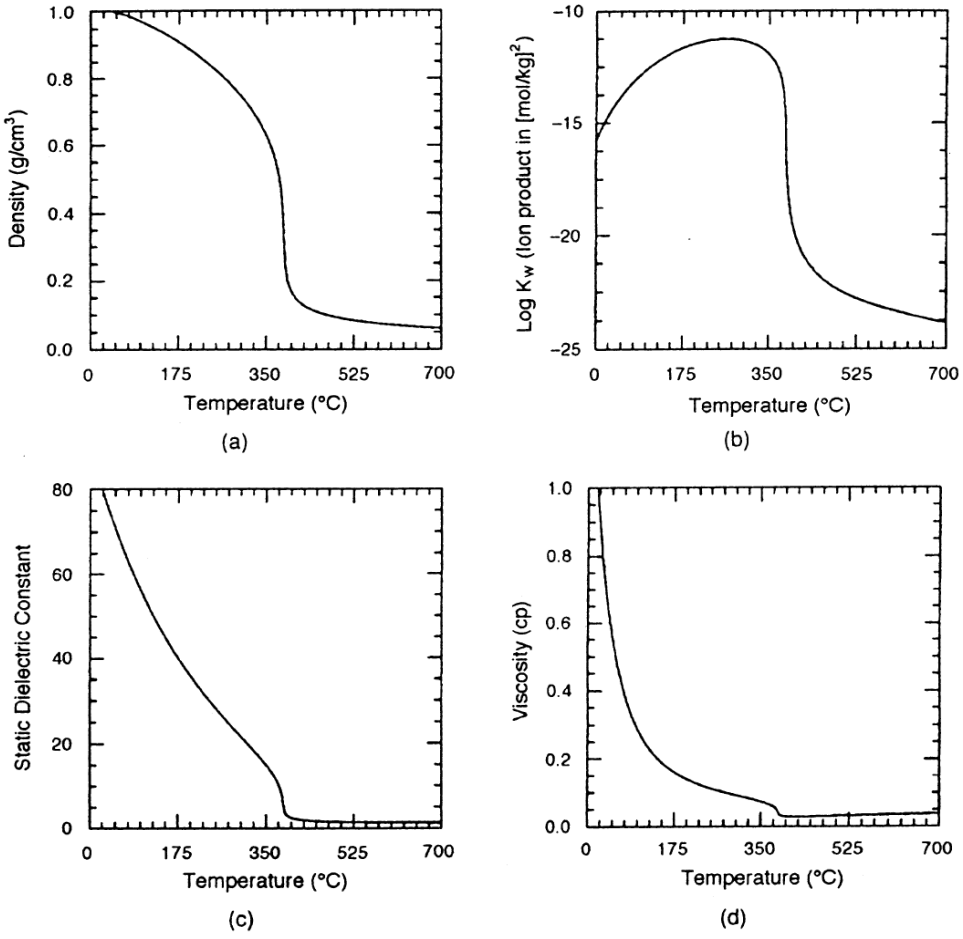


Figure 2-7. The properties of water at 25 MPa as a function of temperature [13]

To reduce chemical consumption, SW can be used as feasible solvent since it is cleaner, cheaper and more environmental friendly. Due to its unique features, SW extraction has been developed by several works for lignocellulosic biomass degradation/hydrolysis and plant proteins extraction [8-10, 73, 74]. Almost researchers carry on their experiments, especially for protein extraction, in batch system. The effects of process parameters such as temperature, pressure, water:feed ratio and retention time were investigated. Some parameters affect the properties of water leading the different involved chemical reactions and related products. The reactions between some components of rice bran such as proteins and carbohydrates and SW would be described in a next section. Then the main process parameters would be proposed as further down.

2.4.2 Reaction of proteins and carbohydrates under SW atmosphere

In case of rice bran, the main components those will be considered for SW extraction are protein and carbohydrate. Protein recovery from rice bran using SW has been demonstrated, since there has some works indicates several advantages of using SW as a solvent; high yields, easy applying for food, no required catalysts and cost-effective technique [75]. However, SW extraction process has to operate at high temperature. The protein degradation is thus simultaneously taken place. It maybe a disadvantage of SW extraction, but it maybe considered as an attractive way for amino acid recovery instead. Besides protein and amino acid recovery, structured carbohydrate such as cellulose and hemi-cellulose can be hydrolyzed to form sugars and some valuable compounds such as furfural and 5-(hydroxymethyl)-2-furfural (5-HMF). The mechanisms would be summarized and shown as follow.

1) Protein and amino acid production

As shown in Fig. 2-7, the dielectric constant of SW is varied with its temperature. The SW is thus useful for dissolving an organic solvent, since the solubility of organic compounds is governed by the dielectric constant of the medium. A decreasing in dielectric constant enhances the solubility of organic compounds and decreases the solubility of inorganic ionic compounds[76]. Many researches used SW

as a promise solvent for extract protein from rice bran and reported that protein could be recovered using this technique [8, 10, 73, 74, 77]. The results are indicated in terms of direct analytical method, Lowry assay which would be introduced in Chapter III, or radical scavenging activity. Some literatures indicated that a greater than 100% of protein contents in the extract due to the appearing of an interfere during protein analysis [76, 77] or a forming of small molecular weight proteins [8].

Since the ability to trap a free radical is main characteristic of antioxidant, the antioxidant activity is determined in term of hydrogen donating ability or the radical scavenging activity. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and 2, 2´-azinobiz (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay are normally used, since they are stable free radical. Many literature reviews those are presented in this part used this assay to determine the activity of antioxidant such as vitamin E (tocopherols and tocotrienols) oryzanol, proteins and amino acids (partially arginine, histidine, cystein and methaionine) which are extracted form rice bran as a protein hydrolysate [8, 77]. The extracted protein with presence of carbohydrate, in form of reducing sugar, gave high antioxidant activity [74, 77]. Some results of these literature reviews presented in Fig. 2-8.

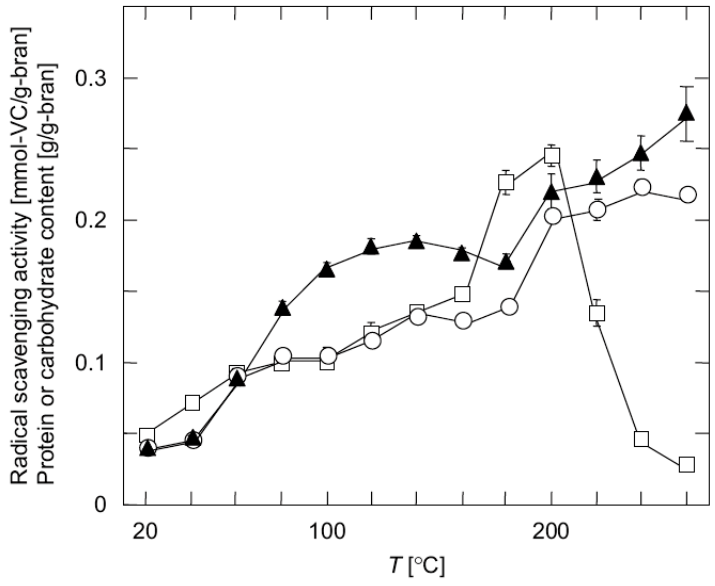


Figure 2-8. Effect of temperature on the radical scavenging activity, protein and carbohydrate content in protein hydrolysate:
▲ DPPH radical activity, ○ protein, □ carbohydrate content [77]

The authors reported that protein in rice bran which is normally insoluble in water becomes more soluble at higher temperature as shown in Fig. 2-8. Although the radical scavenging activity was increased as protein, information from other results indicated that the activity was results from phenolic compounds and maillard reaction products, reaction between proteins and reducing sugars to form brownish products [74, 77].

For amino acid production, since the protein is the other type of bio-polymer which has amino acids as its building blocks, the proteins can be hydrolyzed in order to recover amino acids. The hydrolysis reaction pathway is shown in Fig. 2-9 [78]. The hydrogen atom firstly attacks to nitrogen atom at peptide bond. The carbo-cation and amino acid are produced and then hydroxide ion attaches to the cation to form carboxyl compound.

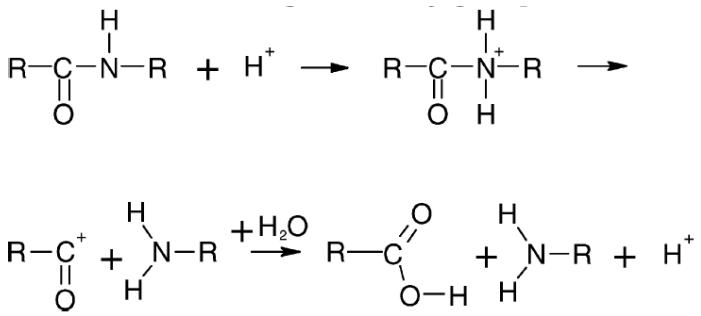


Figure 2-9. Hydrolysis step of protein [78]

The peptide bond is stable than glysocidic bond of cellulose and startch. Only slow hydrolysis is occurred below 230 ^{o}C. [79] Besides, the hydrolysis reaction depends on ion product of water which varies with temperature of water at high pressure as shown in Fig. 2-7. Therefore, the factors those affect the hydrolysis of peptide bond are reaction temperature and time. [78, 80, 81] Anyway, not only amino acids but also many compounds are produced as decomposition products of protein hydrolysis such as formaldehyde, acetaldehyde, glycolic acid, formic acid and ethanol [82].

2) Conversion of carbohydrate, sugars production

Besides of protein, major component of rice bran is carbohydrates such as cellulose and hemicellulose which can be hydrolyzed under high temperature and pressurized water. Cellulose consists of D-glucose unit which is linked together by β(1→4) glycosidic bond. Whilst hemicellulose consists of many sugar monomers such as glucose, xylose, mannose and galactose [79]. At subcritical condition, cellulose and hemicellulose can be dissolved and hydrolyzed to its constituents. The general mechanism of cellulose and hemicellulose is shown in Fig. 2-10. It can be seen that glucose is produced by this reaction and can isomerize itself into fructose. Glucose and fructose then degraded through many reactions such as isomerization, defragmentation and recombination to form furfurals, 5-HMF, phenol and coke under subcritical condition. Some researches showed that furfural was obtained from D-fructose rather than D-glucose [78, 79].

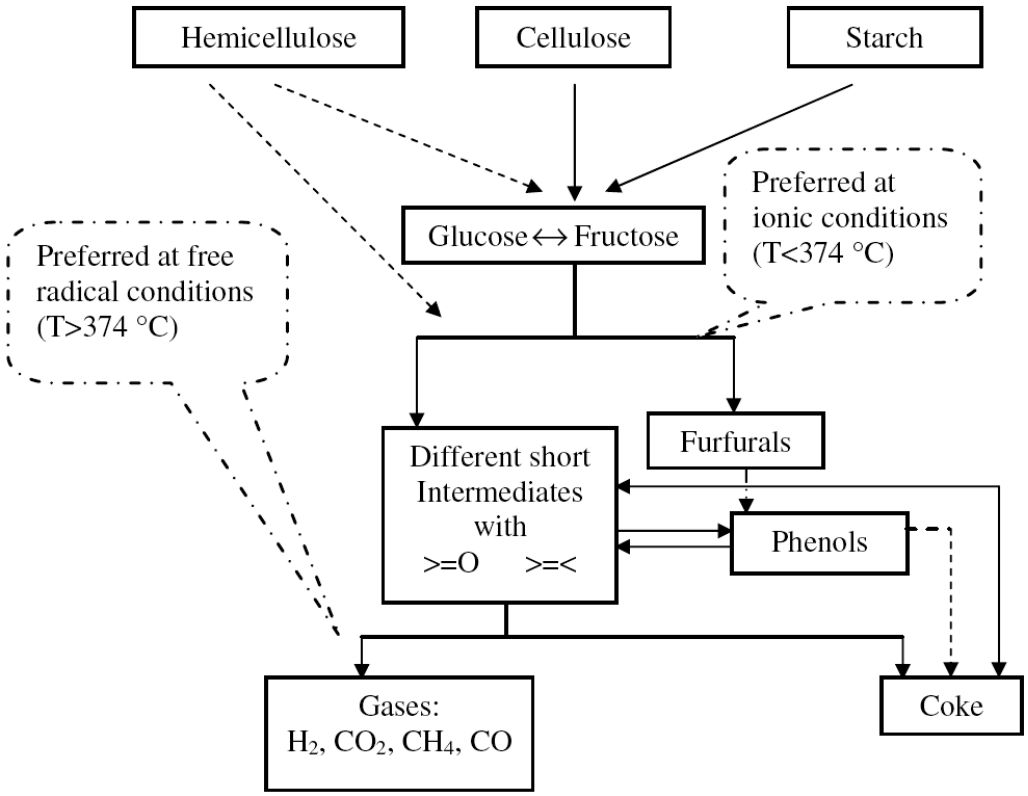


Figure 2-10. A simplified reaction mechanism of carbohydrate hydrolysis and degradation under subcritical and supercritical water condition [79]

2.4.3 Process parameters for SW extraction

As described above, the water properties are varied with its temperature leading many reactions. The effects of each process parameters such as temperature, flow rate, pressure, additives and residence time would be proposed and optimal conditions obtained from many literature reviews would be shown as follow:

1) Temperature and residence time

From Fig. 2-7, it can be seen that the water properties such as polarity (dielectric constant), ion product, viscosity and density are changed with temperature under high pressure. Increasing in temperature of water decreases its polarity in term of static dielectric constant and leads more rice bran protein solution. It thus can be seen that protein containing in the extract increases with temperature. However, besides decrement of polarity, increasing in temperature increases water's ion product which enhances hydrolysis reaction. The hydrolysis products such as amino acids and sugars are thus increases with temperature. Some reviews indicated the interaction between temperature and residence time. In case of residence time, when water's temperature is increased, it leads a decrement of not only hydrogen bonding cohesion (decreasing in static dielectric constant), but also surface tension [83]. The reduced surface tension improves solute-solvent contact resulting in using shorter residence time. The residence time using in many literature reviews is thus less than 30 min at relatively high temperature in order to maximize extract yields for rice bran and de-oiled rice bran extraction as shown below.

Watchararuji and coworkers [7] studied the extraction of value-added product from rice bran and de-oiled rice bran using subcritical water. The experiments were carried out in 8.8-mL batch reactor and the range of temperature between 200 – 220^{o}C, reaction time of 10 – 30 min. The authors reported that 75% and 84% of protein in rice bran and de-oiled rice bran, respectively, could be extracted at temperature of 200^{o}C and 30 min of extraction time. The soluble protein was increased by increasing temperature and reaction time. However, at 20 – 30 min and over 210 ^{o}C, protein yield was decreased at longer reaction time and higher temperature due to increment of water ion product concentration leading the

secondary reaction. Sereewattanawut and coworkers [8] also studied the protein and amino acids extraction from de-oiled rice bran in 8.8-batch reactor. The experiments were carried out in the temperature range of 100 – 220^{o}C and reaction time of 0 – 30 min. The authors reported the same suitable condition as previous literature review for achieving highest protein yield (200 ^{o}C and 30 min). Soluble protein, amino acid and reducing sugars increased with temperature and time. At lower temperature and time, these two factors did not affect the yields. At higher temperature and time, large amount of rice bran was dissolved and converted to hydrolysis products.

Hata and coworkers [9] studied the de-oiled rice bran treated with subcritical water in 9 and 117-mL reactor at the temperature of 180 – 280^{o}C, 5 min of extraction and raw material: water ratio of 1:50. The results showed that protein yield reached the maximum at 240^{o}C while the total sugar was significantly decreased at temperature higher than 200^{o}C in both reactors. In this work, the results demonstrated that sugar is thermal labile substrate which was decomposed at longer extraction time while protein concentration was not changed during experiments. Pourali and coworkers [10] investigated the application of subcritical water for rice bran hydrolysis and rice bran oil extraction under the temperature range of 100 – 360^{o}C, raw material: water of 1:6 and residence time of 5 min. The authors indicated that SW was not suitable for oil extraction. All amino acids including 8 essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine) and 6 non- and/or conditionally essential amino acids (glutamic acid, alanine, tyrosine, serine, glycine, and asparatic acid) reached the maximum yield at temperature of 120 – 150^{o}C and could not be detected at higher temperature (227^{o}C).

Wiboonsirikul and coworkers [73, 77] studied the functional substances production from black rice bran in subcritical water. This work showed that protein yield and radical scavenging activity were increased with treatment temperature while carbohydrate content was decreased at temperature above 200 ^{o}C, corresponding with other literature reviews. On the other hand, at a temperature range of 200 – 260 ^{o}C, the shorter reaction time was suggested for more beneficial valuable substrates.

2) Pressure

The effect of pressure is usually negligible in many process of SW extraction of biomass. The effect of pressure is mostly confirmed by conducting the essential oil extraction and hydrolysis products (amino acids and sugars) recovery from plant materials using SW [78, 83-86]. The effect of pressure on dielectric constant of water is less than that of temperature. In static extraction, pressure used in the reactor is desired to be saturated pressure determined by steam table. In dynamic extraction, the extraction or hydrolysis process are operated at a pressure range of saturated pressure to 25 – 27 MPa and the result demonstrates no significantly effect of pressure on extraction yields. However, the valuable compounds extraction using flow-through system is still operated with varying of pressure and temperature, some works reported that organic pollutants recovery from solid samples has a little dependence on pressure since subcritical water can be forced through a sample cell and desired compounds can be taken out [87].

3) Flow rate

In static extraction, the extraction efficiency depends on solubility and partition-equilibrium constant of analytes and SW. The high concentrated and/or low solubility products are thus incomplete extracted. In order to enhance extraction efficiency the dynamic extraction mode is chosen in many studies to increase volume of water [87]. In dynamic extraction, the increment in water flow rate provides lower extraction time at constant temperature [83]. Flow rate of 1 – 1.5 mL/min of water is generally used in this extraction mode. The higher flow rate improves the extraction efficiencies since the total volume of water is increased and the physical mass transfer of substrates from solid matrix is increased. Not only extraction time but also flow rate depends on nature of analytes and solid matrix. The extraction time or flow rate has to be optimized for each process [87]. Liu and Wyman [88] studied the effect of flow rate on xylan, lignin and total mass removal of corn stover and reported the results those were obtained from using static mode and dynamic mode at different flow rate of water (1 and 10 mL/min). The authors found that original mass was dissolved from about 35 to 45% after 16 min when flow rate was increased from 0 to

1 mL/min (200 ^{o}C) and dissolved about 55% when flow rate was increased to 10 mL/min. In this work, the different mechanisms for static mode and dynamic mode extraction were proposed and the effect of flow rate was also suggested. The flow-through system can reduce the mass transfer resistance and provide more water over solubility limitation. The long chain polymers are firstly released to water and then forming of short chain polymers or monomers is occurred instead of repolymerization. Higher flow rate allowed greater dissolution result.

However, large amount of water consumption may not be a good choice for extraction process and the product concentration can not be optimized since other SW-soluble compounds are also extracted.

4) Additives

Since the hydrolysis reaction is led by H^{+} ion concentration in SW, the additives such as sulfuric acid and CO_2 are added in order to decreasing pH and enhancing hydrolysis products [78, 80, 84]. Yu and coworkers suggested in their work that alkali additives provide isomerization and retro-aldol condensation reactions while acidic additives provide dehydration of glucose [89].

2.4.4 Summarization of optimal conditions

The optimal conditions reported by many researchers who investigated the protein and valuable substrates production from rice bran under SW atmosphere are summarized and shown in Table 2-9. All extraction experiments were carried out using batch/static system. It can be seen that almost rice bran protein content in raw material can be recovered at a temperature range of 150 – 250 ^{o}C and reaction time less than 30 min. At these conditions, the other valuable products such as amino acids and reducing sugars were also produced as hydrolysis products. The radical scavenging activity was analyzed in some works and the authors indicated that rice bran extract have high antioxidant activity.

Table 2-9. Optimal conditions for protein extraction from rice bran and de-oiled rice bran using SW

Researchers	Raw material	Reactor type	T (^{o}C)	P (MPa)	t (min)	Protein yield/ other valuable products
Watchararuji and coworkers [7]	RB and de-oiled RB	8.8-mL batch reactor (1 g in 5 mL water)	220	sat. press.	30	106 mg/g RB (75%) and 130 mg/g de-oiled RB (84%). Maximum results of amino acid, sugar and antioxidant activity
Sereewattanawut and coworkers [8]	de-oiled RB	8.8-mL batch reactor (1 g in 5 mL water)	200	sat. press.	30	219 and 8.0 mg/g of dry RB for protein (~100%) and amino acids
Hata and coworkers [9]	de-oiled RB	9-mL and 117-mL batch reactor	240	sat. press.	5	~3.5 g/L extract in 117-mL reactor. The antioxidant activity obtained from SW was much higher than those obtained from ethanol and ethyl acetate.
Pourali and coworkers [10]	RB	batch reactor (3 g in 18 mL water)	280	sat. press.	5	N/R. Amino acid reached max. value at 127 ^{o}C
Wiboonsirikul and workers [73]	de-oiled RB	10-mL batch reactor (1.5 g in 75 mL water)	200	sat. press.	5	0.1 g/g RB Carbohydrate, phenolic compounds and antioxidant activity were detected
Wiboonsirikul and coworkers [77]	Black RB	10-mL batch reactor (0.14 g in 7 mL water)	200	sat. press.	5	0.2 g/g RB Carbohydrate and antioxidant activity were detected

N/R – not reported, RB – rice bran

2.4.5 Protein aggregation and degradation

Since the protein extraction using SW is performed at relatively high temperature compared to conventional procedure, the change of protein structure may be caused by heating, e.g. protein aggregation and protein degradation into smaller polypeptides and amino acid [90]. When protein is heated, the number of micelle, polymerization of protein, and protein particles those are smaller than micelle are increased. The smaller protein particles include denatured proteins and disaggregated proteins which come from micelles. Singh and Latham [90] reported that the relatively large protein complex was formed at initial heating (140 ^{o}C). The intermediate-size protein complex was then formed at further heating whilst original protein was decreased. The smaller fraction which is defined as thermal degradation product of protein was formed simultaneously at heating time over 10 min. The mechanisms and influencing factors of protein aggregation and protein degradation were proposed in many researches as shown below.

1) Protein aggregation

The protein aggregation is occurred by different pathways. Fig. 2-11 showed major mechanisms which can be divided into 3 pathways [91]:

(1) Through denatured state and unfolding intermediate – Native protein (N) is normally in equilibrium with intermediate (I) which is in equilibrium with denatured states (D). The interaction between intermediates causes the formation of aggregates (A). If these aggregates are larger than certain size and solubility limit, they will be insoluble and precipitate (P).

(2) Through self-association/direct chemical linkage – Without intermediate state, many proteins can aggregate from native state by 2 pathways: association themselves which is caused by electrostatic alone or both of electrostatic and hydrophobic (pathway 2a) and forming chemical linkage such as disulfides linkages, formaldehyde-mediated cross-linking and Maillard type reactions.

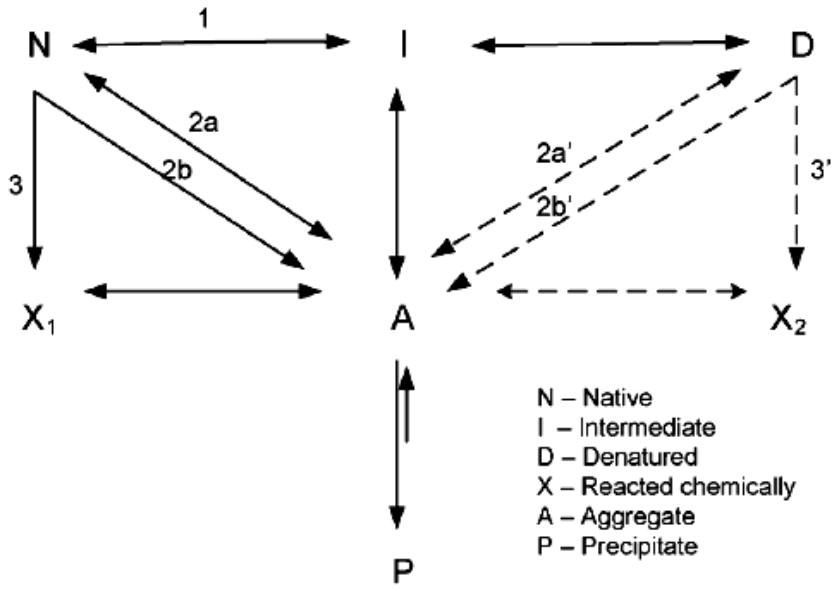


Figure 2-11. Protein aggregation pathways [91]

(3) Through chemical degradation – Some chemicals can change protein properties such as hydrophobicity and protein structures. Protein can thus form an aggregate through reacted form (X) as shown in Fig. 2-11.

Besides of heating time that has effect on protein behavior/ denature as described above, temperature also promote protein aggregation and degradation [91]. Increasing of temperature might has many effects on protein aggregation pathway. Activation energy for I formation in pathway 1, chemical linkage formation in pathway 2b and chemical reaction in pathway 3 may be decreased by increasing temperature. Moreover, increasing in temperature may has other effects such as increasing of protein diffusion and enhance frequency of molecular collisions.

