UTILIZATION OF SOAPSTOCK FROM RICE BRAN OIL PRODUCTION

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ผู้วิจัย : ขนิษฐา หวังดี

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คำสำคัญ : แกมมาโอรีซานอล ไขสบู่ สภาวะที่เหมาะสม การสกัด เอนแคปซูล

งานวิจัยนี้มีวัตถุประสงค์ เพื่อศึกษาการใช้ประโยชน์จากไขสบู่ที่ได้จากกระบวนการผลิตน้ำมันรำข้าว ซึ่งไขสบู่เป็นผลพลอยได้จากกระบวนการทำให้น้ำมันบริสุทธิ์ มีปริมาณแกมมาโอรีซานอลร้อยละ 9.91 โดยน้ำหนักแห้ง โดยนำไขสบู่มาทำการสกัดแยกแกมมาโอรีซานอลด้วยวิธีการซอกห์เลต (soxhlet extraction) วิธีการใช้คลื่นอัลตราโซนิก (ultrasonic assisted extraction) และวิธีการร่วมกันระหว่างการใช้คลื่นอัลตราโซนิกและวิธีซอกห์เลต ศึกษาสภาวะที่เหมาะสมด้วยเทคนิค Response surface methodology (RSM) ออกแบบการทดลองด้วยวิธี Box-Benhken experimental design (BBD) พบว่าสภาวะที่เหมาะสมในการสกัดด้วยวิธีซอกห์เลต คือ อัตราส่วนไขสบู่ต่อตัวทำละลายเอทิลอะซีเตท 1:12 อุณหภูมิการสกัด 70.1 องศาเซลเซียส และสกัดนาน 7.26 ชั่วโมง สามารถสกัดแกมมาโอรีซานอลได้ร้อยละ 8.74 และสภาวะที่เหมาะสมสำหรับวิธีการใช้คลื่นอัลตราโซนิก คือ ความถี่คลื่นอัลตราโซนิก 12 กิโลเฮิรตซ์ อุณหภูมิ 74.1 องศาเซลเซียส และสกัดนาน 60 นาที สามารถสกัดแกมมาโอรีซานอลได้ร้อยละ 5.50 และศึกษาการสกัดแกมมาโอรีซานอลด้วยการใช้คลื่นอัลตราโซนิกร่วมกับวิธีซอกห์เลท โดยทำการสกัดแกมมาโอรีซานอลจากไขสบู่ด้วยสภาวะที่เหมาะสมจากวิธีการใช้คลื่นอัลตราโซนิก จากนั้นนำกากไขสบู่ที่เหลือไปสกัดด้วยสภาวะที่เหมาะสมของวิธีซอกห์เลทต่อ พบว่า สามารถสกัดแกมมาโอรีซานอลจากได้ร้อยละ 9.05 อย่างไรก็ตามสารที่สกัดได้มีความบริสุทธิ์ของแกมมาโอรีซานอลต่ำ จำเป็นต้องนำไปผ่านกระบวนการทำให้บริสุทธิ์ด้วยการตกผลึก 2 ขั้นตอน พบว่า การใช้สารละลายผสมระหว่าง เอทิลอะซีเตทและเมทานอลในอัตราส่วน 3:7 เก็บที่อุณหภูมิ -22 องศาเซลเซียส นาน 24 ชั่วโมง จากนั้นแยกส่วนของแข็งออกและเก็บสารละลายส่วนใสที่อุณหภูมิ 5 องศาเซลเซียส นาน 48 ชั่วโมงสามารถเกิดผลึกแกมมาโอรีซานอลได้ความบริสุทธิ์ประมาณร้อยละ 87 แต่อย่างไรก็ตามแกมมาโอรีซานอลเป็นสารที่มีคุณสมบัติละลายในตัวทำละลายที่ไม่มีขั้ว ดังนั้นการนำไปประยุกต์ใช้ในอาหารจึงเป็นไปอย่างจำกัด จึงได้ทำการศึกษาการเอนแคปซูลแกมมาโอรีซานอลที่สกัดได้ โดยการเตรียมอิมัลชันชนิดน้ำในน้ำมัน (Oil-in-water) ด้วยอัตราส่วนน้ำต่อน้ำมัน 80:20 ส่วนของน้ำมัน ประกอบด้วยแกมมาโอรีซานอลที่