Weijers and coworkers [92] studied kinetics of the heat-induced denaturation of ovalbumin. In this work, the authors proposed mechanism which is correspond with pathway 1 and found that the denaturation rate is strongly depended on temperature. For high purity ovalbumin, the decrease in native protein could be described by first-order kinetics. For the effect of heating time, Mleko and Foegeding [93] studied effect of extended heating time and pH on whey protein gel formation and reported that increasing of heating time and decreasing of pH increased optical density of protein gel by increasing whey protein polymer size.

The formation of visible protein aggregate is normally occurred by nucleation-controlled aggregation [91, 94]. When a sufficient-size protein aggregate is formed, an addition of monomers is strongly favored leading formation of larger particles. Fig. 2-12 shows an addition of native protein into critical nucleus or protein aggregates. The visible protein aggregate is then simultaneously formed. The temperature may also affect on this process to different degree [91].

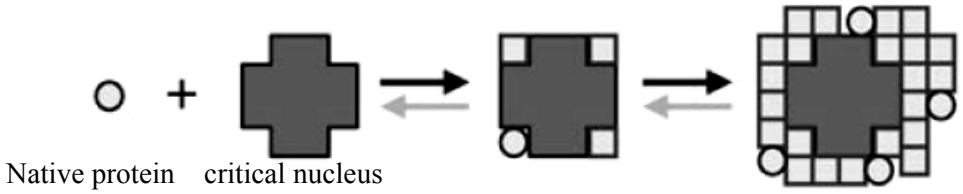


Figure 2-12. Protein aggregation through nucleation-controlled aggregation [94]

2) Protein degradation

In subcritical water regime, biopolymers such as protein and some carbohydrates react with water within short reaction time. Many works studied the mechanism and kinetics of protein and amino acids hydrolysis to produce valuable compounds [95-98], since this information is very important for the design of the main equipment such as a reactor. However, the complicated reaction of complex substance and structure of biomass makes difficulty study of kinetic model. The simplified model is thus used to describe the reaction.

Rogalinski and coworkers [95, 96] studied the hydrolysis kinetics of corn starch and cellulose and bovin serum albumin which was used as protein model in continuous system and used the simplified kinetic model for explanation of the degradation reaction of single component as shown below:

$$f = 1 - \exp(-k_{S,j}t) \qquad (2\text{-}1)$$

It is under the assumptions as an ideal plug flow reactor, irreversible reaction and first order reaction [96]. Where f is substrate liquefaction, $k_{S,j}$ is rate constant of substrate degradation, t is the residence time in the reactor.

A general approach for temperature dependence of the reaction rate constant is the Arrhenius law as shown in Eq. (2-2):

$$k_{S,j}(T) = k_{S,j,0} \exp(-\frac{E_{A,j}}{RT}) \qquad (2\text{-}2)$$

Where $k_{S,j,0}$ is the pre-exponential factor, $E_{A,j}$ is the activation energy of reaction, R is gas constant and T is temperature.

By plotting the logarithm of the rate constant against the temperature, the results of the Arrhenius Eq. (2-2) can be illustrated in the form of a straight line, the so-called Arrhenius plot (Eq. (2-3)), from which the activation energy as well as the pre-exponential factor can be determined.

$$\ln k_{i,j} = \ln k_{i,j,0} - \frac{E_A}{RT} \qquad (2\text{-}3)$$

The simplified reaction model that the authors used in their works is:

Protein $\longrightarrow$ amino acids $\longrightarrow$ degraded products

The amino acids were main products of protein hydrolysis whilst degradation products were found to be aldehyde, organic acids and gaseous products such as CO, CO_2 and H_2.

Another model has been proposed in Esteban and coworkers' work [97]. The author proposed a simplified kinetic model to describe the amino acid production during hog hair hydrolysis in subcritical water. This study performed the hog hair hydrolysis in 500-mL batch reactor at 250^{o}C. Before the first 45 min, the reaction followed the zero-order kinetic, after that first order kinetic was taken place. After 60 min, the amino acids were dramatically decreased due to they are thermal labile for degrading to other products. The hydrolysis models were the following:

$$\frac{[aa]}{[S]_0} = \frac{k_A}{[S]_0} t;\ 0 \le t \le 45\,\text{min} \qquad (2\text{-}4)$$

$$\frac{[aa]}{[S]_0} = \frac{[aa]_{45}}{[S]_0} e^{-k_c(t-45)} + \frac{k_B}{k_C - k_B}(e^{-k_B(t-45)} - e^{(-k_C(t-45)}); \quad t > 45\,\text{min} \qquad (2\text{-}5)$$

The authors indicated that these models could describe both individual and total amount of amino acids.

CHAPTER III
EXPERIMENTAL APPARATUS AND ANALYTICAL METHOD

3.1 Equipment

The schematic diagram of experimental apparatus is shown in Fig. 3-1. The experiments are divided into 4 parts: SC-CO_2 extraction, SW extraction, kinetics study of rice bran protein aggregation and degradation and, finally, integrated process of SC-CO_2 and SW extraction. The picture and model of each apparatus those are used in each part will be shown as following down.

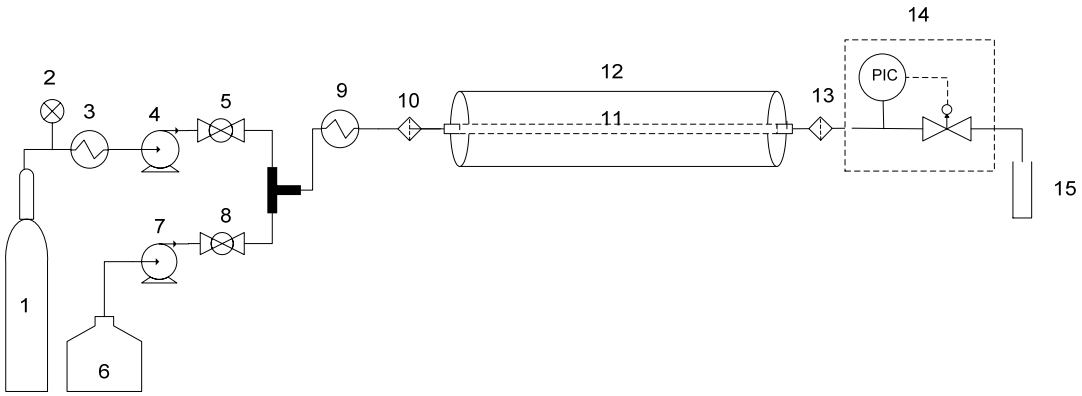


Figure 3-1. Experimental apparatus consists of CO_2 cylinder (1), Pressure gauge (2), Cooler (3), High-pressure pump (4 and 7), Ball valve (5 and 8), Water reservoir (6), Pre-heater (9), Inline-filter (7 micron) (10 and 13), Tube reactor (11), Tube furnace (12), Automatic back-pressure regulator (14) and Sample tube (15).

3.1.1 Supercritical carbon dioxide extraction process

This process consists of CO_2 cylinder, cooling bath (HETO CBN 18-30), The HPLC pump model JASCO PU-1580 (Fig. 3-2), pre-heater (water bath model Heto OBN 18), reactor which was made from 1/2 inch OD-stainless steel with 0.083 inch of thickness and tubular furnace model PTF 12/38/500 (Fig. 3-3) and automatic back-pressure regulator model JASCO BP-1580-81 (Fig. 3-4).

Figure 3-2. High pressure pump

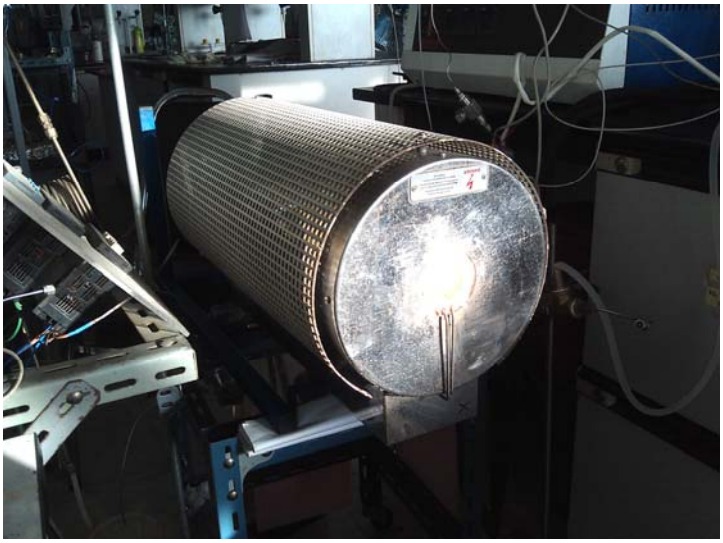


Figure 3-3. Tubular furnace

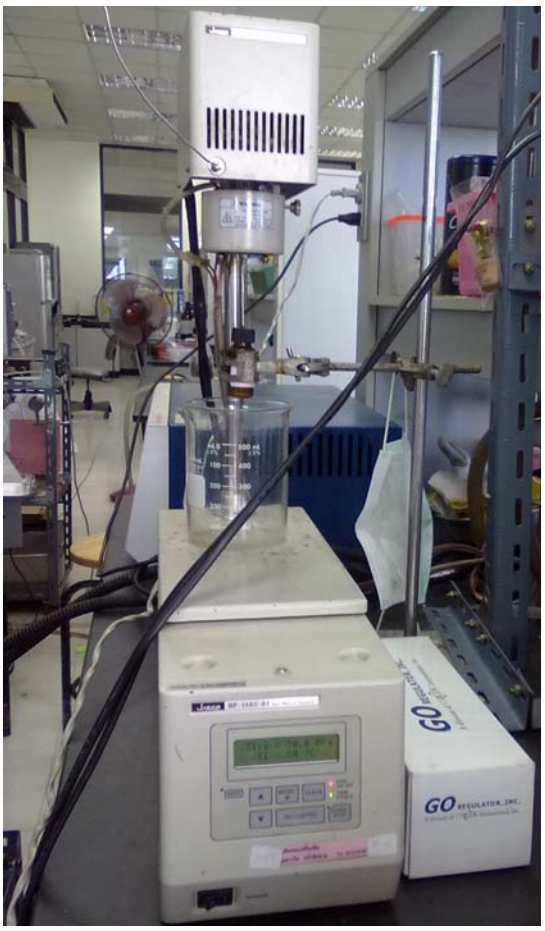


Figure 3-4. Automatic back-pressure regulator model BP1580-81 (JASCO)

3.1.2 Subcritical water extraction process

The system consists of 30-mL reactor (SUS316, OD: 1/2 inch and 0.083 inch of thickness), HPLC pump and tubular furnace as shown in Fig. 3-5. The HPLC pump model PU-1580 (JASCO) used in this process is shown in Fig 3-6.

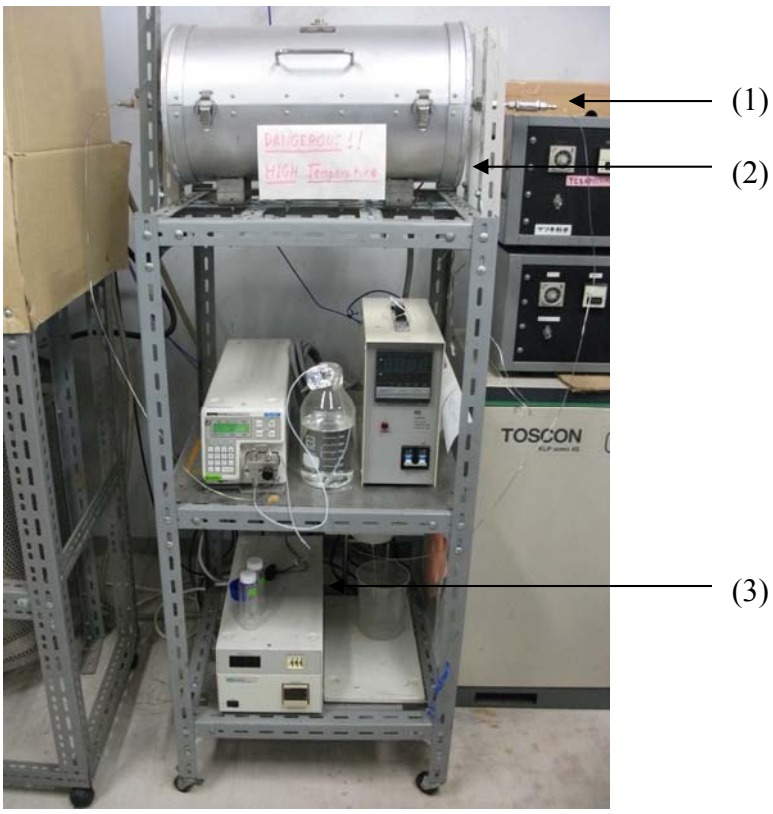


Figure 3-5. Reactor (1), tubular furnace (2) and back-pressure regulator (3)

Figure 3-6. HPLC pump

3.1.3 Aggregation and degradation of rice bran protein

The kinetics study experiments were carried out in batch type reactor. 4 mL-batch reactor was made from 3/8 inch OD-stainless steel with 0.049 inch of thickness. The sand bath was used to heat up the reactor and protein solution.

Figure 3-7. Sand bath with batch type reactors put inside.

3.1.4 Integrated process of SC-CO_2 and SW extraction

In this part, the schematic diagram shown in Fig. 3-1 is used. The main equipments consist of high pressure pump for CO_2 (Fig. 3-2), reactor and tubular furnace (Fig. 3-3), automatic back-pressure regulator (Fig. 3-4) and HPLC pump for water (Fig. 3-6).

3.1.5 Analytical equipment

- High performance liquid chromatography (HPLC ShimadzuLC-9A) was used to analyze glucose, xylose fructose, furfural and 5-HMF (5-(Hydroxymethyl) furfural) concentration.

Figure 3-8. HPLC Shimadzu

- High performance liquid chromatography (HPLC Varian model 400) was used to analyze γ-oryzanol concentraction.

Figure 3-9. HPLC Varian

- UV spectrophotometer (JASCO V-360) was used to analyze total proteins, total sugars and amino acids concentration.

Figure 3-10. UV spectrophotometer JASCO

3.2 Raw materials and Chemicals

The rice bran was obtained from the Thai Edible Oil Co., Ltd., Thailand. The composition was analyzed by NIR spectrometer and its result was shown in Table 3-1.

Bovine serum albumin (BSA) was purchased from Acros organics, USA. Folin and Ciocalteu's phenol reagent was purchased from Sigma-Aldrich, USA. Sulfuric acid, sodium hydroxide, phenol and copper sulfate were purchased from Merck, UK. Sodium carbonate was purchased from Fisher Scientific, UK. Sodium tartrate dehydrate and D-glucose were purchased from BDH, UK. Furfural and 5-HMF (5-(Hydroxymethyl) furfural) were purchased from Carlo Erba Reagent. γ-oryzanol was purchased from Wako Pure Chemical Industries, Japan.

Table 3-1. The composition of the rice bran and de-oiled rice bran (on wet basis)

Composition (%wt)	Rice bran[a]	De-oiled rice bran[a]
Results from NIR spectroscopy[b]		
Moisture content	9.1	10.8
Total oil	20.2	1.5
Protein	12.7	16.9
Fiber	7.3	9.6
Other[d]	50.7	61.2
Results from analytic method described in Section 3.5 [c]		
Cellulose	15.5 ± 0.3	16.0 ± 0.3
Hemicellulose	31.1 ± 0.2	29.8 ± 0.1
Lignin	11.5 ± 0.1	12.7 ± 0.5
Ash	13.2 ± 0.1	14.3 ± 0.1
Other[e]	28.6 ± 0.1	27.2 ± 0.3

[a]Data are shown as the mean ± 1 SD.

[b, c] Note the contents were evaluated in two separate batches, where [b] is from the wet weight and [c] is from the dry weight.

[d] Other compositions include other carbohydrate, lignin and ash.

[e] Other compositions include protein, oil, pectin and other form of carbohydrates.

3.3 Procedure

3.3.1 SC-CO_2 extraction

A schematic diagram representation of the semi-continuous extraction apparatus was shown in Fig. 3-1. The gravel was packed into both ends of the reactor so as to keep the raw material in the center of the reactor. The raw material and gravel were mixed at a (w/w) ratio of 3:20 (or a 1:1 (v/v) ratio) and then filled into the reactor. Packing material is added to raw material in order to increase heat transfer and a chance of solvent contact to raw material. After loading raw material and packing material and checking all fitting part to prevent gas leak, CO_2 was pumped into the reactor at a flow rate of 3 g/min by high pressure pump. The pressure and

temperature were then adjusted to the desired conditions using back-pressure regulator and a three-zone tube furnace. The product depressurized; rice bran oil was collected in an amber glass bottle.

3.3.2 SW extraction

As schematic diagram that was shown in Fig. 3-1, the gravel and raw material mixed with gravel were packed into the reactor as described in Section 3.3.1. In case of SW extraction, packing material is used in order to improve a well mixing of SW and raw material. The water: raw material might be decreased. In this process, the gravel-packing zone in the end of the reactor provided a pre-heating zone. The raw materials were mixed with gravel and then filled into the reactor to prevent the packing of dense raw material after water feeding. Distilled water was pumped into the reactor at a flow rate of 0.5 mL/min. The pressure was adjusted to 4 – 10 MPa using a back pressure regulator and then the reactor was heated to the desired temperature in a three-zone tube furnace. The liquid product discharged from the reactor was depressurized, and then was collected in an amber glass bottle and kept in the refrigerator. The remaining solid was filtered and dried overnight at 90 ^{o}C. The volume of the extract and weight of the dried solid were measured.

3.3.3 Aggregation and degradation of rice bran protein

1) Sample preparation

Rice bran protein concentrate was firstly extracted from rice bran before making a protein solution. The protein was extracted from rice bran by the method that described by Lew and coworkers [99] with some modifications. Rice bran protein was prepared from rice bran by stirring with aqueous NaOH (pH 11) at rice bran: solvent ratio of 1:7.5 (w: v) for 2 hrs. Then the solution was centrifuged at 3400 rpm using tabletop centrifuge, model 2420 – KUBOTA. Supernatant was separated and adjusted pH to 4.5 by 1 N HCl. The precipitated protein was separated using centrifuge and dried by vacuum oven at room temperature after adjusting pH to neutral by washing with distillation water. The dried protein was collected at 277 K until further use.

2) SW treatment of protein solution

Rice bran protein was dissolved in distillation water at 308 K in an ultrasonic bath for 1 hr. Then the protein solution was left and cool at room temperature. The aggregation and decomposition of protein were studied at 423, 448, 473, 498 and 523 K in 4-mL batch reactor (SUS316, OD: 0.375 inch and 0.049 inch of thickness). Protein solution was loaded into 12 batch reactors and then the reactors were sealed with end caps in order to keep water in its liquid state. These reactors were put in the sand bath at the desired temperature and each reactor was taken out every 5 min and cooled in the water. Then the products were kept at 277 K before analysis.

3.3.4 Integrated process of SC-CO_2 and SW extraction

In the reactor, the gravel was packed into ends of the reactor whilst rice bran and gravel was mixed as described in Section 3.3.1. In the SC-CO_2 process, CO_2 was pumped into the reactor at a flow rate of 3 g/min. The pressure and temperature were then adjusted to the desired conditions using back-pressure regulator and a three-zone tube furnace. The product, rice bran oil, was released at the outlet, depressurized and collected in an amber glass bottle. After completion of SC-CO_2 extraction, the distilled water was pumped into the reactor at a flow rate of 0.5 mL/min. The pressure and temperature were adjusted to desired condition to achieve its subcritical state using the same equipments. The liquid product discharged from the reactor was depressurized and collected in an amber glass bottle and kept in the refrigerator. The remaining solid was separated from gravel and dried overnight at 100 ^{o}C. The weight of each product and the dried solid were measured.

3.4 Experimental Design

The 2^k factorial design was used as a factor screening for analysis of the significance of various process variables whilst the central composite design (CCD) was used to predict the optimum experimental condition. In this work, three factors such as temperature (T), time (t) and pressure (P) were investigated. Then regression

model representation of the 2^3 factorial design and three-level central composite design could be written as Eqs. (3-1) and (3-2), respectively [100].

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \quad (3\text{-}1)$$

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i,j=1,i\neq j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (3\text{-}2)$$

Where y = response

β = parameters whose values are to be determined

x_i = process variables (x_1, x_2 and x_3 represents T, t and P, respectively)

ε = normal random variable.

The 2 replicates were done at each point. The analysis of variance (ANOVA) was used to indicate significance of three main effects and their interaction those affect on the response. In this work, the P value = 0.05 which was corresponded to a 95% confidence level for a test of the hypothesis that the effects were equal to zero was used in order to find very accurate results. The small P-value (<0.05) indicated that the model terms were significant.

In case of simultaneous considering of the multiple results, the desirability function was used to simultaneously optimize the multiple responses [101]. The proposed desirability function transforms each response to a value between 0 and 1. The total desirability is defined as a geometric mean of the individual desirability as shown in Eq. (3-3) [102]:

$$D = (d_1 x d_2 x ... x d_n)^{\frac{1}{n}} = \left(\prod_{i-1}^{n} d_i \right)^{\frac{1}{n}} \quad (3\text{-}3)$$

Where D = the total desirability

d_i = the i^{th} desirability

n = number of response in the measure, in this case, n = 2

The d_i is 1 when the response comes beyond its highest values while the d_i is 0 when the response is lower than its lowest value. Thus, when all of the responses reached the maximum value, then D is 1, whilst D is 0 if none of the response reached its required value. If some of the responses did not reach its ideal value, d_i is lower than 1, and then D is also lower than 1.

3.5 Analytical Methods

3.5.1 Analysis of holocellulose, alphacellulose and lignin

The lignin was analyzed by Tappi T222om-98 [103]. The amount of holocellulose (cellulose and hemicellulose) was analyzed by the Browning method of wood chemistry [104], whilst the amount of α-cellulose was analyzed by Tappi T203om-88 [105].

3.5.2 Ash determination

According to ASTM E1755-01 [106], ash content in raw material and solid residue were determined after ashing at 575 ^{o}C for 3 hrs in muffle furnace.

3.5.3 Determination of γ-oryzanol content

The γ-oryzanol content in rice bran oil product was analyzed by Reverse-phase HPLC (Varian model 400) according to Xu and Godber [107]. The analytical system was composed of: 250 x 4.6 mm Column (Pinnacle II C18 5um), UV detector (model 325), pump model 230. The peak was observed at 330 nm. The mobile phase was methanol: acetonitrile: dichloromethane: acetic acid = 50:44:3:3, by volume, and the flow rate was 1.4 mL/min.

3.5.4 Analysis of protein content in liquid product

The total protein content in liquid product was analyzed by Lowry's method using BSA as a standard [108]. The solution was prepared and mixed well with diluted sample. To analyzing the total protein concentration, the absorbance at 750 nm was measured using a UV spectrophotometer. In addition, the procedure and calibration curve were shown in Appendix A.

3.5.5 Analysis of total sugar in liquid product

The total sugar content in liquid product was analyzed by a modified phenol-sulfuric method using D-glucose as a standard [109]. The sample was mixed with 5% (v/v) phenol solution and then conc. sulfuric acid was added, mixed well and the total sugar concentration was measured by means of absorbance value at the wavelength of

490 nm using a UV spectrophotometer. In addition, the procedure and calibration curve were shown in Appendix A.

3.5.6 Analysis of total amino acid in liquid product

The total amino acid content was analyzed by a ninhydrin method using glycine as the standard [110]. The amino acid concentration was measured by means of absorbance value at a wavelength of 570 nm using a UV spectrophotometer. In addition, the procedure and calibration curve were shown in Appendix A.

3.5.7 Determination of xylose and fructose content

The xylose, fructose and glucose content in rice bran oil product were analyzed by HPLC (Shimadzu LC-9A). The analytical system was composed of: 250 x 4.6 mm Column (GL Science), RI detector (Shimadzu, RID-10A). Column temperature was 45 ^{o}C. The mobile phase was acetonitrile: water = 95:5, by volume, and the flow rate was 1 mL/min.

3.5.8 Determination of furfural and 5-HMF content

The furfural and 5-HMF content in rice bran oil product were analyzed by HPLC (Shimadzu, UV-Vis). The analytical system was composed of: 250 x 4.6 mm Column (GL Science). Column temperature was 85 ^{o}C. The peak was observed at 285 nm. The mobile phase was acetonitrile: water = 10:90, by volume, and the flow rate was 0.8 mL/min.

3.5.9 Determination of glucose in solid residue

The total sugars content in solid residue was determined according to Sluiter and coworkers' work [111]. Solid was hydrolyzed by 72% H_2SO_4 for 2 hrs at 30 ^{o}C. Then the sample was diluted to 4% H_2SO_4 and autoclaved for 1 hr at 121 ^{o}C. After completion of autoclave procedure, the liquor was neutralized and glucose was measured by HPLC with same condition as xylose and fructose determination using D-glucose as the standard.

3.5.10 Determination of elemental components

The carbon, hydrogen and nitrogen content in solid and liquid were analyzed by CHN analyzer. The analytical condition was a sample weight of 0.1 – 0.2 g, combustion temperature of 950 ^{o}C, IR oven temperature of 48 ^{o}C, helium flow rate of 200 mL/min and oxygen flow rate of 5 L/min.

CHAPTER IV

RICE BRAN OIL AND γ-ORYZANOL EXTRACTION BY SUPERCRITICAL CARBON DIOXIDE

The γ-oryzanol and rice bran oil were extracted from rice bran and de-oiled rice bran by SC-CO_2. The experiments were performed in semi-continuous reactor as described in Chapter III. The results of 2^3 experimental design experiments and their analysis were shown in this chapter. The investigated three process variables were the operating temperature (40 – 100 ^oC), time (5 – 60 min) and pressure (10 – 30 MPa). The effect of each parameter, suitable condition and regression model for γ-oryzanol and rice bran oil extraction were determined based on analysis of variance (ANOVA). The effect of time was finally optimized by using suitable temperature and pressure obtained from 2^3 experimental design experiments and their analysis.

4.1 Experimental Results and Effects of Experimental Conditions

Table 4-1 shows the 2^3 experimental conditions including the actual factors and coded values (in parentheses) and experimental results including the amount of extracted rice bran oil and extracted γ-oryzanol (mg/g of raw materials) for the SC-CO_2 extraction of rice bran and de-oiled rice bran. However, most of γ-oryzanol containing in trace amount of the oil obtained from de-oiled rice bran extraction (Exp. nos. 1, 3, 5, 6 and 7) could not be detected. The ANOVA and response surface for only γ-oryzanol extraction of rice bran was thus shown and discussed.

With respect to these results, it could be seen that the oil and γ-oryzanol yields were increased with increasing extraction time and pressure, but reducing temperature. It was mainly due to an increase in density of CO_2 which were also shown in Table 4-1. When the density increased from 620 kg/m^3 (Exp. Nos. 1 and 3) to 910 kg/m^3 (Exp. Nos. 2 and 4) with increasing pressure, the oil yield and γ-oryzanol yield were increased obviously. These results indicated that increasing pressure increased the

solvating power of SC-CO_2. It was consistent with the change of apparent solubility with pressure reported by Shen and coworkers [64]. The authors reported that an apparent solubility of rice bran oil increased from 2.50 to 6.93 g/kg CO_2 when pressure increased from 17 to 31 MPa (at 40 ^oC), respectively. The oil yields reached their maximum values at the highest operating pressure (30 MPa). A little experimental error due to small-scale measurement was occurred when the experiment was operated at lower extraction time (5 min) as can be seen in the results of oil yields obtained from Exp. Nos. 1 and 2 for de-oiled rice bran extraction and γ-oryzanol yields obtained from Exp. Nos. 5 and 6 for rice bran extraction.

The operating time also had a positive effect on oil yield and γ-oryzanol yield as expected since increasing operating time increased amount of supplied CO_2. Comparison of lower and higher extraction time, the oil yield and γ-oryzanol yield increased more than 2 times when operating time increased from 5 (Exp. Nos. 1 and 2) to 60 min (Exp. Nos. 3 and 4). However, the operating time could not be optimized by this experimental design method.

Table 4-1. The amount of total oil and γ-oryzanol after rice bran and de-oiled rice bran extraction using SC-CO_2

Experiment No.	Factors: Actual value (coded value)				Rice bran**		De-oiled rice bran**	
	Temperature, ^oC (T)	Time, min (t)	Pressure, MPa (P)	Density*, kg/m^3	Extracted oil (mg/g)	γ-oryzanol (μg/g)	Extracted oil (mg/g)	γ-oryzanol (μg/g)
1	40 (-1)	5 (-1)	10 (-1)	620	5.0 ± 1.7	136.0 ± 6.6	1.5 ± 0.2	BDL***
2	40 (-1)	5 (-1)	30 (1)	910	76.7 ± 10.0	393.3 ± 55.0	1.3 ± 0.0	19.7 ± 0.0
3	40 (-1)	60 (1)	10 (-1)	620	23.4 ± 3.5	956.9 ± 26.6	3.3 ± 0.0	BDL
4	40 (-1)	60 (1)	30 (1)	910	188.3 ± 18.3	3,181.2 ± 30.0	11.7 ± 1.7	54.8 ± 0.4
5	100 (1)	5 (-1)	10 (-1)	190	11.7 ± 1.7	32.2 ± 7.0	1.5 ± 0.2	BDL
6	100 (1)	5 (-1)	30 (1)	660	20.0 ± 10.0	104.6 ± 9.2	8.3 ± 1.7	BDL
7	100 (1)	60 (1)	10 (-1)	190	6.7 ± 3.3	47.0 ± 4.5	1.7 ± 0.0	BDL
8	100 (1)	60 (1)	30 (1)	660	176.7 ± 3.3	165.4 ± 7.0	5.0 ± 1.7	30.7 ± 2.3

* Read from Fig. 2-5 (a)

** Average from two replicates which reported in mg/g raw materials and μg/g of raw materials

*** BDL = Below detection limit

In contrast with effect of pressure and time, the temperature had negative effect on both oil yields and γ-oryzanol yield since increasing temperature decreased density of CO_2, leading to a reduction in its solvent power. The best extraction yields were reached at the lowest temperature (40 ^oC). At 40 ^oC, 60 min and 30 MPa, the maximum oil yields were obtained and reported to be 188.3 mg/g rice bran and 11.7 mg/g de-oiled rice bran which were about 94% and 80% extraction of rice bran and de-oiled rice bran, respectively. The γ-oryzanol concentration in rice bran oil was around 17,000 ppm at this condition.

4.2 Statistical Analysis for the Effects of Operating Factors

4.2.1 For the oil yield

Table 4-2 shows the results of the analysis of variance (ANOVA). The *p*-value < 0.05 indicated the significant of each term in the models. As shown in Table 4-2 (a), all independent parameters including temperature (T), time (t) and pressure (P) had a significant effect on the oil yield obtained from rice bran extraction whilst temperature had no significant effect on the yield obtained from de-oiled rice bran extraction as demonstrated in Table 4-2 (b). Meanwhile, the interaction terms of temperature with pressure (TP) and time with pressure (tP) had lesser but significant effect on oil yield obtained from rice bran oil extraction. In addition, the interaction terms of temperature with time (Tt) and interaction between all factors (TtP) had a significant effect on oil yield obtained from de-oiled rice bran extraction.

As described in Section 4.1, pressure and temperature affected the oil yield by means of changing CO_2's density whilst an increase in operating time increased amount of solvent. In case of de-oiled rice bran extraction, the temperature had less effect in de-oiled rice bran extraction compared to operating time and pressure. However, its effect could be detected by using longer time as shown in form of interaction terms.

Table 4-2. ANOVA test for the oil yields

a) Rice bran extract

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob > F
Model	81,722.2	7	11,674.6	82.4	< 0.0001*
T	1,344.5	1	1,344.5	9.5	0.0151*
t	20,544.5	1	20,544.5	145.0	< 0.0001*
P	42,025.0	1	42,025.0	296.6	< 0.0001*
Tt	177.8	1	177.8	1.3	0.2951
TP	1,002.8	1	1,002.8	7.1	0.0288*
tP	15,625.0	1	15,625.0	110.3	< 0.0001*
TtP	1,002.8	1	1,002.8	7.1	0.0288
Residue	1,133.3	8	141.7		
Total	82,855.5	15			

b) De-oiled rice bran extract

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob > F
Model	206.7	7	29.5	14.1	0.0006*
T	0.4	1	0.4	0.2	0.6575
t	20.2	1	20.2	9.7	0.0145*
P	84.0	1	84.0	40.1	0.0002*
Tt	58.8	1	58.8	28.0	0.0007*
TP	1.0	1	1.0	0.5	0.5094
tP	6.3	1	6.3	3.0	0.1226
TtP	36.0	1	36.0	17.2	0.0032*
Residue	16.8	8	2.1		
Total	223.5	15			

* Significant F-values at the 95% confidence level. T, P and t represent temperature, pressure and time, respectively

Table 4-3 shows the coded regression models which were generated based on ANOVA for oil extraction from rice bran and de-oiled rice bran. The regression coefficients (R^2) were close to unity (0.99 for rice bran extraction and 0.92 for de-oiled rice bran extraction) which indicated a good agreement between predicted values and experimental values. Additionally, the normal probability plot of residuals and the plots of the residuals versus the predicted response were given in Appendix B. The coefficients of variables those were shown in Eqs. (4-1) and (4-2) confirmed that the temperature had negative effect on the yield whilst time and pressure were the dominant factors which had positive effect on the yield.

According to an observation, ANOVA and regression model, the lowest temperature (40 ^{o}C) was chosen to minimize operating cost and maximize extraction yield. Thus, the interaction between operating time and pressure was considered in order to indicate the suitable condition. Fig. 4-2 illustrates the response surface and contour plots on the oil yields for rice bran extraction ((a) and (b)) and de-oiled rice bran extraction ((c) and (d)), respectively. The figure showed the oil yields with an influence of pressure and time at fixed temperature of 40 ^{o}C. It could be seen that increasing pressure favors an increase in extractable oil yield for both rice bran extraction and de-oiled rice bran extraction as described in Section 4.1. Prolonging the extraction time led an increase in oil yield as well. The interaction of time and pressure could be observed in this figure. At lower extraction time, effect of pressure on oil yield was not significant as that obtained by operating at higher extraction time. The maximum oil yield was reached at the edge of experimental design condition (30 MPa and 60 min). Although the highest pressure provided the maximum oil yield, the optimum operating time could not be indentified by using this model of experimental design. The further experiments were carried in order to indicate the suitable extraction time and reported as further down.

Table 4-3. Regression models for oil yield

Raw materials	Regression model	R^2	Eq.
Rice bran:	Y = 64.2 – 9.17T + 35.8t + 51.2P + 3.3Tt – 7.9TP + 31.2 tP +7.9TtP	0.99	(4-1)
De-oiled rice bran:	Y = 4.3 – 0.2T + 1.1t + 2.3P – 1.9Tt + 0.3TP +0.6tP – 1.5TtP	0.92	(4-2)

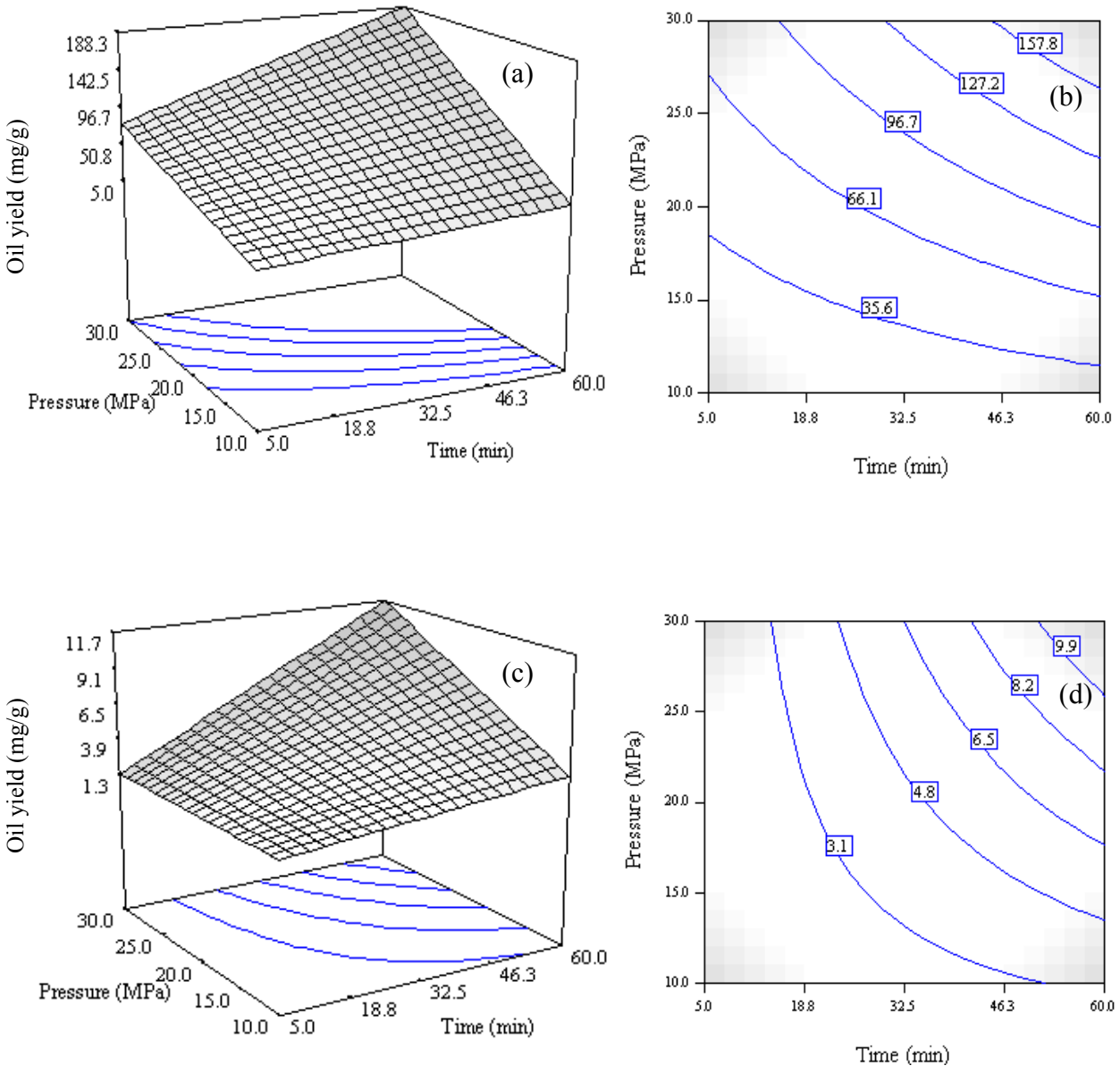


Figure 4-1. The (a, c) response surface and (b, d) contour plot for the oil yield obtained from (a, b) rice bran and (c, d) de-oiled rice bran extractions, as function of the operating pressure and time, all at temperature of 40 ^{o}C.

4.2.2 For the γ-oryzanol yield

Table 4-4 shows the results of ANOVA for rice bran extraction and it presented that all operating factors and their interaction have significant effect on the γ-oryzanol yield. Eq. 4-3 showed the coded regression model for γ-oryzanol extraction from rice bran with high regression coefficient (R^2 = 1.00). This equation was generated based on ANOVA. Additionally, the normal probability plot of residuals and the plots of the residuals versus the predicted response were given in Appendix B which indicated that the proposed models are adequate.

Eq. 4-3 shows that temperature had negative effect whilst operating time and pressure had positive effect on γ-oryzanol yield. The change of operating factors including reducing temperature and increasing pressure affected the yield by means of changing CO_2's density as described in Section 4.1. The longer time increased the amount of solvent and provided the completion of solvent diffusion into solid matrix. The effect of a change in temperature and pressure was thus obviously observed with using longer time as shown in interaction terms (Tt and tP).

Table 4-4. ANOVA test for the γ-oryzanol yields from rice bran extract

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob > F
Model	16.2	7	2.3	1,899.6	< 0.0001*
T	4.7	1	4.7	3,824.2	< 0.0001*
t	3.4	1	3.4	2,783.9	< 0.0001*
P	1.8	1	1.8	1,464.7	< 0.0001*
Tt	3.1	1	3.1	2,560.1	< 0.0001*
TP	1.3	1	1.3	1,076.2	< 0.0001*
tP	1.0	1	1.0	831.0	< 0.0001*
TtP	0.9	1	0.9	756.7	< 0.0001*
Residue	0.0	8	0.0		
Total	16.2	15			

* Significant F-values at the 95% confidence level. T, P and t represent temperature, pressure and time, respectively

$$Y = 627.1 – 539.8T + 460.6t + 334.1P – 441.7Tt - 286.4TP + 251.6tP – 240.12TtP \quad (4\text{-}3)$$

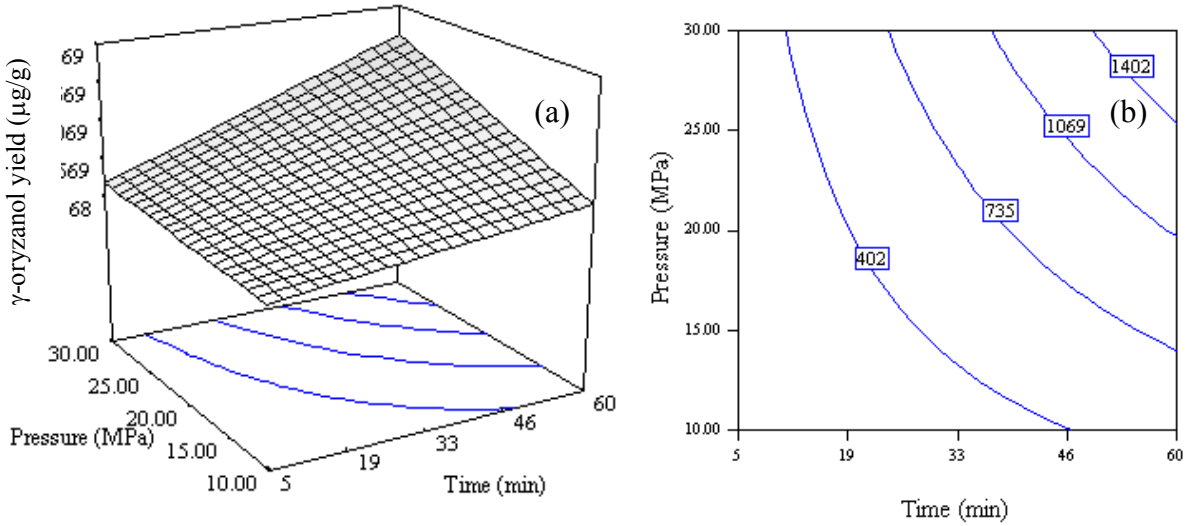


Figure 4-2. The (a) response surface and (b) contour plot for the γ-oryzanol yield obtained from rice bran extractions, as function of the operating pressure and time, all at temperature of 40 ^{o}C.

Fig. 4-2 illustrates the response surface and contour plot which was generated by Eq. 4-3 for γ-oryzanol yield. The figure showed the γ-oryzanol yield with an influence of pressure and time at lower temperature (40 ^{o}C). The lower temperature was fixed because it provided the higher γ-oryzanol yield and lower energy consumption. As can be seen in Fig. 4-2 and described above, the effect of pressure was more dominated by longer time. The maximum γ-oryzanol yield was obtained at the edge of experimental condition (30 MPa and 60 min).

4.3 Optimization of Oil and γ-oryzanol Yield

The results of Section 4.2 indicated the suitable temperature and pressure which should be 40 ^{o}C and 30 MPa, respectively, for achieving the highest oil and γ-oryzanol yields. This section studied the effect of time for rice bran oil and γ-oryzanol extraction. The oil and γ-oryzanol was extracted from rice bran by SC-CO_2 at temperature of 40 ^{o}C and pressure of 30 MPa at different operating time. From Fig. 4-3, it could be seen that rice bran oil and γ-oryzanol yield was gradually increased

within firstly 20 min and then slightly increased with time. At 20 min of operation, oil yield and γ-oryzanol yield were about 82 mg/g rice bran and 1,260 μg/g rice bran, respectively. The concentration of γ-oryzanol in rice bran oil was about 15,400 ppm at this condition. The optimum condition for rice bran oil with relatively high γ-oryzanol was thus temperature of 40 °C, pressure of 30 MPa and operating time of 20 min.

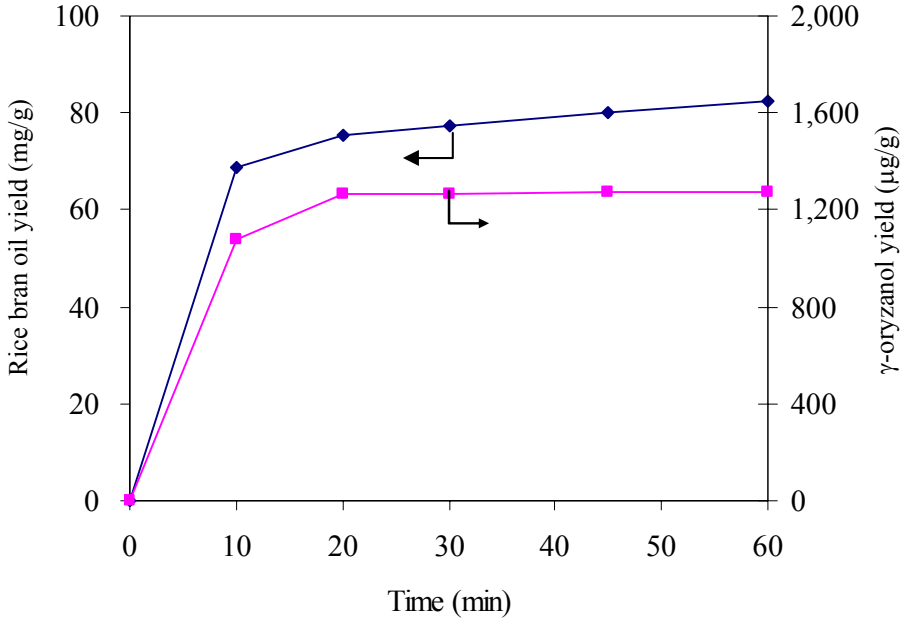


Figure 4-3. Extracted rice bran oil and γ-oryzanol at different times, all at temperature of 40 °C and pressure of 30 MPa.

One of advantages of this technique is reducing energy consumption by reducing temperature. The conventional process operates at 50 °C but the SC-CO_2 extraction can be operated at 40 °C. In conventional process, higher temperature provides higher solvent power and higher oil yield. In SC-CO_2 extraction process, lower temperature provides higher CO_2's density which favors oil solubility. However, the operating temperature should be maintained over its critical point (31.1 °C). The temperature of 40 °C was chosen to make sure that CO_2 maintains its supercritical state and prevent the temperature drop due to error of temperature controller.

Compared to literature reviews, the rice bran oil that was obtained by this work had relatively high γ-oryzanol concentration. Moreover, optimum operating parameters including temperature, pressure, time and CO_2 flow rate were relatively low. The γ-oryzanol concentration reached 15,400 – 17,000 ppm at 40 ^{o}C, 30 MPa, 20 min and CO_2 flow rate of 3 g/min. It was probably due to the use of high ratio of packing material to raw material (20:3 wt:wt). The higher ratio of packing material to raw material provided higher chance of a solvent contact to raw material. Moreover, many works [2, 11, 62] studied the extraction process at high temperature (more than 60 ^{o}C). Those processes were thus operated at higher pressure in order to increase density of CO_2. Therefore, reducing temperature can reduce pressure as shown in this work.

CHAPTER V

PROTEIN AND SUGAR EXTRACTION BY SUBCRITICAL WATER

In this chapter, valuable chemicals such as proteins and sugars were extracted by SW in semi-continuous reactor. Since the de-oiled rice bran has low level of oil but relatively high level of protein compared to rice bran, it was also used as raw material and compared its results with those obtained from using rice bran as raw material. The CCD experiments were performed in order to get preliminary results that are more applicable to a larger commercial scale production. Analysis of variance (ANOVA) and response surface methodology were used for determining the variable(s) that significantly contributed to the prediction model. The investigated three process variables were the operating temperature (150 – 250 ^{o}C), time (5 – 60 min) and pressure (4 – 10 MPa). The optimum conditions for maximum protein and sugar yield were determined from these for each component alone and both together.

5.1 Effects of Raw Materials on the Colors of the Extract

The photographs of the extract obtained from experiments those are representative of 40 independent experiments of the rice bran and de-oiled rice bran extractions are shown in Fig. 5-1. The experimental conditions including operating temperatures, times and pressure were shown in the pictures. It could be seen that the extract was transparent at the lower temperature and shorter times and then changed to yellow and to dark brown at the higher operating temperatures and times, representing more severe conditions. The extract obtained from treatment at 250 ^{o}C also had a burnt smell, somewhat like coffee. This phenomenon is the browning reaction, like maillard reaction and caramelization [9, 112] that would have occurred as the extracted proteins and carbohydrates partially decompose into amino acids and simple sugars during the extraction and hydrolysis in SW conditions.

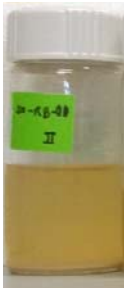
Rice bran				De-oiled rice bran			
150 °C, 5 min	150 °C, 60 min	250 °C, 5 min	250 °C, 60 min	150 °C, 5 min	150 °C, 60 min	250 °C, 5 min	250 °C, 60 min
							

Figure 5-1. Photographs of the extracts obtained from SW extraction (all at 4 MPa) of rice bran and de-oiled rice bran as a function of the temperature and time.

In addition, the color of the extracts obtained from each of the rice bran extractions was darker than those obtained from the de-oiled rice bran extraction under the same conditions due to the color of the extracted yellow-brown rice bran oil content. This is because when the extraction temperature was increased, the static dielectric constant of the SW is decreased allowing non-polar molecules, such as rice bran oil, to dissolve into the SW phase giving the turbid brown color of the extract.

5.2 Experimental Results and Effect of the Operating Factors

The experimental conditions, including the actual values and their coded values which were shown in parentheses, and the amount of total extracted protein and sugar (mg/g of raw materials) for the SW extraction of rice bran and de-oiled rice bran are summarized in Table 5-1.

Table 5-1. The amount of total protein and total sugar recovered from rice bran and de-oiled rice bran.

Experiment No.	Factors: Actual value (coded value)			Rice bran		De-oiled rice bran	
	Temperature, °C (T)	Time, min (t)	Pressure, MPa (P)	Protein* (mg/g)	Total sugar* (mg/g)	Protein* (mg/g)	Total sugar* (mg/g)
1	150 (-1)	5 (-1)	4 (-1)	11.2 ± 1.1	18.3 ± 10.8	45.6 ± 5.7	20.9 ± 7.0
2	150 (-1)	5 (-1)	10 (1)	11.3 ± 11.2	23.0 ± 8.5	15.3 ± 8.3	43.6 ± 6.7
3	150 (-1)	60 (1)	4 (-1)	49.5 ± 5.1	165.1 ± 21.6	23.3 ± 2.1	137.6 ± 0.3
4	150 (-1)	60 (1)	10 (1)	43.9 ± 2.2	142.4 ± 8.8	20.1 ± 5.0	144.4 ± 9.6
5	250 (1)	5 (-1)	4 (-1)	31.6 ± 0.2	94.3 ± 18.7	26.4 ± 3.0	124.8 ± 27.3
6	250 (1)	5 (-1)	10 (1)	32.9 ± 6.5	94.8 ± 29.2	14.0 ± 13.0	96.2 ± 26.4
7	250 (1)	60 (1)	4 (-1)	146.4 ± 0.4	119.0 ± 39.8	178.6 ± 6.3	247.8 ± 4.7
8	250 (1)	60 (1)	10 (1)	145.6 ± 5.3	186.1 ± 32.5	116.2 ± 22.5	255.0 ± 33.4
9	200 (0)	32.5 (0)	7 (0)	67.9 ± 0.2	254.7 ± 21.3	49.2 ± 11.7	278.9 ± 25.7
10	116 (-1.68)	32.5 (0)	7 (0)	6.2 ± 1.1	50.5 ± 13.8	0.0 ± 0.0	52.5 ± 13.5
11	284 (1.68)	32.5 (0)	7 (0)	78.5 ± 12.2	131.8 ± 15.9	71.9 ± 7.9	129.4 ± 18.3
12	200 (0)	0 (-1.68)	7 (0)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
13	200 (0)	79 (1.68)	7 (0)	153.0 ± 25.8	215.3 ± 28.4	62.6 ± 2.9	399.8 ± 24.7
14	200 (0)	32.5 (0)	2 (-1.68)	19.6 ± 2.6	170.6 ± 6.3	17.5 ± 2.1	187.9 ± 5.5
15	200 (0)	32.5 (0)	12 (1.68)	10.1 ± 1.1	179.6 ± 11.0	13.1 ± 3.1	234.2 ± 29.2

* Average from two replicates which reported in mg/g raw materials

The rice bran was composed of protein, carbohydrates and other polysaccharides such as cellulose and hemicellulose (Table 3-1) which could be extracted and partially decomposed under the SW conditions. As can be seen in Table 5-1, the protein and sugar content in the extract increased as both the operating temperature and time increased. For example, an increase in the temperature from 150 °C to 250 °C, at the same pressure and operating time (No. 1 and 5), led to a 2.8- and 5.1-fold increase in the total protein and sugar yields, respectively, whilst the yields obtained were also dramatically increased when the operating time was increased in all experiments.

Under the highest operating temperature and time (250 °C for 60 min), the highest yields of protein (146.4 mg / g rice bran and 178.6 mg / g de-oiled rice bran)

and sugars (186.1 mg / g rice bran and 255.0 mg / g de-oiled rice bran) were obtained. When the total protein yields were compared to the original amount of protein in the raw materials (Table 3-1: 127 mg/g of rice bran, 169 mg/g de-oiled rice bran), it is clear that protein can be totally extracted by SW. The overestimate is probably due to analytical errors from the interference caused by the sugars in the solution. Some reports indicated some interfering compounds for Lowry's assay including some sugars such as sucrose, fructose and glucose [113-115].

In general, the hydrogen bonding of water becomes weak and less persistent as the temperature is increased and the pressure is decreased [13], which corresponds to the decrease in the static dielectric constant and the increase in the ion product (K_w) of the water. The decreased static dielectric constant leads to an increased solubility of organic non-polar compounds into the water under these SW conditions. Moreover, as the ion product (K_w) increases, H^+ ion-based reactions, such as hydrolysis, are favored and take place [116], leading to the breakdown of the protein peptide bonds and the polysaccharide polymer chains and so the accumulation of smaller proteins and amino acids, sugars and other decomposition products, such as formic acid and acetic acid [117]. This leads to the higher level of protein and sugar extraction at the higher operating temperatures of the extraction process. With respect to the operating time, longer operating times result in an increased protein yield because the ratio of water, and also H^+ ions, to raw materials is increased allowing the protein to be extracted and hydrolyzed more efficiently.

Anyway, no consistent unequivocal trend of any significant effect of pressure on the total protein and sugar yields obtained in the extract were observed. This case was in accord with the fact that pressure, which is over the water saturated pressure, has only a small effect on the dielectric constant of the SW compared to the effects of the operating temperature and time [85]. However, it was plausible that as the pressure was increased from 2 MPa the extraction efficiency was increased to a maximal level at around 7 MPa, and then decreased with further increases in the pressure (nos. 9, 14 and 15), but this phenomenon could be observed only at 200^{o}C.

5.3 Statistical Analysis for the Effects of Operating Factors

5.3.1 For the total protein yield

The results of the ANOVA, including the effect estimates, of the CCD based evaluation into the total protein yields (Table 5-1) are summarized in Tables 5-2 and reported by means of the *p*-value. As shown in Table 5-2, the operating time and interaction term between temperature and times had a significant effect (*p*-value < 0.05) on the protein yields obtained from the rice bran extraction (Table 5-2 (a)), whilst only the interaction term between temperature and time had a significant effect on the protein extraction from the de-oiled rice bran extraction (Table 5-2 (b)). In fact that oil containing in de-oiled rice bran is lower than that containing in rice bran, water molecule thus can penetrate into solid matrix easier and effect of only operating time became less.

With respect to the effect of interaction term, the change in the water's properties can not affect the extracted protein yield within a short operating time. In the SW medium, the relative dielectric constant decreases from about 40 at 150 ^{o}C to 30 at 250 ^{o}C, which favors the increased solubility of proteins with a hydrophobic portion in their structure. However, the interaction term indicated that SW needs longer time to penetrate into the solid matrix. These interaction effects are illustrated in the interaction effect plots between the operating temperature and time for the protein extraction from rice bran (Fig. 5-2 (a)) and de-oiled rice bran (Fig. 5-2 (b)), and reveal that the operating time interacted strongly with temperature. When the temperature was increased to 250 ^{o}C, an increase in the operating time from 5 to 60 min caused a significant increase in the protein yield from 50.5 to 154.3 mg/g of rice bran and 13.2 to 140.0 mg/g of de-oiled rice bran. However, even 2^{k} factorial design was carried out under assumption of linearity, the curvilinear trend could be seen from this figure. The curvature was the result from the twisting of plane that was induced by interaction terms [100].

Table 5-2. ANOVA test for the total protein yields

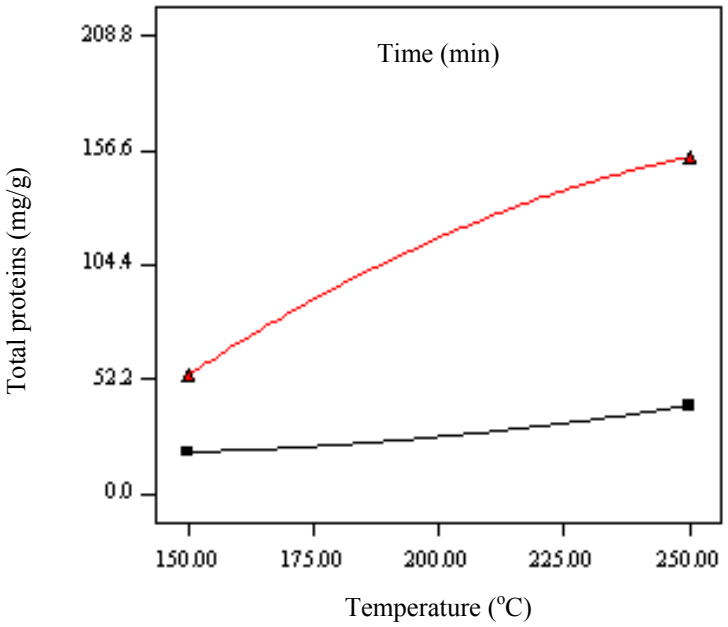
a) Rice bran extract

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob > F
Model	79,558.9	13	6,119.9	4.7	0.0023*
T	5,239.6	1	5,239.6	4.0	0.0625
t	23,419.7	1	23,419.7	17.9	0.0006*
P	89.9	1	89.9	0.1	0.7964
T^2	281.5	1	281.5	0.2	0.6488
t^2	637.5	1	637.5	0.5	0.4948
P^2	2,560.7	1	2,560.7	2.0	0.1806
Tt	6,134.4	1	6,134.4	4.7	0.0457*
TP	8.7	1	8.7	0.0	0.9358
tP	15.4	1	15.4	0.0	0.9148
T^2t	444.6	1	444.6	0.3	0.5678
T^2P	32.0	1	32.0	0.0	0.8775
Tt^2	485.4	1	485.4	0.4	0.5507
TtP	3.1	1	3.1	0.0	0.9616
Residual	20,902.3	16	1306.4		
Total	100,461.2	29			

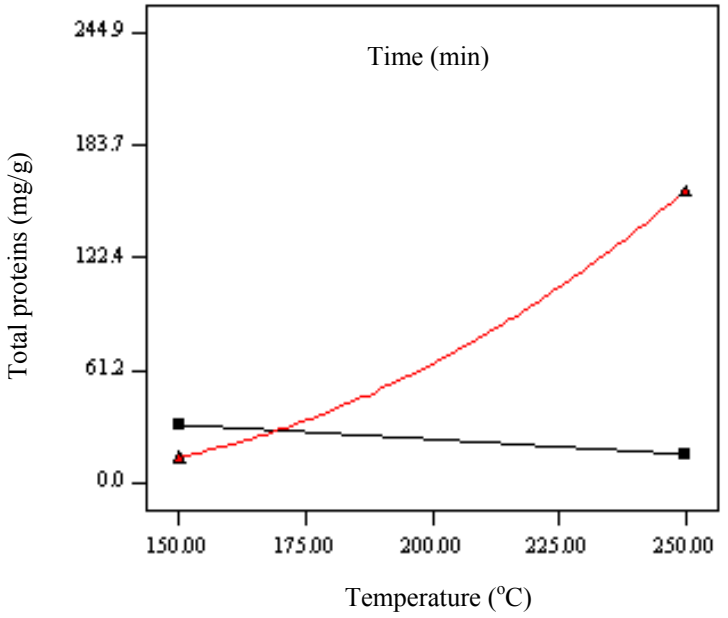
b) De-oiled rice bran extract

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob > F
Model	60,878.5	13	4,683.0	2.0	0.0946
T	5,175.4	1	5,175.4	2.2	0.1563
t	3,913.1	1	3,913.1	1.7	0.2142
P	19.1	1	19.1	0.0	0.9292
T^2	105.3	1	105.3	0.0	0.8347
t^2	20.1	1	20.1	0.0	0.9272
P^2	230.9	1	230.9	0.1	0.7575
Tt	18,485.8	1	18,485.8	7.9	0.0126*
TP	423.8	1	423.8	0.2	0.6760
tP	132.0	1	132.0	0.1	0.8153
T^2t	805.4	1	805.4	0.3	0.5655
T^2P	990.9	1	990.9	0.4	0.5244
Tt^2	371.0	1	371.0	0.2	0.6957
TtP	1,486.3	1	1,486.3	0.6	0.4371
Residual	37,428.5	16	2,339.3		
Total	98,307.0	29			

* Significant F-values at the 95% confidence level. T, P and t represent temperature, pressure and time, respectively



(a)



(b)

Figure 5-2. Interaction plot between the operating temperature and time for protein extraction from (a) rice bran and (b) de-oiled rice bran. Filled square = 5 min of operation and filled triangle = 60 min of operation.

5.3.2 For the total sugar yield

The results of the ANOVA, including the effect estimates, of the CCD based evaluation into the total protein yields (Table 5-1) are summarized in Tables 5-3 and reported by means of the *p*-value. As shown in Table 5-3, the temperature (T), operating time (t) and pressure (P) had a curvilinear significant effect (*p*-value < 0.05) on the total sugar yield with represented second-order terms (T^2, t^2 and P^2). The operating time also had a significant linear effect, while the interaction between these factors was not statistically significant (*p*-value > 0.05).

This phenomenon was quite different from the results for the total protein yield because of the different reactions that are required in order to produce sugar and extract the protein. In the SW medium, the change in water properties favors different reactions. The extraction or dissolution of proteins, the main mechanism of obtaining soluble proteins, is affected by the change in the relative dielectric constant, whilst the hydrolysis of largely insoluble polysaccharides to produce soluble sugars is affected by the change in the ion product (K_w).

As described in Section 5.2, higher temperatures increased the concentration of the H^+ and OH^- ions, by means of increasing the K_w from 10^{-12} mol^2/kg^2 at 150 ^oC to 10^{-11} mol^2/kg^2 at 250 ^oC. The complex network structure of the cell walls which is composed of hemicellulose and other polysaccharides could be extracted by this change in the water properties. When the operating time was increased, the water to raw material ratio was increased allowing the polysaccharides to be more completely hydrolyzed and dissolved which resulted in a major effect on the sugar yield obtained.

Table 5-3. ANOVA test for the total sugar yield

a) Rice bran extract

Source	Sum of Square	Degree of Freedom	Mean Square	F-Value	p-Value
Model	160237.5	13	12326.0	14.4	< 0.0001*
T	6617.8	1	6617.8	7.7	0.0133*
t	46354.1	1	46354.1	54.2	< 0.0001*
P	81.0	1	81.0	0.1	0.7622
T^2	44822.1	1	44822.1	52.4	< 0.0001*
t^2	36628.1	1	36628.1	42.8	< 0.0001*
P^2	11766.6	1	11766.6	13.8	0.0019*
Tt	5643.8	1	5643.8	6.6	0.0206*
TP	1829.7	1	1829.7	2.1	0.1628
tP	385.1	1	385.1	0.5	0.5117
T^2t	1745.3	1	1745.3	2.0	0.1723
T^2P	81.4	1	81.4	0.1	0.7616
Tt^2	239.9	1	239.9	0.3	0.6036
TtP	2206.7	1	2206.7	2.6	0.1277
Residual	13678.1	16	854.9		
Cor Total	173915.6	29			

b) De-oiled rice bran extract

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob > F
Model	338618.4	13	26047.6	15.6	< 0.0001*
T	5913.6	1	5913.6	3.5	0.0784
t	159840.0	1	159840.0	95.5	< 0.0001*
P	2143.7	1	2143.7	1.3	0.2744
T^2	65502.8	1	65502.8	39.1	< 0.0001*
t^2	14862.8	1	14862.8	8.9	0.0088*
P^2	11706.6	1	11706.6	7.0	0.0176*
Tt	1032.0	1	1032.0	0.6	0.4437
TP	646.4	1	646.4	0.4	0.543
tP	97.5	1	97.5	0.1	0.8123
T^2t	21113.3	1	21113.3	12.6	0.0027
T^2P	1078.9	1	1078.9	0.6	0.4337
Tt^2	3915.4	1	3915.4	2.3	0.1456
TtP	666.9	1	666.9	0.4	0.5367
Residual	26771.0	16	1673.2		
Cor Total	365389.4	29			

* Significant F-values at the 95% confidence level. T, P and t represent temperature, pressure and time, respectively

5.4 Optimization of the Extraction Process

5.4.1 Protein extraction

In order to optimize the process condition for protein extraction, the coded regression models were generated, based on the ANOVA analysis, and shown in Table 5-4 as Eqs. (5-1) and (5-2) for the rice bran and de-oiled rice bran extractions, respectively. Statistical analysis indicated that these proposed models were adequate, with high R^2 values (0.98 for rice bran and 0.94 for de-oiled rice bran). Additionally, the normal probability plot of residuals and the plots of the residuals versus the predicted response were given in Appendix B. The response surfaces and contour maps for rice bran extraction and de-oiled rice bran extraction were shown in Fig. 5-3. It could be found that total protein yield was a function of temperature and time. Thus, the protein yield significantly increased as the temperature increased at longer operating times and reached a maximum at 250 ^{o}C and 7 MPa for 60 min. Although the maximum protein yield was obtained at edge of the experimental design condition, the yield attained was 100% of the original protein level in the two raw materials, and so no extension of the experimental conditions was required to be carried out.

Table 5-4. Regression models for protein yield

Raw materials	Regression model	R^2	Eq.
Rice bran:	$Y = 64.9 + 21.5T + 45.5t - 2.8P - 4.8T^2 + 7.3t^2 - 14.5P^2 + 19.6Tt + 0.7TP - 1.0tP - 8.2T^2t + 2.2T^2P + 8.6Tt^2 + 0.4TtP$	0.98	(5-1)
De-oiled rice bran:	$Y = 43.7 + 21.4T + 18.6t - 1.3P + 2.9T^2 + 1.3t^2 - 4.4P^2 + 34.0Tt - 5.1TP - 2.9tP + 11.0T^2t - 12.2T^2P + 7.5Tt^2 - 9.6TtP$	0.94	(5-2)

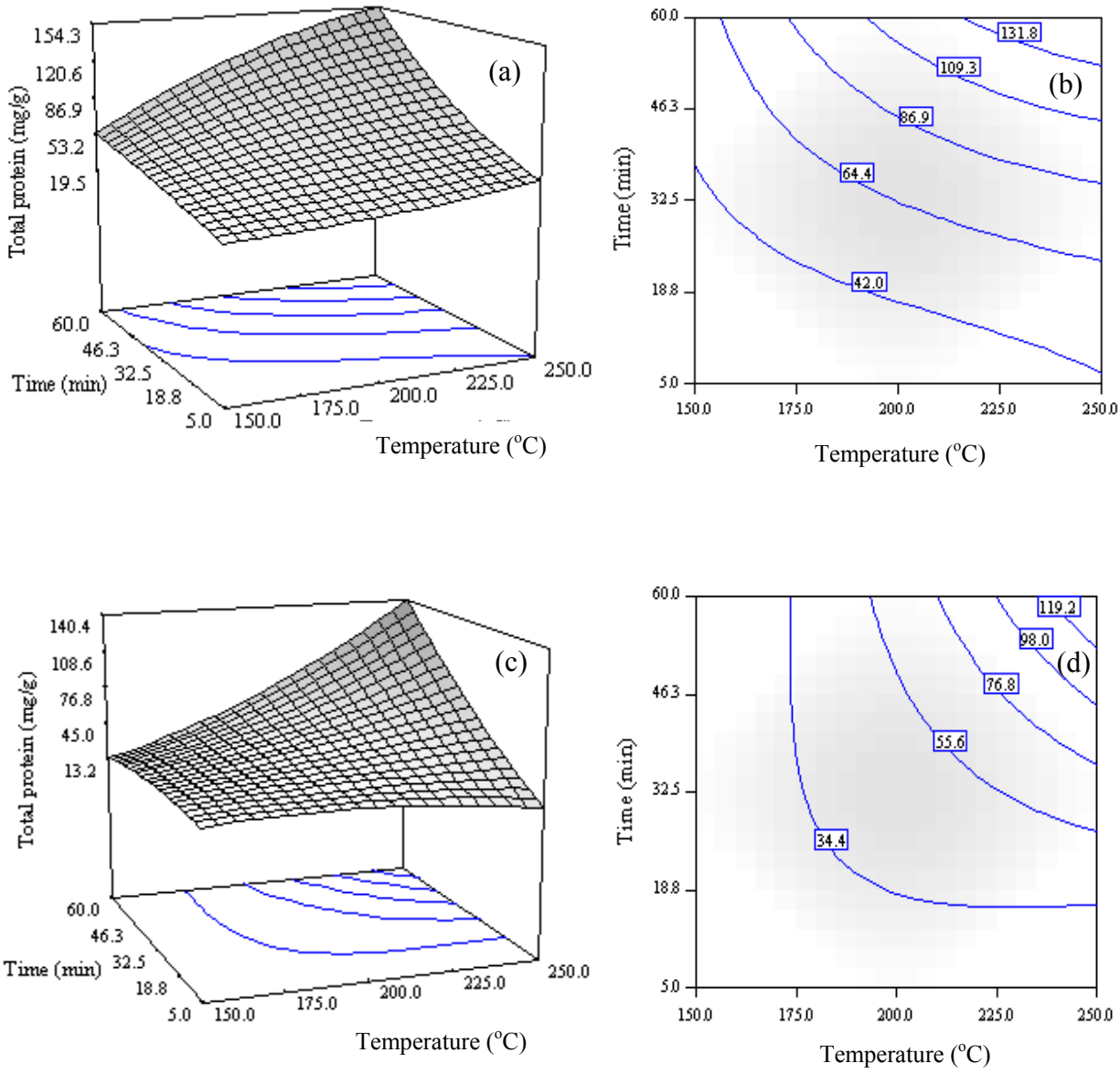


Figure 5-3. The (a, c) response surface and (b, d) contour plot for the total protein yield in (a, b) rice bran and (c, d) de-oiled rice bran extractions, as function of the operating temperature and time, all at 7 MPa of pressure.

5.4.2 Sugar extraction

In order to optimize the process conditions for sugar production, the coded regression model of the sugar yields obtained from rice bran and de-oiled rice bran extraction was generated as Eqs. (5-3) and (5-4), respectively (Table 5-5), from the statistical analysis based on the ANOVA test. The regression coefficient for each extraction was significant and close to unity which indicated a good performance of the model. Additionally, the normal probability plot of residuals and the plots of the residuals versus the predicted response were given in Appendix B. The response surface and contour plots were generated (Fig. 5-4) using these equations and used to illustrate the effect of temperature and operating time on the sugar yield at 7 MPa. From this figure, the maximum extraction yield under different conditions could be predicted. The sugar yields obtained from both the rice bran (Fig. 5-4 (a) and (b)) and the de-oiled rice bran (Fig. 5-4 (c) and (d)) extractions were positively curvilinear in relation to the interaction between temperature and operating time. The optimum conditions for the two different raw material extractions were slightly different. For rice bran extraction, the contour map (Fig. 5-4 (b)) indicated that the sugar yield reached the high value at temperature range of 175 – 240 ^{o}C at longer operating times and reached the maximum value at 210 ^{o}C, 50 min and 7 MPa. For the de-oiled rice bran extraction, the contour map (Fig. 5-4 (d)) demonstrated that the maximum sugar yield was also obtained at 210 ^{o}C and 7 MPa.

However, as shown in Fig. 5-4, when temperature did beyond an optimum temperature, the sugar yield was decreased as a sign of product degradation. During extraction process, hemicellulose was removed from raw material matrix in form of xylose which could be changed to furfural at high temperature, generally more than 200^{o}C [118]. This degraded product would act as an inhibitor in some processes such as bioethanol production, thus the process condition had to be chosen carefully.

Table 5-5. Regression models for sugar yield

Raw materials	Regression model	R^2	Eq.
Rice bran:	$Y = 256.9 + 24.2T + 64.0t + 2.7P - 60.8T^2 - 55.0t^2 - 31.2P^2 + 18.8Tt + 10.7TP + 4.9tP - 16.2T^2t + 3.5T^2P - 6.0Tt^2 + 11.7TtP$	0.92	(5-3)
De-oiled rice bran:	$Y = 284.0 + 22.9T + 118.9t + 13.8P - 73.6T^2 - 35.0t^2 - 31.1P^2 + 8.0Tt - 6.4TP + 2.5tP - 56.4T^2t - 12.8T^2P + 24.3Tt^2 + 6.5TtP$	0.93	(5-4)

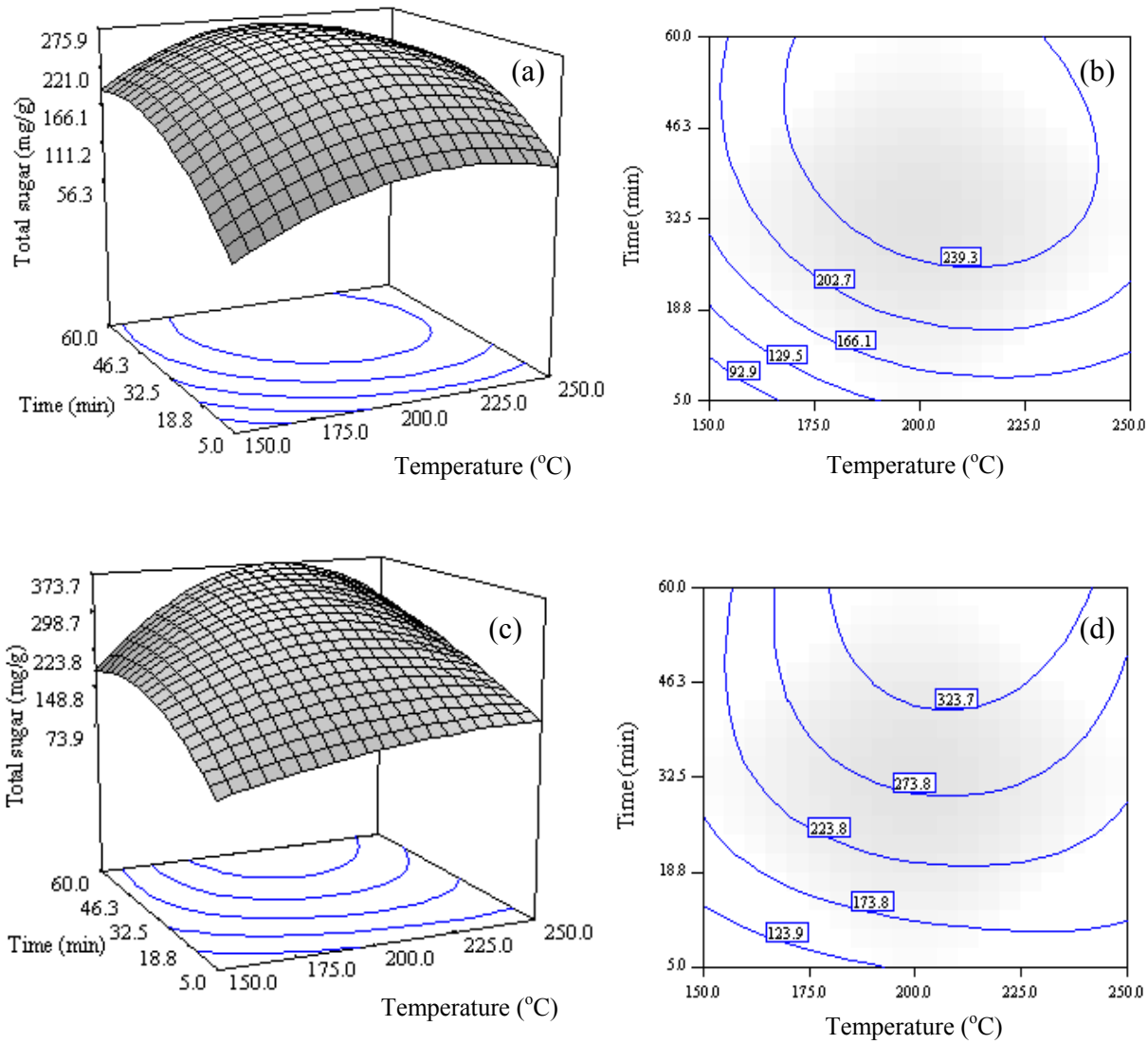


Figure 5-4. The (a, c) response surface and (b, d) contour plot for the total sugar yield in (a, b) rice bran and (c, d) de-oiled rice bran extractions, as function of the operating temperature and time, all at 7 MPa of pressure.

5.4.3 Simultaneous considering of both protein and sugar extraction

Since the desired extraction products are both sugars and proteins, then in order to simultaneously maximize the total sugar and protein yields, their individual response surfaces were combined into one (Fig. 5-5), according to method which was described in Section 3.4. The combined optimum for the extraction from rice bran, with a desirability of 0.80, was an operating temperature and time of 223 ^{o}C and 60 min, to give a theoretical maximum sugar and protein yields of 250.6 mg/g and 137.4 mg/g rice bran, respectively. The extraction from de-oiled rice bran was somewhat similar with the same combined optimum time and pressure but with a higher operating temperature of 250 ^{o}C (desirability as 0.70), giving maximum sugar and protein yields of 277.3 mg/g and 159.0 mg/g de-oiled rice bran. These yields increased with increasing operating temperature and time.

From Fig. 5-5, as described above, it could be seen that the protein and sugar yield did not reach their respective maximum value at the combined optimum condition (desirability as 0.70 – 0.80), compared to the values obtained from the separate optimum conditions for protein and sugar (Figs. 5-3 and 5-4). Of course, this combined optimum condition is based upon the same priority for the protein and sugar yields, but in reality, these may change with different applications. For example, the sugar yield and its concentration should be the desired product for bioethanol production, therefore the optimum condition for sugar yield (210 ^{o}C, 50 min and 7 MPa) must be used in this case to achieve the maximum value of desired product and avoid the effect of inhibitors in term of degraded products during bioethanol production. This work provides the condition that can serve as the preliminary starting point to reach the aim of protein or sugar extraction from rice bran and de-oiled rice bran.

As mention earlier, the sugar and protein yield were increased with temperature and operating time according to the decrease in the static dielectric constant and the increase in the ion product of the water. The optimum conditions for each product were then shown in Figs. 5-3 and 5-4. The optimum conditions for protein extraction was occurred at the edge of selected range of experimental conditions whilst the optimum condition for sugar extraction was occurred around 200^{o}C since some products could be degraded, for example, xylose could be changed

to furfural at high temperature, generally more than 200°C. Finally, in order to simultaneously maximize both of two products, their individual response surfaces were combined as shown in Fig. 5-5. The optimum condition of this case was reported by means of maximum desirability.

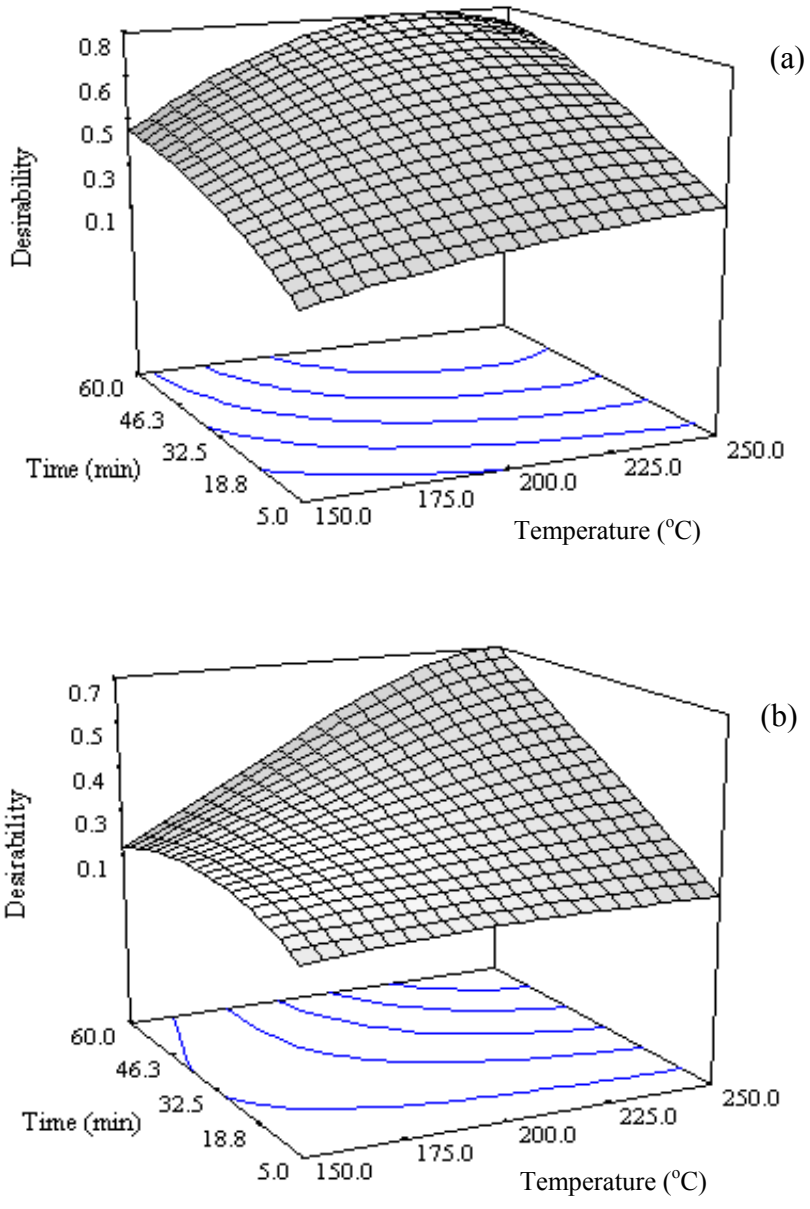


Figure 5-5. Optimization of the SW extraction of (a) rice bran and (b) de-oiled rice bran as a function of the operating temperature and time at 7 MPa of pressure

Compared to literature reviews, this work used higher temperature and operating time. However, this technique provided high protein yield which was about 100% extraction. Moreover, it could be operated with higher amount of raw material. Compared to some works [73, 77], using semi-continuous reactor reduced ratio of water to raw material. In batch reactor, ratio of water to raw material should be high enough in order to control the density and viscosity of the mixture. The lower ratio of water to raw material causes an increase in density and viscosity of the mixture. The poor mixing of water and raw material can be occurred in this case [7]. The accessibility of water to raw material is thus reduced. In semi-continuous reactor, the water is forced to flow through the sample resulting in well mixing and increased accessibility of water into raw material.

In addition, the reactor that was used in this part was the same as using in SC-CO_2 process. Therefore, the sequential process for combining SC-CO_2 process and SW process could be carried out easily.

CHAPTER VI

KINETICS OF RICE BRAN PROTEIN HYDROLYSIS IN SUBCRITICAL WATER

Since the proteins were extracted from rice bran and de-oiled rice bran by SW at quite severe condition, some of them might be degraded into other products. Although the protein aggregation, protein extraction and amino acid production from agricultural biomass feedstock have been investigated as described in Chapter II, the overall reaction of proteins during SW extraction has not been established. Moreover, knowledge of the kinetics of such processes is essential in order to optimize the extraction process of proteins, their derived polypeptides and the subsequent production of amino acids from rice bran. Thus, this chapter not only focuses on the rate determination of protein aggregation and disaggregation with polypeptide production, but it also investigates the production of amino acids from rice bran extracted proteins in SW.

To study the protein aggregation or degradation, the protein was extracted directly from the rice bran, dissolved in water and then heated under a SW atmosphere in a batch reactor as described in Chapter III. The total protein/ polypeptide and amino acid concentrations were analyzed. This chapter reported the elementary mechanism of changes in the proteins during SW hydrolysis. The kinetic parameters were obtained for protein aggregation, disaggregation and hydrolysis to amino acids within the temperature range of 150 – 250 ^{o}C (423 – 523 K), a reaction time of 0 – 60 min and two different initial protein concentrations.

6.1 Proposed Reaction Models and Data Evaluation

Fig. 6-1 shows the photographs of the rice bran protein solution after pretreatment by SW at 250 ^{o}C from 5 min to 60 min. The protein was aggregated to produce large particulate flocculants within 5 min, from the first sample bottle, and then the large matter decreased with increasing reaction time. The apparent viscosity of the protein/polypeptide solution seemed to be increased, which is consistent with that observed and reported by Mleko and Foegeding [93] on the temperature and pH-induced aggregation of whey protein. Moreover, the color of the protein solution became brownish due to the reaction so-called Maillard reaction which occurred between the produced amino acids and the residual sugar in the extracted rice bran protein.

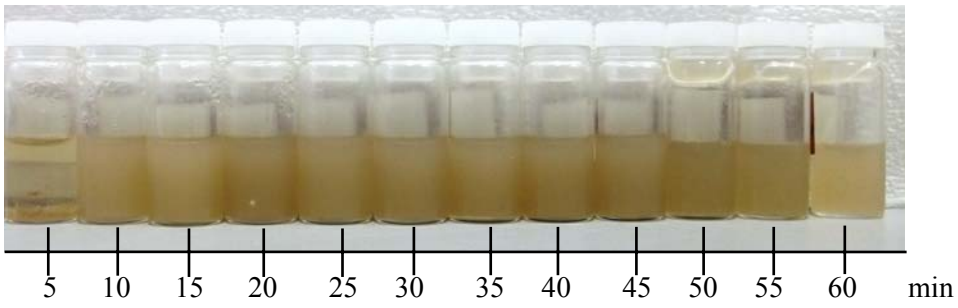


Figure 6-1. Photographs of the rice bran protein solution after pretreatment by SW at 250 ^{o}C

In order to confirm this observation, the protein/polypeptide and amino acid concentrations were analyzed as described in Chapter III, and the results are summarized in Fig. 6-2 for two different initial concentrations of protein of 1.46 and 1.67 g/L. The result showed the trend of protein/polypeptide conversion and amino acid production. Protein concentrations decreased within 5 min as the proteins aggregated and became solid particles, as shown in Fig. 6-1, only a small amount of protein thus remained in the liquid solution. After that, when the reaction time was increased, the disaggregated protein/polypeptide and amino acid concentrations increased since the aggregated protein was decomposed and hydrolyzed to form smaller soluble polypeptides and amino acids. These results were used to generate the

reaction scheme of protein aggregation and disaggregation/decomposition as schematically shown in Fig. 6-3. In this figure, a simplified reaction model was suggested according to the pattern of parallel and consecutive reactions. It was assumed that all reactions were irreversible.

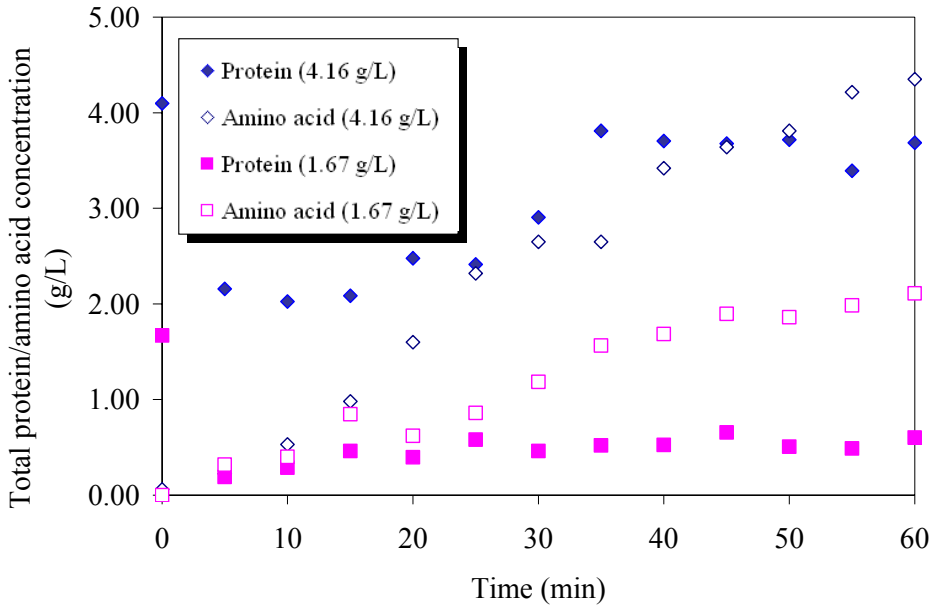


Figure 6-2. Protein/polypeptide and amino acid concentrations of the rice bran protein solutions following SW extraction at 250 °C with the residence time. The initial concentration of proteins in g/L is shown in parenthesis.

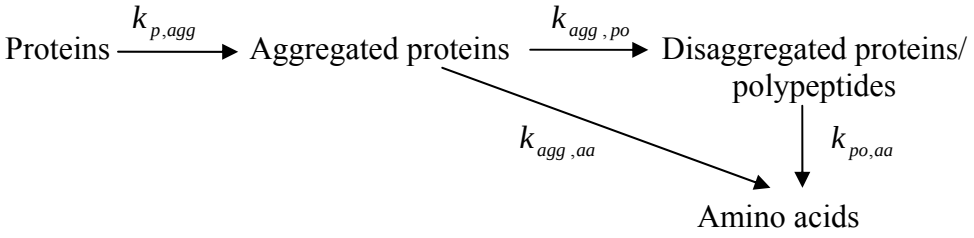


Figure 6-3. Simplified reaction scheme for protein aggregation and degradation and their hydrolysis to polypeptides and amino acids.

This reaction pathway was used to determine the rate constants for the protein aggregation ($k_{p,agg}$), aggregated protein decomposition and formation of soluble polypeptides ($k_{agg,po}$), amino acid production from the disaggregated protein/polypeptides ($k_{po,aa}$) and amino acid production from the aggregated protein ($k_{agg,aa}$). The formation of aggregated proteins could not be quantified. However, it could be predicted from an observation and using the experimental data of the disaggregated protein/polypeptide concentration in the liquid solution. The aggregated protein formation was assumed to occur as the first step of the reaction as shown in Fig. 6-3. The temporal decrease in the rice bran protein was then described by Eq. (6-1)

$$\frac{dC_p}{dt} = -k_{p,agg} C_p^m \tag{6-1}$$

The aggregated protein was then decomposed or disaggregated to produce smaller soluble polypeptides and then hydrolyzed to from amino acids. The calculated concentration of the aggregated proteins changed due to two decomposition reactions to produce proteins/polypeptides and amino acids, as shown in Eq. (6-2). For the concentration of the disaggregated proteins/polypeptides, it was affected by two reactions including the aggregated protein decomposition and the disaggregated proteins/polypeptides hydrolysis to amino acids, as illustrated by Eq. (6-3).

$$\frac{dC_{agg}}{dt} = -k_{agg,po} C_{agg}^n - k_{agg,aa} C_{agg}^o \tag{6-2}$$

$$\frac{dC_{po}}{dt} = k_{agg,po} C_{agg}^n - k_{po,aa} C_{po}^l \tag{6-3}$$

The concentration of amino acid changed due to two reactions including the disaggregated proteins/polypeptides decomposition (e.g. to amino acids) and the aggregated protein decomposition, as shown in Eq. (6-4).

$$\frac{dC_{aa}}{dt} = k_{po,aa} C_{po}^l + k_{agg,aa} C_{agg}^o \tag{6-4}$$

In order to determine the rate constants; $k_{agg,aa}$, $k_{agg,po}$, $k_{po,aa}$ and $k_{p,agg}$, and the reaction order; *l*, *m*, *n* and *o*, the protein/polypeptide and amino acid concentration curves were reconstructed by fitting the estimated concentration of protein/ polypeptides and amino acids with the observed protein concentration and amino acid

concentration curves, and solved by using numerical method. The Heun's method was chosen since it is developed from Euler's method which is easy to understand. The process steps of Heun's method are shown as Eqs. (6-5) and (6-6) [119]:

$$p_{k+1} = y_k + hf(t_k, y_k) \quad , \; t_{k+1} = t_k + h \tag{6-5}$$

$$y_{k+1} = y_k + \frac{h}{2}(f(t_k, y_k) + f(t_{k+1}, p_{k+1})) \tag{6-6}$$

The numerical results obtained from this method were fitted to experimental results directly until the best fit model could be identified. In order to fitting the experimental data with the numerical results which would be defined as theoretical predictions, the method of least square was used as an optimization method. The best fit model which minimized the sum of squared residue was then obtained and all kinetic parameters including reaction orders and rate constants were determined as would be described below.

The linear relationship between the protein concentration and reaction time, the first step shown in Fig. 6-3, suggested zero-order kinetics and so Eq. (6-7) became:

$$\frac{dC_p}{dt} = -k_{p,agg} \quad ; \quad at \quad t = 0, C_p = C_{p0} \tag{6-7}$$

Integration of Eq. (6-7) then provided the relationship between C_p and t, as shown in Eq. (6-8):

$$C_p = -k_{p,agg} t + C_{p0} \tag{6-8}$$

The concentration of the aggregated protein (C_{agg}) could then be calculated by the decrease in the rice bran protein concentration as shown in Eq. (6-9):

$$C_{agg} = C_{p0} - C_p \tag{6-9}$$

According to kinetic equation shown in Eq. (6-8), the rice bran protein concentration decreased to zero when $t = \frac{C_{p0}}{k_{p,agg}}$. After that, when $t > \frac{C_{p0}}{k_{p,agg}}$, the proteins/polypeptides concentration increased with time as both of the aggregated protein and soluble polypeptides were split by the cleavage of peptide bond to form amino acids under the SW condition. After fitting the Heun's method to the experimental data, the reaction order and reaction rate were determined. The

differential equation of each reaction step, such as the aggregated protein decomposition, polypeptides production and decomposition and amino acids production, were illustrated as shown in Eqs. (6-10) – (6-12), respectively, and the kinetic parameters are shown in Table 6-1.

$$\frac{dC_{agg}}{d\tau} = -k_{agg,po}C_{agg}^2 - k_{agg,aa}, \quad (6\text{-}10)$$

$$\frac{dC_{po}}{d\tau} = k_{agg,po}C_{agg}^2 - k_{po,aa} \quad \text{and} \quad (6\text{-}11)$$

$$\frac{dC_{aa}}{d\tau} = k_{po,aa} + k_{agg,aa}, \quad (6\text{-}12)$$

where $\tau = t - \frac{C_{po}}{k_{p,agg}}$, since these models were valid after $t = \frac{C_{p0}}{k_{p,agg}}$, with the initial condition according to Eq. (6-13):

$$\text{at } \tau = 0 \quad ; \quad C_{agg} \approx C_{p0},\ C_{po} \approx 0, C_{aa} \approx C_{aa0} \quad (6\text{-}13)$$

Table 6-1. Kinetic parameters of rice bran protein aggregation, disaggregation and degradation in batch reactor under SW conditions

T (^oC)	P = P_{sat} (kPa)	Initial protein concentration (g/L)	$k_{p,agg}$	$k_{agg,po}$	$k_{po,aa}$	$k_{agg,aa}$
150	475.8	1.67	0.2820	0.0007	0.0014	0.0009
		4.16	0.8449	0.0028	0.0065	0.0010
175	892.0	1.67	0.4282	0.0031	0.0017	0.0020
		4.16	1.0615	0.0102	0.0111	0.0022
200	1,553.8	1.67	0.5759	0.0085	0.0090	0.0029
		4.16	1.4276	0.1005	0.0263	0.0099
225	2,556.5	1.67	0.8789	0.0244	0.0169	0.0096
		4.16	2.1789	0.1391	0.0502	0.0239
250	3,973.0	1.67	1.5667	0.0308	0.0178	0.0214
		4.16	4.6000	0.1857	0.0603	0.0245

$k_{p,agg}$, $k_{po,aa}$, $k_{agg,aa}$ have units of g/L•min

$k_{agg,po}$ has units of L/g•min

Eqs. (6-10) – (6-12) demonstrated that the aggregated protein was decomposed into smaller polypeptides with a second order rate law, whilst the amino acid production obeyed a zero order rate law. These reaction models could be used to describe at least the trends of the experimental data, as shown in Sections 6.2 and 6.3.

6.2 Protein Aggregation and Disaggregation or Decomposition

Figure 6-4 showed the experimentally derived data (symbols) and the theoretical predictions (lines) those were obtained by numerical solution, Heun's methods, using Eqs. (6-10) and (6-11) and the parameters listed in Table 6-1. It could be seen that protein aggregation changed with temperature and initial concentration. The dramatic decrease in the protein concentration over time was increased at higher temperatures (225 ^{o}C and 250 ^{o}C), with complete protein aggregation before 5 min. This phenomenon reflected the fact that the protein stability is decreased with increasing temperature in different ways, such as the reduction of activation energy or an increasing frequency of molecular collision [91]. The increase in the initial protein concentration increased the protein aggregation as simply due to the increased chance of protein-protein association as they denature since many proteins can directly associate into protein aggregates physically from the native state [91].

From the rate constants, more process information of protein aggregation could be obtained. At $t \le \frac{C_{p0}}{k_{p,agg}}$, the values of $k_{p,agg}$ confirmed that the protein aggregation rate increased with increasing temperature and initial protein concentration. As can be seen in Table 6-1, the rate constants clearly increased about five times as temperature was increased from 150 to 250 ^{o}C (423 to 523 K) and about two- to three-fold as the initial protein concentration was increased about 2.5-fold from 1.67 to 4.16 g/L.

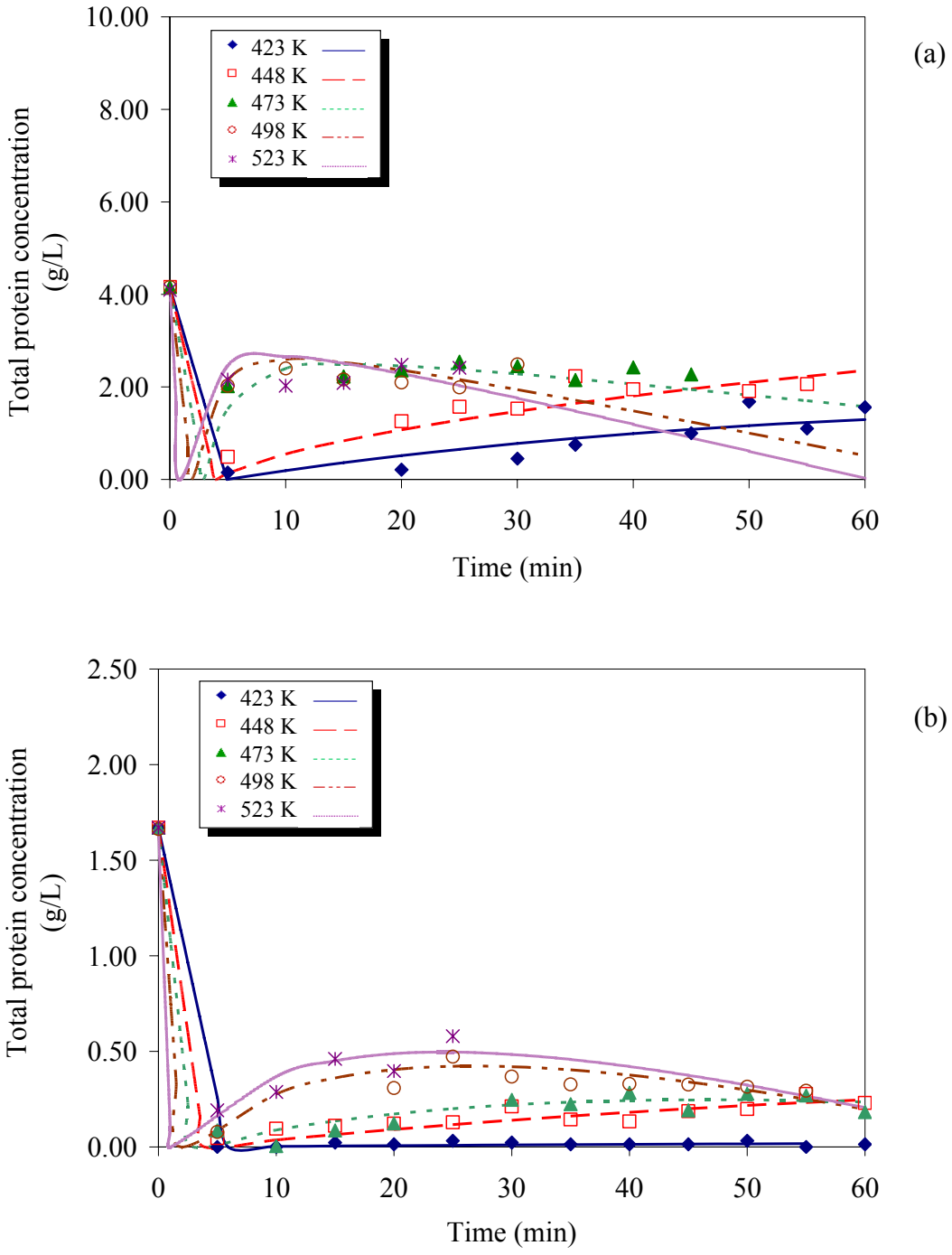


Figure 6-4. Effect of temperature and reaction time on the protein aggregation and disaggregation at an initial protein concentration of (a) 4.16 g/L and (b) 1.67 g/L.

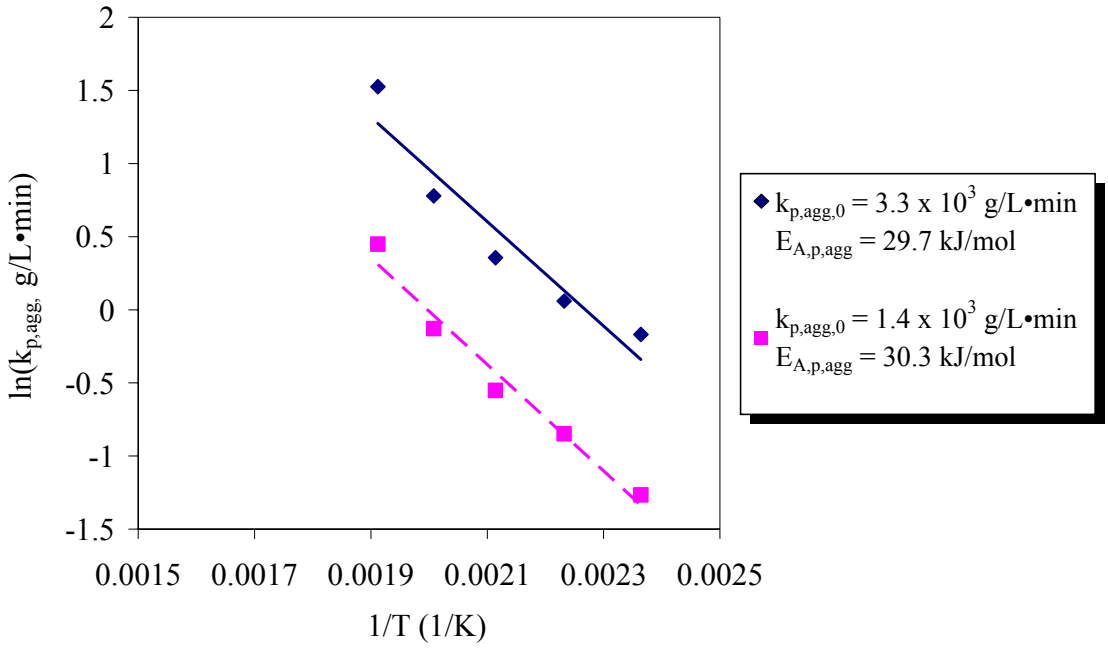


Figure 6-5. Determination of the kinetic parameters from the Arrhenius approach for the protein aggregation at initial protein concentrations of (◆) 4.16 g/L and (■) 1.67 g/L.

Fig. 6-5 shows the Arrhenius plot with the respective values of the pre-exponential factor. The protein aggregation rate clearly varies with the temperature, which the protein aggregation seems to follow the Arrhenius relationship with high regression coefficient. As can be seen in this figure, the fitting lines were shown as solid line with R^2 = 0.91 and dash line with R^2 = 0.97 for the initial protein concentration of 4.16 and 1.67 g/L, respectively. In addition, it indicates that the average activation energy for protein aggregation is 30.0 ± 0.3 kJ/mol.

After protein aggregation as shown in Fig. 6-4, the protein/polypeptides concentration was then increased as the aggregated protein disaggregated and was partly hydrolyzed under SW condition to produce smaller polypeptides and amino acids. Both of these product yields were strongly affected by the temperature and reaction time. As can be seen in Fig. 6-4 (lines), at lower operating temperature and after $t > \frac{C_{p0}}{k_{p,agg}}$, the polypeptides concentration increased with increasing reaction time. As the operating temperature was increased, the maximum proteins/poly-

peptides concentration was observed. The maximum values were found at a shorter reaction time as temperature was increased. In addition, the proteins/polypeptides concentrations were decreased in magnitude at higher temperatures and reaction times due to thermal decomposition and hydrolysis into amino acids. As exemplified in Fig. 6-4 (a), at 150 ^{o}C (423 K) and 175 ^{o}C (448 K), the maximum polypeptide yield was still not achieved after a reaction time of 60 min. However, the optimum polypeptide yield was obtained at higher temperatures (200 – 250 ^{o}C or 473 – 523 K), at a reaction time of less than 15 min, indicating that around 65% of the aggregated protein was decomposed to polypeptides and amino acids. At 250 ^{o}C (523 K), the theoretical (model) prediction predicted that the total proteins and polypeptides were almost completely decomposed after 60 min.

The actual polypeptide yields obtained from the decomposition of the aggregated proteins at the lower initial protein concentration (1.67 g/L) suggested that only 25 – 30% of the aggregated protein could be decomposed into polypeptides at 225 ^{o}C (498 K) and 250 ^{o}C (523 K) and a reaction time of 25 min (Fig. 6-4 (b)). In this case, some of aggregated proteins and produced polypeptides could be decomposed into amino acids as another product which can be seen in a simplified reaction model and the results those were shown in Section 3.3 (Fig. 6-7 (b)). On the other hand, the rate constants for aggregated protein decomposition ($k_{agg,po}$) as shown in Table 1, indicated that increasing the initial protein reactant concentration from 1.67 to 4.16 g/L increased the disaggregation rate obviously.

Fig. 6-6 shows the Arrhenius plot for the aggregated protein decomposition. The rate constants for the two different initial protein concentrations could be described by straight lines with reasonable confidence, with high linear regression coefficients. As can be seen in this figure, the fitting lines were shown as solid line with R^2 = 0.91 and dash line with R^2 = 0.97 for the initial protein concentration of 4.16 and 1.67 g/L, respectively. The activation energy and frequency factor were calculated by these fitting curves and were reported in Fig. 6-6. It indicates that the average activation energy for aggregated protein degradation is 77.1 ± 5.1 kJ/mol. A difference of activation energy for different initial protein concentrations was probably due to complicated reaction mechanism of protein disaggregation and decomposition.

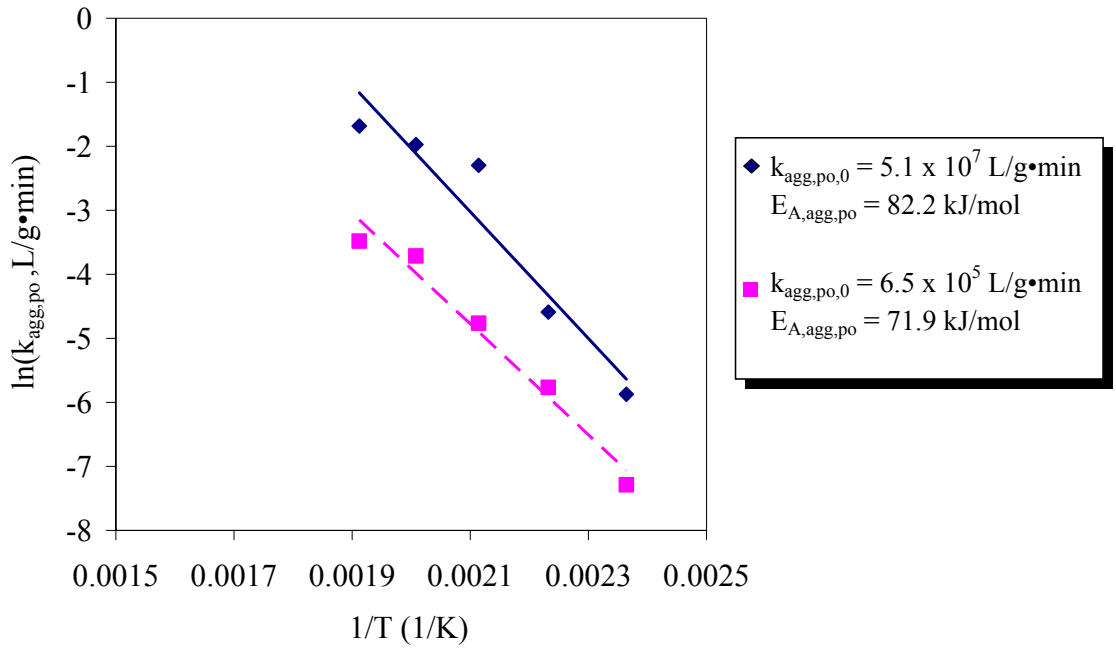


Figure 6-6. Determination of the kinetic parameters from the Arrhenius approach for the aggregated protein degradation at initial protein concentrations of (◆) 4.16 g/L and (■) 1.67 g/L.

6.3 Amino Acids Production

Since the protein and polypeptide peptide bonds can be hydrolyzed under the SW conditions, the decomposition of the aggregated proteins and polypeptides to amino acids was also evaluated. Fig. 6-7 shows the experimentally derived data (symbols) and the theoretical predictions (lines) of the amino acid concentrations those were produced during the reaction times of 5 to 60 min, at five temperatures (range of 150 – 250 ^{o}C or 423–523 K). It could be seen that the amino acids those were produced at higher temperature were greater than those obtained at lower temperature since they are thermal labile compounds. The process information could be more obtained when considered with the model predictions those shown in Fig. 6-7 (lines) which follow zero order kinetics as shown in Eq. (6-12).

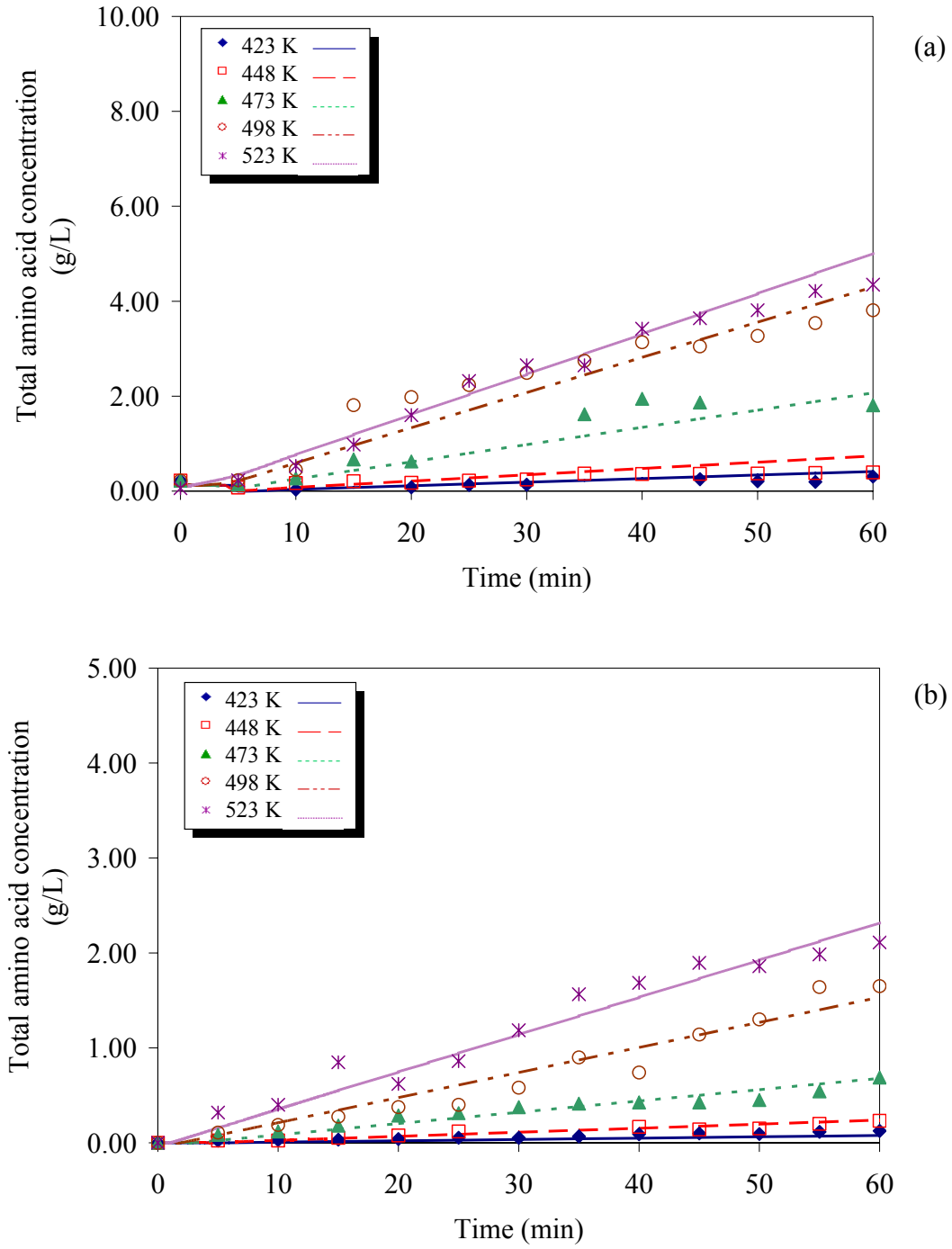


Figure 6-7. Effect of the temperature and reaction time on the production of amino acids at an initial protein concentration of (a) 4.16 g/L and (b) 1.67 g/L.

The regression coefficients (R^2) obtained from linear regression analysis were in a range of 0.87 – 0.97 for an initial protein concentration of 4.16 g/L and more than 0.91 for an initial protein concentration of 1.67 g/L. The R^2 value nearer to unity indicated the model fitting of the amino acid production mechanism. The maximum amino acid concentration was not observed in either the experimental or the theoretical data. Thus, amino acids decomposition might not occur efficiently under these experimental conditions.

From the obtained rate constants and other kinetic parameters those were shown in Table 6-1 and Fig. 6-8, information about the mechanism could be deduced. Fig. 6-8 shows the Arrhenius plot for the amino acids production with the values of the activation energy and frequency factor. Fig. 6-8 (a) indicates that the average activation energy for polypeptide decomposition is 49.3 ± 5.3 kJ/mol. For aggregated protein decomposition, Fig. 6-8 (b) indicates that the average activation energy 59.3 ± 6.5 kJ/mol The rate constants for the two different initial protein concentrations could be described by straight lines with reasonable confidence, with high linear regression coefficients. In Fig. 6-8 (a), the fitting lines were shown as solid line with $R^2 = 0.98$ and dash line with $R^2 = 0.96$ for the initial protein concentration of 4.16 and 1.67 g/L, respectively. In Fig. 6-8 (b), the fitting lines were shown as solid line with $R^2 = 0.90$ and dash line with $R^2 = 0.95$ for the initial protein concentration of 4.16 and 1.67 g/L, respectively. The values of $k_{po,aa}$ and $k_{agg,aa}$ indicated that the rate constants of amino acid production depended on the temperature and decomposition pathway. The amino acids were produced from the soluble proteins/polypeptides at a greater rate and amount than those produced from the aggregated proteins. This is likely to be because of the different thermal stabilities and less complicated structure, and also the greater water solubility of the polypeptides compared to the aggregated proteins with their hydrophobic pockets.

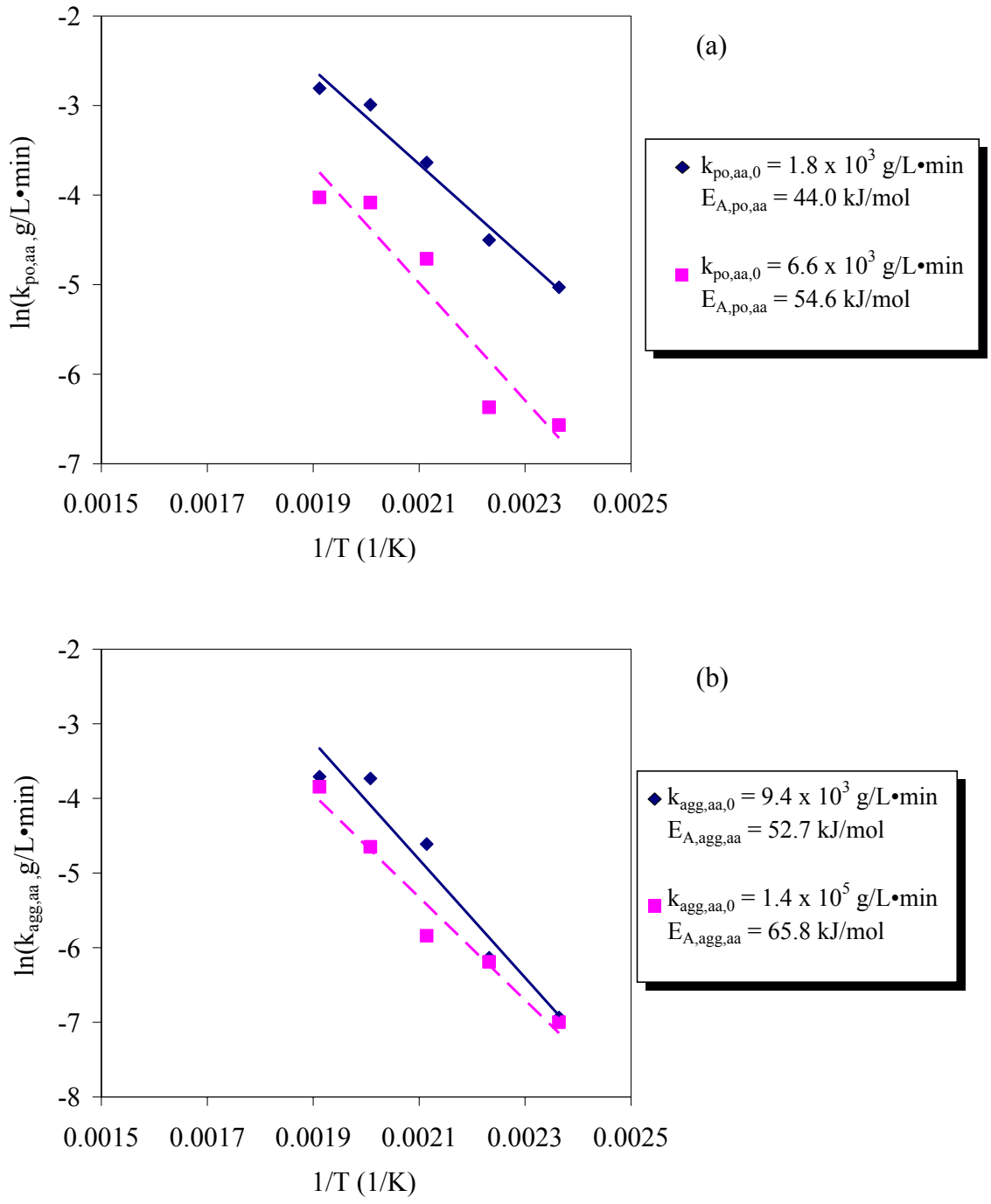


Figure 6-8. Determination of the kinetic parameters from the Arrhenius approach for the amino acid production from (a) deaggregated protein/polypeptide degradation and (b) aggregated protein decomposition at initial protein concentrations of (◆) 4.16 g/L and (■) 1.67 g/L.

CHAPTER VII

INTEGRATED PROCESS FOR γ-ORYZANOL AND PROTEIN EXTRACTION BY SUBCRITICAL AND SUPERCRITICAL FLUIDS

In this chapter, the possibility of performing the integrated process for extracting rice bran oil contented γ-oryzanol and protein consecutively was studied. This process is combined process of experiments in Chapter IV and V. The rice bran oil and γ-oryzanol were extracted firstly from rice bran by SC-CO_2, the protein solution was then extracted from remained solid by SW as experimental procedure described in Chapter III. The extracted valuable compounds were analyzed and shown in this chapter whilst the conclusion and recommendation for further developments would be reported in the next chapter.

7.1 SEM Micrographs of Raw Materials and Solid Residues

Before carrying on experiments of this part, the raw materials including rice bran and de-oiled rice bran, remained solids obtained from SC-CO_2 extraction of rice bran and de-oiled rice bran and remained solids obtained from SW extraction of rice bran and de-oiled rice bran were examined by Scanning Electron Microscope (SEM). The SEM images of 500X magnification are shown in Fig. 7-1. Additionally, the SEM images of 1000x magnification and photographs of raw materials and solid residues were shown in Appendix C. Compare to raw materials (Fig. 7-1 (a) and (b)), the surface crack of solid residue obtained from SC-CO_2 extraction (Fig. 7-1 (c) and (d)) could not be observed. However, there were the presence of pores or surface cracks on solid residue obtained from SW extraction (Fig. 7-1 (e) and (f)). It could be concluded that the SC-CO_2 does not have any effect on the surface or structure of raw materials whilst SW obviously changed the structures of raw materials since hemicellulose mostly dissolved in SW during SW extraction.

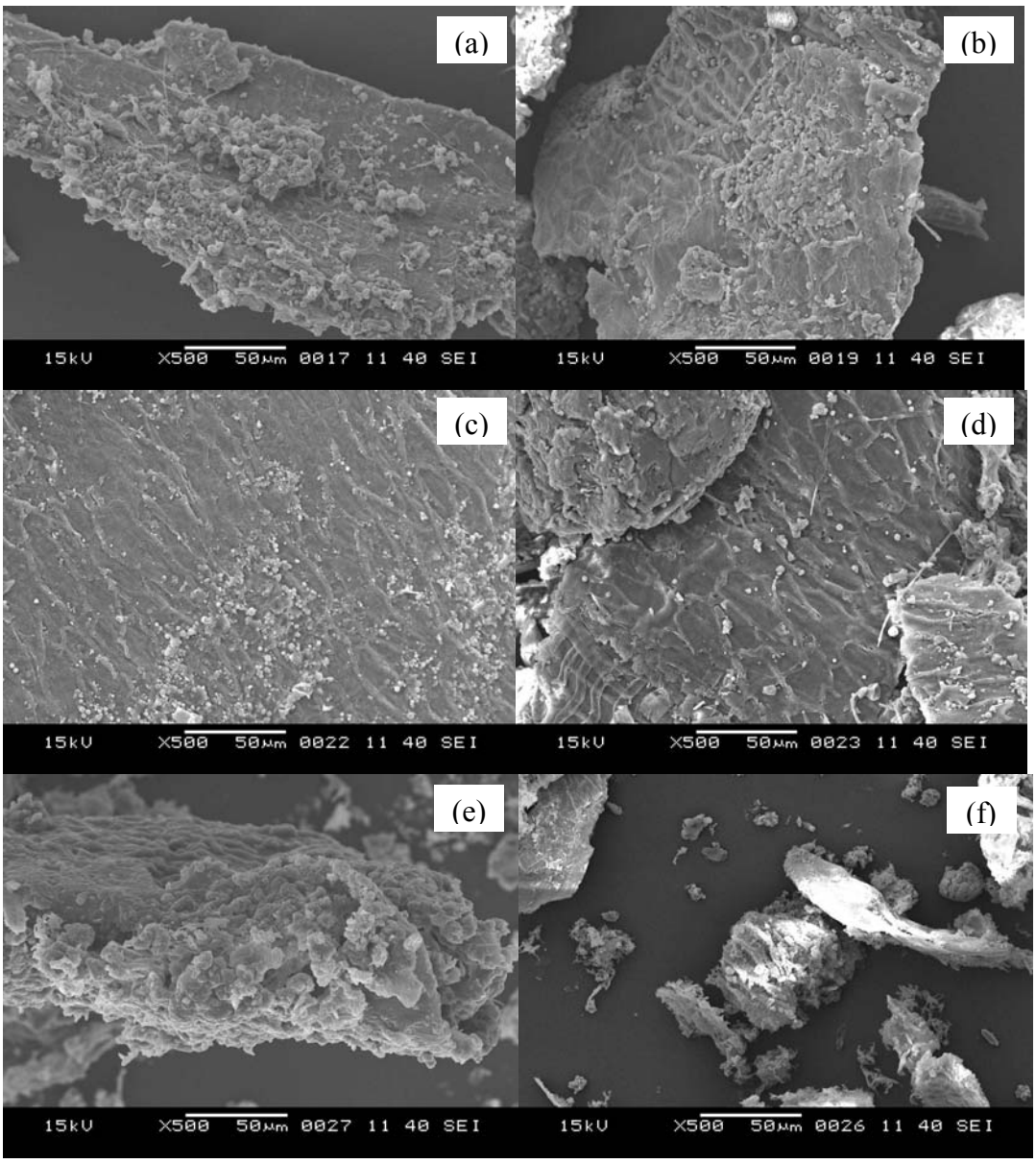


Figure 7-1. SEM images (500X magnification) of
(a) and (b) rice bran and de-oiled rice bran,
(c) and (d) rice bran residue and de-oiled rice bran residue after SC-CO_2 extraction,
(e) and (f) rice bran residue and de-oiled rice bran residue after SW extraction.

In the last decade, there were many literature reviews about hydrothermal pretreatment of lignocellulosic biomass [120, 121]. Steam pretreatment and liquid hot water pretreatment, alternative term of subcritical water or pressurized water, could remove large part of hemicellulose caused the increase in cellulose fiber reactivity. The enzymatic hydrolysis is increased after pretreatment by these pretreatment methods. Thus, the SW could be used to pretreat raw materials before using the other processes.

7.2 Integrated Process

In general use, subcritical and supercritical fluid extractions have been operated in single unit. This newer development was investigated in order to achieve multiple extractable products with sequentially extraction process using different kinds of subcritical and supercritical fluids. In this work, the integrated process for extracting rice bran with relatively high concentration γ-oryzanol and proteins from rice bran was proposed, as illustrated in Fig. 7-2 with the total mass balance. The SC-CO_2 extraction was firstly performed to extract rice bran oil and γ-oryzanol. The SW extraction was then carried out to extract proteins and other by-products. The experimental conditions of each step, the optimal conditions obtained from Chapter IV and V, are shown in Table 7-1. For rice bran oil extraction, the operating time of 60 min was chosen in order to ensure that the product was totally removed from extraction system.

It demonstrated that 11.4%wt of dry rice bran (0.31g/2.73g) could be recovered as rice bran oil at the first-step extraction whilst 68.1%wt of dry rice bran could be recovered as the protein solution at the second-step extraction. Finally, the solid which was 20.5% wt of dry rice bran (0.56g/2.73g) was remained in the reactor and collected as solid residue. The weight lost indicated in Fig. 7-2 was defined as the water that remained in the reactor after SW extraction step.

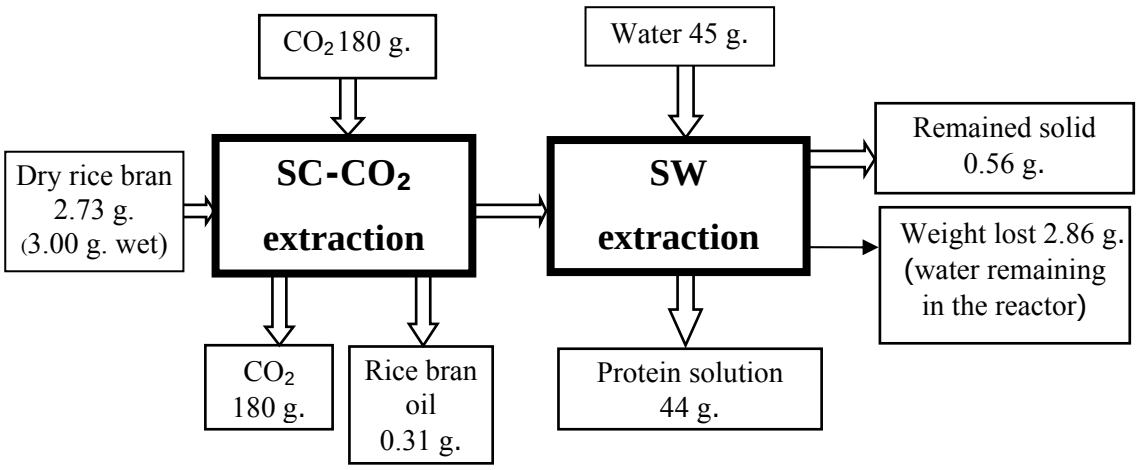


Figure 7-2. Overall process and mass balance

Table 7-1. Conditions of the integrated process

Conditions	**SC-CO_2 extraction**	**SW extraction**
Flow rate (mL/min)	3.3*	0.5
Residence time (min)	1.5	13
Temperature (^oC)	40	250
Pressure (MPa)	25 – 29	4
Operating time (min)	60	60**

* converted from 3 g/min (CO_2 density = 910 kg/m^3)

** excluding 0.5 hrs for pressure and temperature adjustment

Table 7-2. Elemental analysis of rice bran, oil and residue

Component	**Rice bran (g.)**	**Rice bran oil (g.)**	**Solid residue (g.)**
Carbon	1.02	0.23	0.18
Hydrogen	0.17	0.04	0.02
Nitrogen	0.09	0.00	0.01
Others	1.45	0.04	0.35
Total	2.73	0.31	0.56

The elemental composition of rice bran, extracted rice bran oil and the residue were analyzed by CHN analyzer and shown in Table 7-2. It could be seen that about 40% of original carbon content in rice bran (1.02 g.) was in form of rice bran oil (0.23 g.) and residue (0.18 g.). Thus, more than half of the original carbon was contained in protein solution in form of proteins/polypeptides, amino acid, polysaccharides, sugars and other degraded products since cellulose, hemicellulose and proteins can be dissolved and/or hydrolyzed by SW. Only small amount of nitrogen was detected in residue, but the nitrogen could not be detected in rice bran oil. It could be assumed that almost 90% of original nitrogen content in rice bran was contained in the protein solution in form of proteins/polypeptides and amino acids. In addition, it could be implied that there has no protein in rice bran oil and SC-CO_2 thus can not be used to extract protein from rice bran.

The composition (on dry basis) of raw materials, products and solid residue were summarized and shown in Table 7-3. Table 7-3 (a), (b), (c) and (d) reports the composition of raw rice bran, rice bran oil obtained from SC-CO_2 extraction, protein solution obtained from SW extraction and final residue, respectively. From table 7-3 (a) and (b), it could be seen that 51% of rice bran oil was extracted from rice bran with about 19,000 ppm of γ-oryzanol content. The concentration of γ-oryzanol in rice bran oil obtained by this technique was relatively high compared to normal rice bran oil which contains about 2,000 – 4,000 ppm of γ-oryzanol.

From Table 7-3 (c), almost 100% of protein could be recovered from remained solid of SC-CO_2 extraction. Other valuable chemicals such as furfural, 5-HMF, xylose and fructose were also produced during SW extraction. Even if the amount of total sugar demonstrated that almost 100% of hemicellulose was recovered, the only small amount of monosaccharide including xylose and fructose was detected. Thus, the sugars might dissolve in SW in form of di-/tri-/polysaccharide.

Table 7-3. Composition of rice bran and products (on dry basis)

a) Rice bran

Component	**%wt**	**wt (g.)**
Results from NIR spectroscopy		
Total oil	22.2	0.61
Protein	14.0	0.38
Fiber	8.0	0.22
Other	55.8	1.52
Total	100	2.73
Results from analytic method		
Cellulose	15.5	0.42
Hemicellulose	31.1	0.85
Lignin	11.5	0.31
Ash	13.2	0.36
Other	28.6	0.78
Total	100	2.73

b) Rice bran oil

Component	**%wt**	**wt (g.)**
γ-oryzanol	1.9	5.89×10^{-3}
Oil + other	98.1	0.30
Total	100 .0	0.31

c) Protein solution

Component	**%wt**	**wt (g.)**
Proteins	0.77	0.34
Total Sugars	1.68	0.74
- Glucose	BDL	BDL
- Xylose	2.40×10^{-3}	1.10×10^{-4}
- Fructose	6.90×10^{-3}	3.00×10^{-4}
Furfural	1.53×10^{-2}	6.88×10^{-3}
5-HMF	1.81×10^{-2}	8.00×10^{-3}
Other	97.52	42.91
Total	100	44

BDL – Below detection limit

d) Solid residue

Component	**%wt**	**wt (g.)**
Sugars	BDL	BDL
Cellulose	1.49	0.01
Hemicellulose	4.22	0.02
Lignin	28.61	0.16
Ash	61.06	0.34
Other	4.62	0.03
Total	100	0.56

BDL – Below detection limit

For protein solution, it can be dried and then mixed with drink [7]. Watchararuji and coworkers mixed the dried rice bran hydrolysate with milk and coffee. The authors reported that testers preferred the milk with added hydrolysis product more than the milk control. However, there was different result for coffee test. It was probably due to age or gender of testers. Anyway, it proved that SW product can be potentially applied for human food additives. The solution might be mixed as an ingredient for bakery. In addition, the dried protein might be obtained by freeze drying and then mixed up with bakery or drink.

The final solid residue was analyzed to determine its composition. The result is shown in Table 7-3 (d). After hydrolyzing by high concentrated sulfuric acid and analyzing the neutralized liquid solution by HPLC, the result illustrated that no sugar could be detected by this method. Cellulose and hemicellulose were also analyzed and found that there are only trace amount of them remained in solid residue. Protein was also not found in the residue. In addition, after ashing, the remaining amount of solid was almost equal to its original ash content in raw rice bran. However, excluding ash, the main component of solid residue was lignin which can be used as an additive for elastomer and binder for paper industry. Therefore, there have some interests in separation and purification of lignin.

The CO_2 can dissociate and form carbonic acid under SW atmosphere by this eq.[66]:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

The addition of CO_2 at first-step extraction was thus expected to realize more hydrolysis efficient and affect more hydrolyzed products of SW extraction. However, the amount of total protein and total sugar was equal to the result that obtained from Chapter V. Moreover, even the trace amount of reducing sugars including xylose and fructose was detected by HPLC, the glucose could not be detected by this method. Thus, the amount of CO_2 remained from first-step extraction might not favor the hydrolysis reaction in the second-step extraction.

CHAPTER VIII

CONCLUSIONS AND RECOMMANDATIONS

8.1 Conclusions

1. Rice bran oil and γ-oryzanol extraction by supercritical carbon dioxide

This work demonstrated that rice bran oil with high concentrated γ-oryzanol was successfully extracted from rice bran and de-oiled rice bran by using SC-CO_2. Based on ANOVA, all operating factors had significant effect on the oil yield and γ-oryzanol yield obtained from rice bran extraction. The pressure and time had positive effect whilst temperature had negative effect on the yields. The suitable condition was found to be 40 ^{o}C and 30 MPa for 60 min which gave almost 100% oil recovery containing about 1.7%wt of γ-oryzanol. The SC-CO_2 thus has high potential to extract the oil containing rich γ-oryzanol from rice bran. For de-oiled rice bran, the oil yield was reached the maximum value at the same condition. However, the γ-oryzanol contained in trace amount of product was not high enough to analyze.

The optimum condition was then determined by varying the operating time. The results indicated that the optimum operating time was 20 min of operation at 40 ^{o}C and 30 MPa.

2. Protein and sugar extraction by SW

Based on the statistical analysis, the operating time and interaction between operating temperature and time had a significant effect on the protein yield. The yield was reached the maximum value at 250 ^{o}C and 7 MPa for 60 min which gave 100% protein recovery for both rice bran extraction and de-oiled rice bran extraction. The three main factors of temperature, time and pressure all had a significant effect on the sugar yield. The sugar yield was reached the maximal level at 210 ^{o}C, 50 min and 7 MPa for rice bran extraction and at 210 ^{o}C, 7 MPa and 60 min for de-oiled rice bran extraction.

With respect to the combination of two response surfaces, for sugar and protein yields of each raw material, the result provided the suitable conditions for rice bran extraction and de-oiled rice bran extraction which gave a high protein and sugar yield. Thus SW extraction has a high potential for use in the recovery of proteins and sugars from rice bran and de-oiled rice bran.

3. Kinetics of rice bran protein hydrolysis in SW

The proposed mechanism for the changes in rice bran proteins during their hydrolysis under a SW atmosphere includes three steps; (i) protein aggregation, (ii) protein disaggregation by hydrolysis to polypeptides and (iii) amino acid production, which follow zero, second and zero order rate kinetics, respectively. The aggregation and disaggregation kinetics of proteins in SW changed with increasing temperature and initial protein concentrations, whilst the amino acid production from polypeptides and aggregated protein hydrolysis changed with the temperature and different production pathways. The protein/polypeptide concentration reached its maximum value, after aggregation, at 20 – 25 min and 523 K, whilst the amino acid production increased with increasing temperature and heating time without an observable maximum value (within the 60 minutes assay period).

4. Integrated process for γ-oryzanol and protein extraction by subcritical and supercritical fluids

The subcritical and supercritical fluids extraction could be operated sequentially in order to extract several products in one process. The extraction process using SC-CO_2 followed by SW was approved in this work. The results showed that SC-CO_2 could extract rice bran oil with relatively high γ-oryzanol concentration, about 19,000 ppm, whilst SW could be used to recover protein, almost 100% recovery, and other by-products. The SC-CO_2 extraction or trace amount of remained CO_2 did not affect the change in structure of raw material or composition of products obtained from SW extraction.

The rice bran was mostly hydrolyzed during SW extraction and left only 20%wt as solid residue. Since rice bran has low level of cellulose and its residue obtained from this integrated process has no remained sugars, the residue was not suitable for using in some further processes such as bioethanol production. However,

there have some interests in separation and purification of lignin and other possible components in this residue as valuable products.

8.2 Recommendations

From the results of this work which studied the valuable products extraction from rice bran and de-oiled rice bran using subcritical and supercritical fluids, it could be seen that it has possibility to combine several fluids in different subcritical and supercritical regimes together for multi-product extraction. Each type of selected subcritical and supercritical fluids could be used to extract desired product with high % recovery. However, considered an economic benefit of this production process and compared SW extraction of proteins to SC-CO_2 extraction of γ-oryzanol, the SW extraction of protein had less priority since there has no report about the essential health benefit including pharmaceutical properties of the extracted protein obtained from this technique. This process might thus not suitable for commercial scale. Anyway, there are some possibilities to apply this integrated process for achieving more valuable products which can be use in other applications. The following are recommendations for this purpose and further research.

1. Protein extraction

Since rice bran has high value proteins including lipase and α-1 antitrypsin, it is high recommended to extract these kinds of protein and purify them for pharmaceutical use. In case of using SW as a solvent, proteins can be extracted using lower temperature. The kinds of extracted proteins are analyzed and then the optimum condition must be identified.

2. Fractionation for optimization of γ-oryzanol concentration

The concentration of γ-oryzanol in rice bran oil is the one of primary reason for its price or application. To achieve higher product concentration, the fractionation using SC-CO_2 can be an alternative technique for this purpose. The amount of extracted γ-oryzanol and rice bran oil were increased with increasing pressure although the higher γ-oryzanol concentration could be obtained at lower operating pressure. Thus, the suggested process would be the rice bran oil with concentrated γ-oryzanol extraction at lower pressure with/without following by an extraction using higher pressure to obtained remained rice bran oil. The SC-CO_2

extraction with added other co-solvent such as ethanol might be performed in order to increase a solvent's polarity which favors an increasing in antioxidant solubility in SC-CO_2.

3. Amino acid production by SW extraction

The results of such kinetic studies are important for aiding an understanding of protein extraction from rice bran during SW extraction and hydrolysis. Even protein could be extracted from rice bran as shown in Chapter V, the SW condition should be optimized since the amount of proteins/polypeptides in the liquid solution could not be completely recovered after aggregation due to its partial decomposition to amino acids. The use of an optimized SW condition, with longer reaction time (over 20 – 25 min), should be an effective way to obtain amino acids recovery during the rice bran extraction process using SW, with respect to the protein extraction as a second process priority. The further researches should focus on amino acids production using SW and purification of the mixed amino acids for food grade.

4. Application to achieve other valuable products or to other lignocellulosic biomass

Since this work proposed that subcritical and supercritical fluids extraction could operate sequentially, it has possibility for achieving other products by this technique. The SC-CO_2 extraction should be performed firstly since it can be operated at milder condition. The SW extraction is then performed to recover other product. For example, after choosing a suitable biomass, the raw material is firstly de-oiled or extracted to recover antioxidants and vitamins using SC-CO_2. The optimum condition maybe different from that proposed in this work. Then, the remained solid is hydrolyzed under suitable SW condition to depolymerize carbohydrates and polysaccharides such as cellulose and hemicellulose. The monomers and smaller oligomers can be recovered and collected as a hydrolyzate in this step. The hydrolyzate is further digested to glucose by SW at another condition or other methods and then converted to bioethanol by enzymatic technique.

For choosing the suitable lignocellulosic biomass, its composition such as vitamin, carbohydrate, polysaccharides and other valuable component should be considered.

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APPENDICES

APPENDIX A

ANALYSIS OF TOTAL PROTEIN, SUGAR AND AMINO ACIDS

1. Analysis of total protein by Lowry assay [108]

Solutions

A. Standard solution: using Bovine Serum Albumin (BSA)

B. 2% sodium carbonate in 0.1N sodium hydroxide (2 g. sodium carbonate in 100 mL of 0.1N sodium hydroxide)

C. 2% sodium potassium tartrate (0.5 g. of sodium potassium tartrate in 100 mL of water)

D. 1% copper sulfate pentahydrate in water (0.5 g. copper sulfate pentahydrate in 100 mL of water)

E. Solution B:C:D = 100:1:1 (prepare immediately, within 1 hr before use)

F. 1N Folin & Ciocalteu's phenol reagent.

G. Sample: unknown protein solution

Procedure

1. Pipette 1 mL of standard A. or sample G. and drop into 16x150 mm tube.

2. Pipette 5 mL of solution E. into tube and incubate for 10 min at room temperature and then add 0.5 mL of solution F. reagent into tube and incubate for 30 min at room temperature.

3. Detect absorbance values at 750 nm of wavelength.

Calibration curve was derived plotting known standard concentration versus absorbance and shown in Fig. A-1.

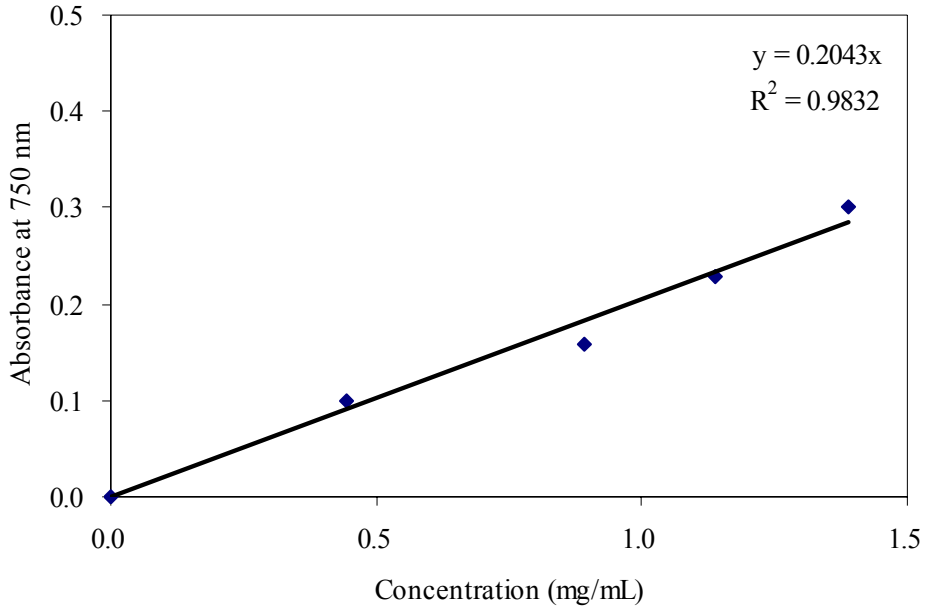

Figure A-1. Calibration curve of BSA standard.

2. Analysis of total sugar by modified-phenol sulfuric method [109]

Solutions

A. Standard solution: using D-glucose

B: 5% phenol in distilled water

C: Concentrated sulfuric acid

D. Sample: unknown sugar solution

Procedure

1. Pipette 0.5 mL of solution A. or sample D. into tube 16x150 mm tube.

2. Pipette 0.5 mL of solution B. into tube and then add 2.5 mL of solution C., mixed well, leave to cool for 20 min to room temperature.

3. Detect absorbance values at 490 nm of wavelength.

Calibration curve was derived plotting known standard concentration versus absorbance and shown in Fig. A-2.

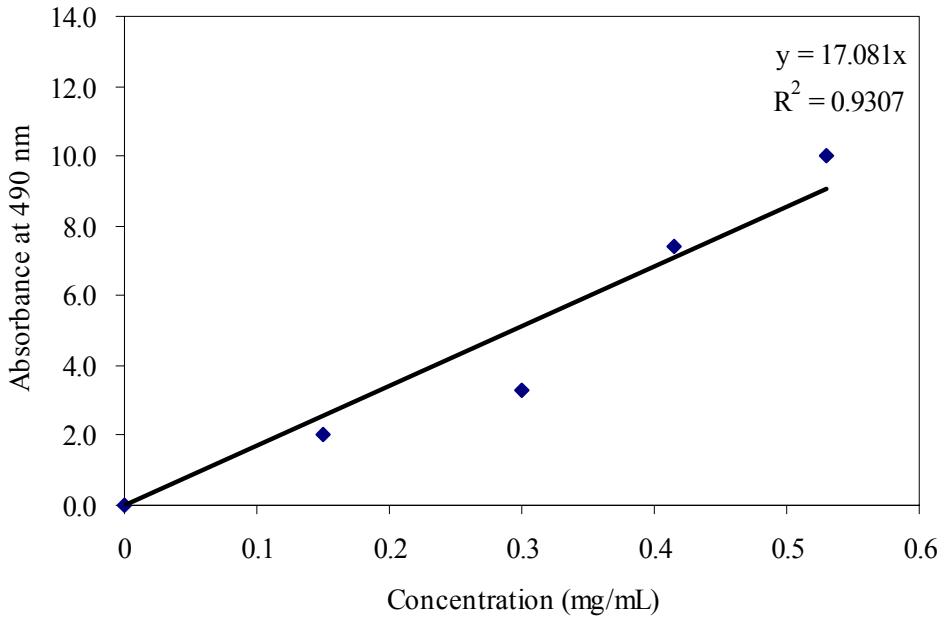

Figure A-2. Calibration curve of glucose standard.

3. Analysis of total amino acid by Ninhydrin method [110]

Solutions

A. 4 M Na-Acetate buffer

- Add 54.4 g. of sodium acetate trihydrate into 60 mL of distilled water.
- Add about 10 mL of glacial acetic acid until the pH is 5.2 (work carefully in hood) and make up to 100 mL with water.

B. Ninhydrin colour reagent (work carefully in hood and prepare immediately before use)

- Add 0.3 g. of hydrindantin and 2 g. of ninhydrin into 75 mL of dimethyl sulfoxide (DMSO) and then add about 25 mL of solution A.

C. Stabilizing solution (50% ethanol)

- Dilute 50 mL of ethanol to 100 mL with water

D. Amino standard: using glycine

Procedure

1. Pipette 2 mL of sample or standard D. into 16x150 mm tube.
2. Add 1.5 mL of solution B. and incubate at 80 ^{o}C for 30 minutes.

3. Cool and then add 2 mL of solution C.

4. Detect absorbance values at 570 nm of wavelength.

Calibration curve was derived plotting known standard concentration versus absorbance and shown in Fig. A-3.

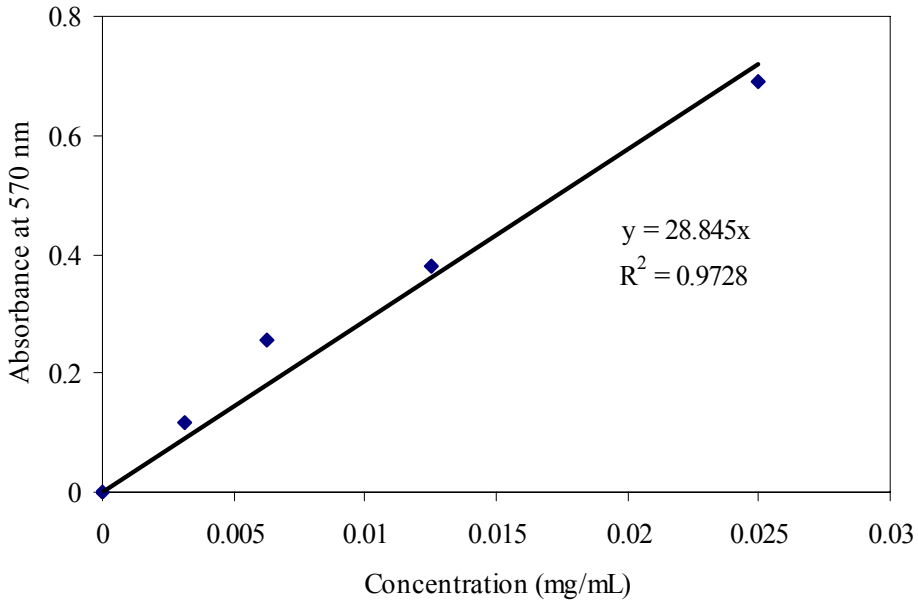

Figure A-3. Calibration curve of glycine standard.

APPENDIX B

THE NORMAL PROBABILITY PLOTS OF RESIDUES AND PLOTS OF RESIDUES VERSUS PREDICTED VALUES FOR SC-CO_2 EXTRACTION AND SW EXTRACTION

As indicated in Chapter V, the normal probability plots of residues and relationship between studentized residues and run number for rice bran oil extraction and γ-oryzanol extraction from rice bran and de-oiled rice bran were shown in Fig. B-1 and B-2, respectively, whilst these plots for protein extraction and sugar extraction were shown in Fig. B-3 and B-4, respectively. All data were obtained from Design Expert ® 6.0 software. These figures (Fig. B-1 – B-4) can be used for checking the model adequacy. The normal probability plot of residues those were presented in Fig. B-1(a,c), B-2(a,c), B-3(a,c) and B-4(a,c) indicated the normality and randomness of the residues. It could be found that the residuals generally follow a straight line which can be implied that the errors are distributed normally.

Moreover, the unusual structure and pattern could not be observed from the relationship between studentized residues and run number those were presented in Fig. B-1(b,d), B-2(b,d), B-3(b,d) and B-4(b,d). It can be implied that the proposed models are adequate and not have to suppose the violation of independence or constant variance assumption.

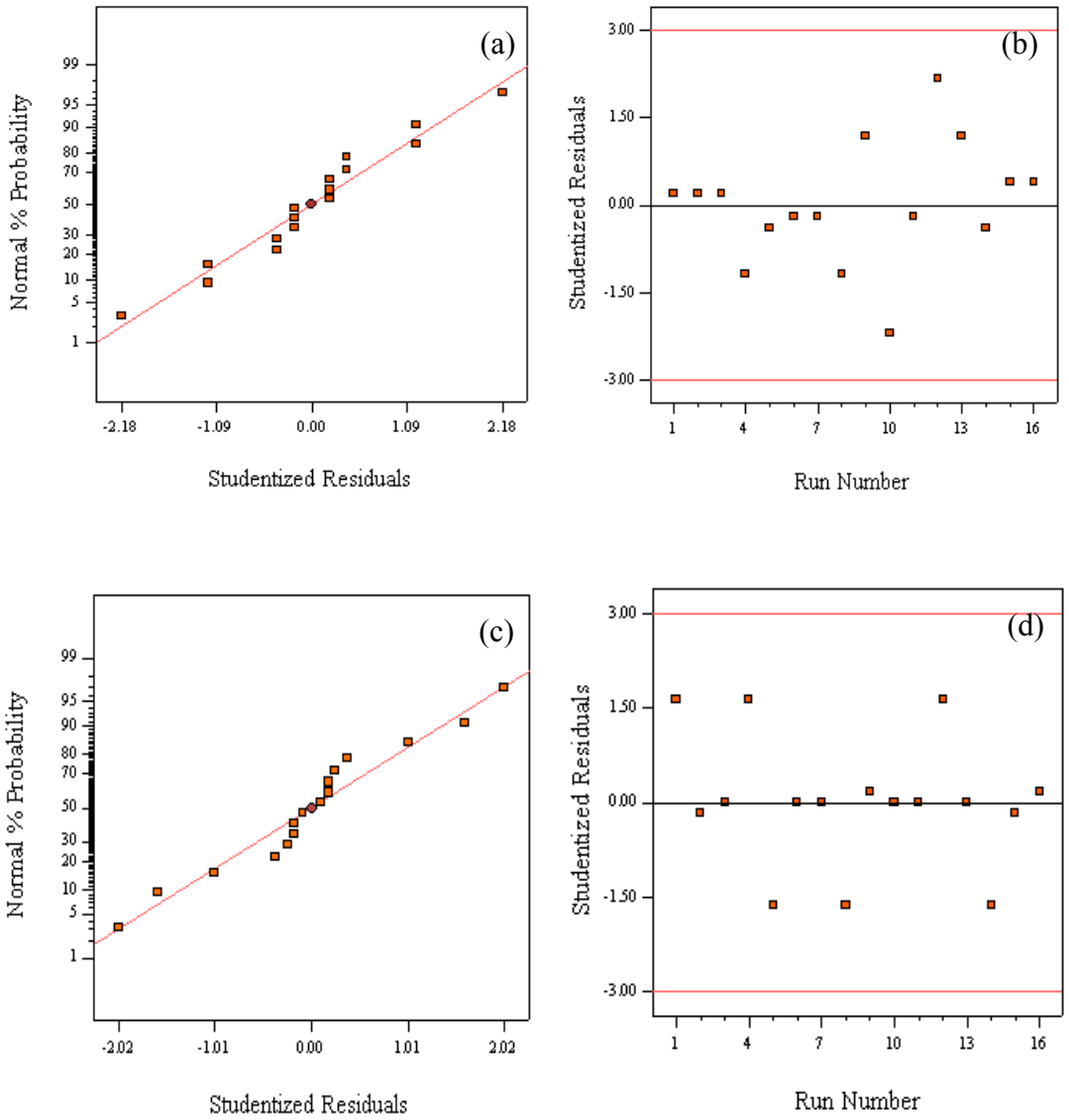

Figure B-1. The Normal probability plots of residues and the relationship between residues and run number for oil extraction from (a,b) rice bran and (c,d) de-oiled rice bran.

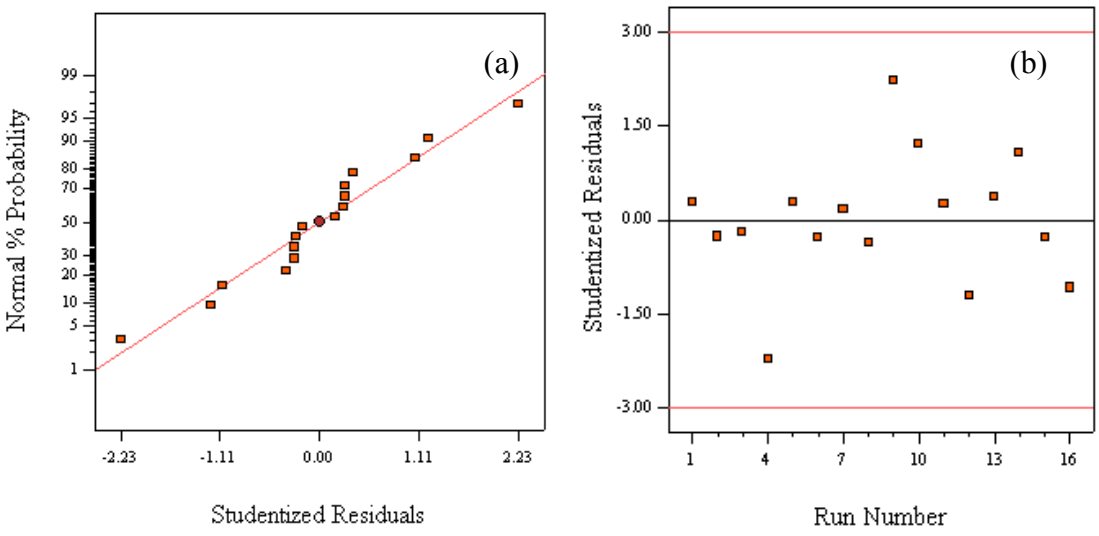

Figure B-2. The Normal probability plots of residues and the relationship between residues and run number for γ-oryzanol extraction from rice bran.

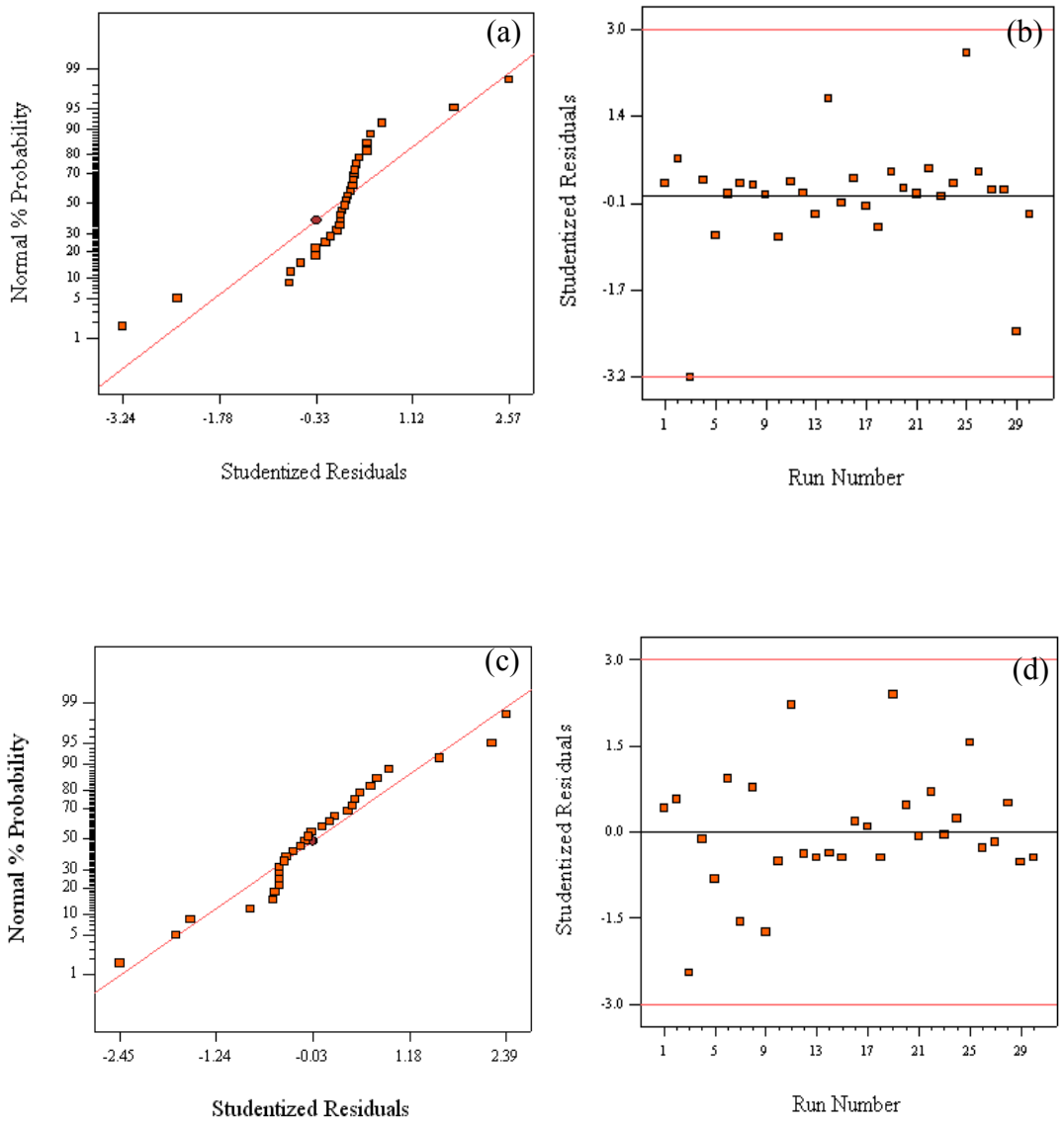

Figure B-3. The Normal probability plots of residues and the relationship between residues and run number for protein extraction from (a,b) rice bran and (c,d) de-oiled rice bran.

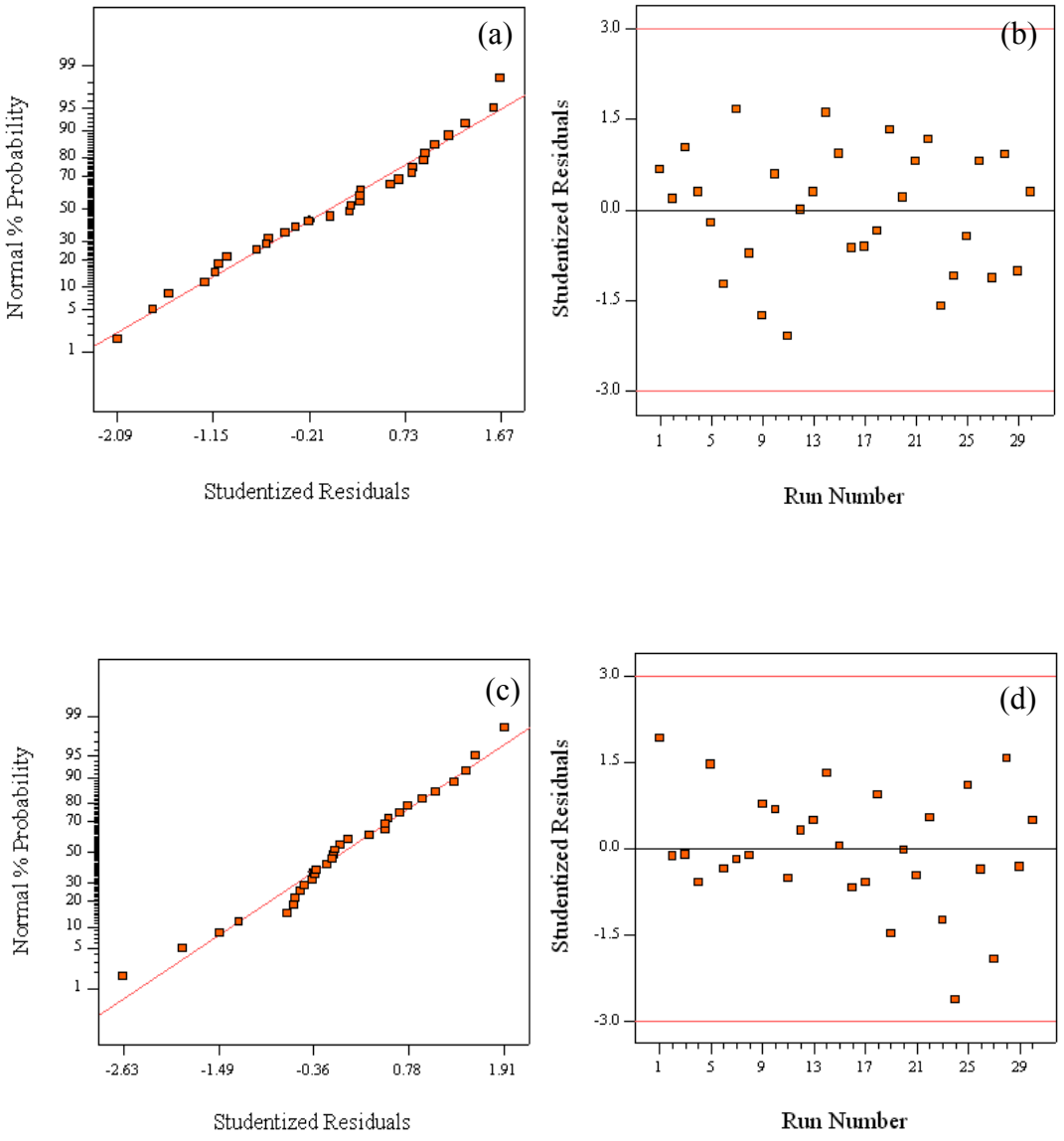

Figure B-4. The Normal probability plots of residues and the relationship between residues and run number for sugar extraction from (a,b) rice bran and (c,d) de-oiled rice bran.

APPENDIX C

PHOTOGRAPHS AND SEM IMAGES OF RAW MATERIALS AND SOLID RESIDUES

As indicated in Chapter VII, additional photographs and SEM images of 1000X of raw materials and solid residues are shown in Appendix C. Fig. C-1 shows photographs of rice bran and de-oiled rice bran and their residues after extraction using SC-CO_2 and SW whilst Fig. C-2 shows their SEM images.

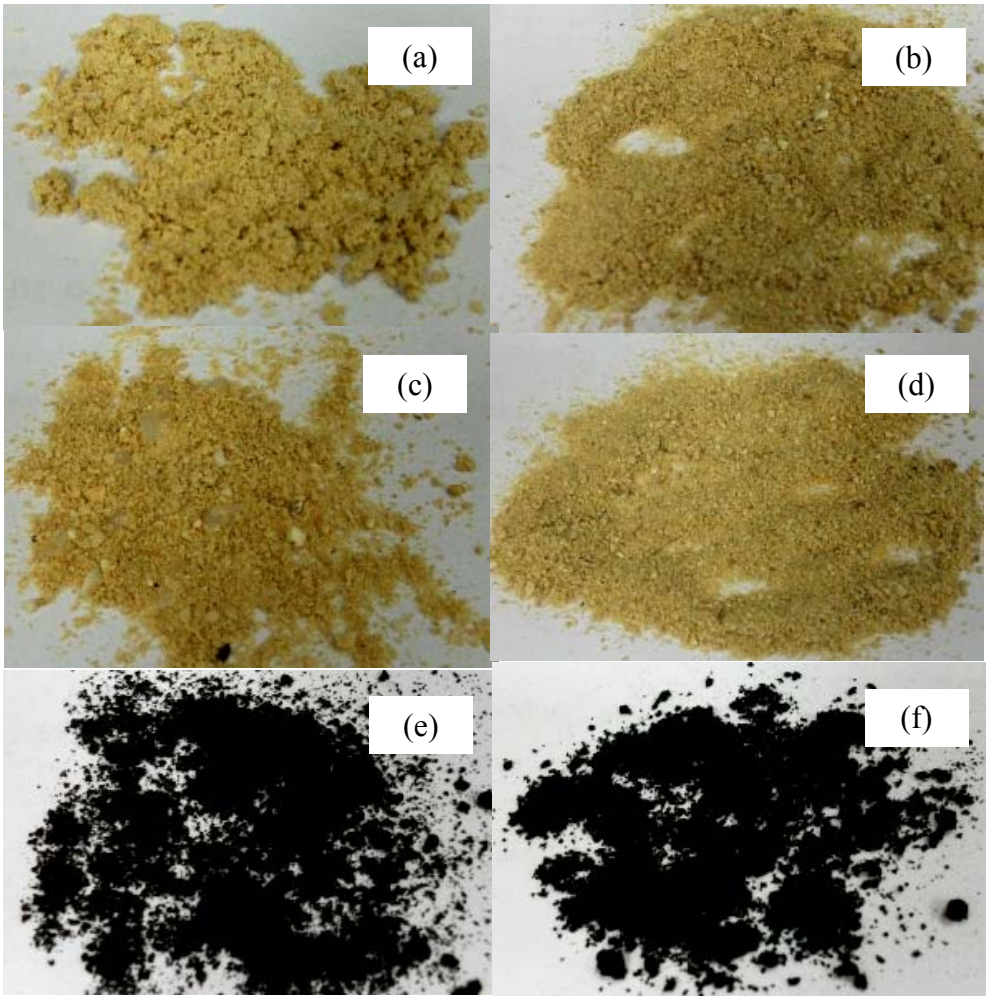

Figure C-1. Photographs of (a) and (b) rice bran and de-oiled rice bran, (c) and (d) rice bran residue and de-oiled rice bran residue after SC-CO_2 extraction, (e) and (f) rice bran residue and de-oiled rice bran residue after SW extraction.

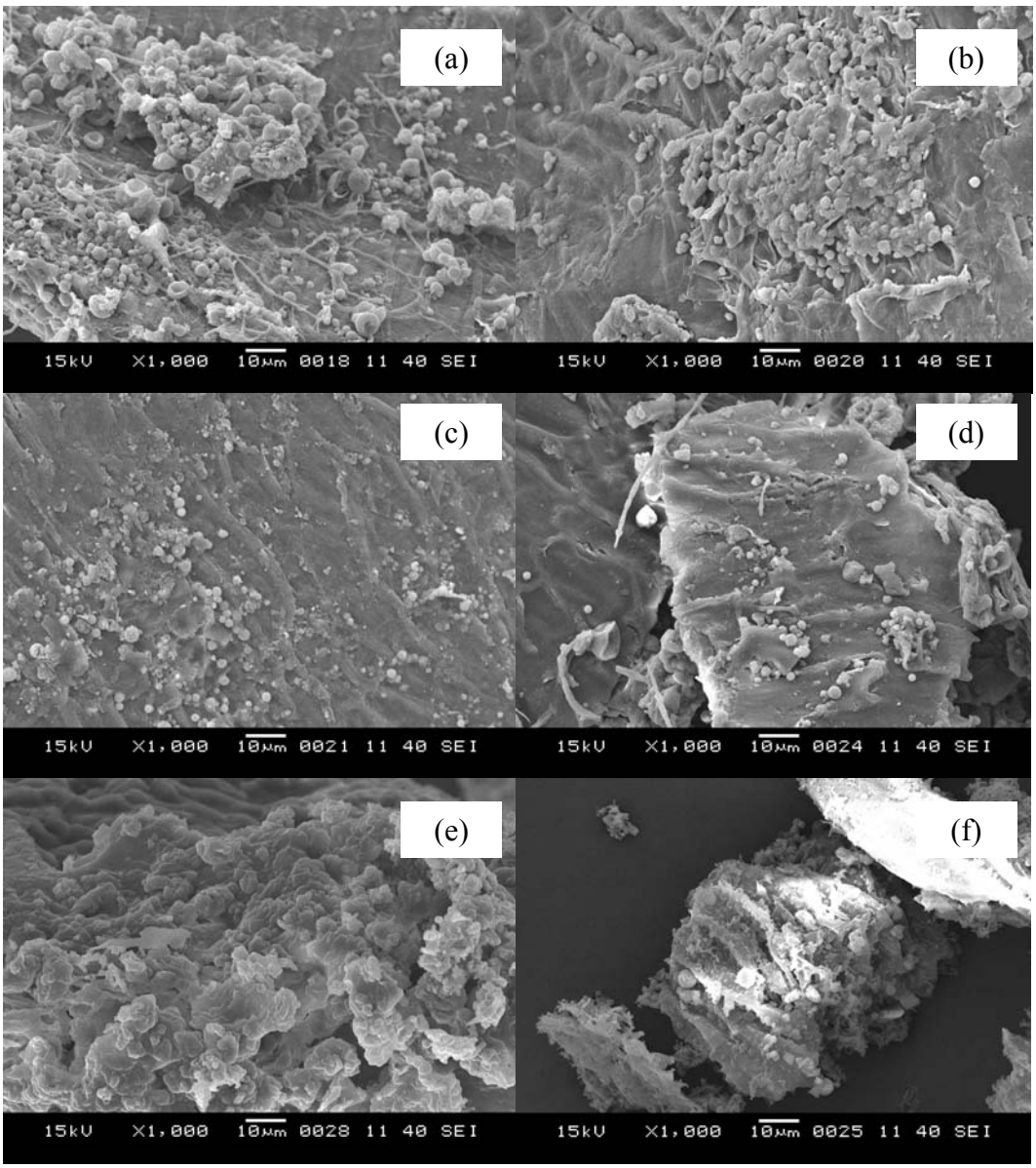

Figure C-2. SEM images (1000X magnification) of
(a) and (b) rice bran and de-oiled rice bran,
(c) and (d) rice bran residue and de-oiled rice bran residue after SC-CO_2 extraction,
(e) and (f) rice bran residue and de-oiled rice bran residue after SW extraction.

BIOGRAPHY

Miss Sasithorn Sunphorka was born on November 4th, 1982 at Bangkok, Thailand. She received her B.Sc. degree and M.Sc. degree in chemical engineering from Chulalongkorn University. Sasithorn joined the department of chemical technology, Chulalongkorn University, as a doctoral student in 2008. She has received the Royal Golden Jubilee Scholarship from Thailand Research Fund (2008 – 2010) and Teaching Assistantship Scholarship from Graduate School, Chulalongkorn University (2011) for her Ph.D. Study. Sasithorn also served as a teaching assistant for undergraduate course "Material and Energy Balances", Thermodynamics", "Operating Laboratory Unit I" and "Heat and Mass Transfer".

During graduate study, Sasithorn spent 6 months (2010) for doing research in Oshima & Otomo Laboratory at Department of Environment Systems in The University of Tokyo, Japan. Her first paper entitled "Protein and sugar extraction from rice bran and de-oiled rice bran using subcritical water in a semi-continuous reactor: Optimization by response surface methodology" has been accepted for publication in "International Journal of Food Engineering" and her second paper entitled "Kinetic Studies on Rice Bran Protein Hydrolysis in Subcritical Water" has been accepted for publication in "The Journal of Supercritical Fluids". She also presented her works at 1 conference in Singapore and 2 conferences in Thailand.