สกัดได้ร้อยละ 2 โดยน้ำหนัก และส่วนของน้ำประกอบด้วยเวย์โปรตีนชนิดเข้มข้น (Whey protein concentrate) ร้อยละ 1 โดยน้ำหนัก ร่วมกับมอลโทเดกซ์ทริน ร้อยละ 10 โดยน้ำหนัก และนำไปทำแห้งด้วยเครื่องทำแห้งแบบพ่นฝอย พบว่า สามารถผลิตสารเอนแคปซูลเลทแกมมาโอรีซานอลที่มีลักษณะเป็นผงสีขาวนวล เมื่อส่องด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด พบว่า อนุภาคมีลักษณะเป็นทรงกลมผิวเรียบ ความชื้นต่ำกว่าร้อยละ 3 มีประสิทธิภาพการห่อหุ้มมากกว่าร้อยละ 95 และค่าการละลายสูงกว่าร้อยละ 75 เมื่อทำการศึกษาอายุการเก็บรักษา พบว่า สารเอนแคปซูลเลทแกมมาโอรีซานอล มีอายุการเก็บรักษา 277 วัน ที่อุณหภูมิ 25 องศาเซลเซียส และ 613 วัน ที่อุณหภูมิ 4 องศาเซลเซียส นอกจากนี้ได้ทำการศึกษาการนำสารเอนแคปซูลเลทแกมมาโอรีซานอลไปประยุกต์ใช้ในน้ำสลัดชนิดครีม พบว่า การเติมสารเอนแคปซูลเลทแกมมาโอรีซานอลไม่มีผลต่อการยอมรับของผู้บริโภค และยังตรวจสอบพบแกมมาโอรีซานอลและกิจกรรมการต้านอนุมูลอิสระของตัวอย่างน้ำสลัดที่เติมสารเอนแคปซูลเลทแกมมาโอรีซานอล

ABSTRACT

TITLE : UTILIZATION OF SOAPSTOCK FROM RICE BRAN OIL PRODUCTION
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DEGREE : DOCTOR OF PHILOSOPHY
MAJOR : FOOD TECHNOLOGY
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KEYWORDS : RICE BRAN OIL SOAPSTOCK, γ-ORYZANOL, EXTRACTION, ENCAPSULATION, ACCERELATED SHELF-LIFE TEST

This research aimed to investigate the optimization of extraction and purification of γ-oryzanol in which rice bran oil soapstock (RBOS) is a product from the chemical refining process of rice bran oil production. The RBOS was dry and contained 9.91% of γ-oryzanol. RBOS was extracted from the γ-oryzanol through soxhlet extraction, ultrasonic assisted extraction, and the integration between the untrasonic assisted method and soxhletex method. Moreover, the RBOS was investigated for optimization by Response Surface Methodology (RSM) and through the Box-Benhken Experimental Design (BBD).

The results indicated that optimization of the soxhlet extraction was identified as 1:2 solid to solvent ratio, 70.1°C extraction temperature and 7.26 h extraction for extracted γ-oryzanol yield of 8.74% dry basis. For ultrasonic-assisted extraction, the process variables were ultrasonic frequency, extraction temperature and extraction period. The optimal ultrasonic assisted extraction conditions were identified as 12 kHz ultrasonic frequency, 74.1 °C extraction temperature and 60 min extraction for maximum γ-oryzanol yield of 5.15% dry basis. Furthermore, the optimal conditions of both soxhlet extraction and ultrasonic assisted extraction were chosen to extract γ-oryzanol which gave the highest γ-oryzanol yield of 9.05 % dry basis. However, purification of γ-oryzanol was low. So, the purification of γ-oryzanol was achieved by a two-step crystallization process to recover γ-oryzanol from enriched extracts. Optimal conditions of a two-step crystallization process were found that the

γ-oryzanol was dissolved in the 3:7 of ethyl acetate: methanol ratio at -22 °C for 24 h for the first crystallization step. Then in the second crystallization step, the supernatant obtained through the first step was kept at 5 °C for 48 h. Under optimal conditions of a two-step crystallization, purity and recovery of γ-oryzanol from all extraction methods were 87 %. However, γ-oryzanol utilization was limited because of γ-oryzanol's dissolving qualities. Then, the extracted γ-oryzanol was investigated through oil-in-water emulsion 80:20 (aqueous phase 80%wt and rice bran oil 20%wt). Rice bran oil consisted of 2%wt γ-oryzanol and aqueous phase consisted of 1.0%wt Whey Protein Concentrate and 10%wt Maltodextrin (DE10). The extracted γ-oryzanol (Encapsulated γ-oryzanol extract power: EOEP) was produced by spray drying. The results indicated that the EOEP were a smooth spheroid powder, had low moisture content that was lower than 3%, the encapsulated efficiency and yields were more than 95%, and the dissolving quality was higher than 75%. For the maintenance, the encapsulated extract γ-oryzanol powder could be kept for 277 days at 25 °C and 613 days at 4 °C. Moreover, the γ-oryzanol powder used in salad dressings was also studied. The results indicated that the encapsulated γ-oryzanol extract powder can be used as a food ingredient in salad dressings, which had a good effect on γ-oryzanol content and antioxidant activity.

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CHAPTER 1
INTRODUCTION

1.1 Motivation

Rice (*Oryza sativa* L.) is the second largest cereal grain produced in the world, after wheat. The United States Department of Agriculture has been estimated that the world rice has produced approximately 741.3 million metric tons in 2016/2017. Most of the rice production is cultivated in the countries in Asia. Thailand produces 30 million metric tons of paddy or 19.8 million tons of milled rice, divided between domestic consumption and exports (Food and Agriculture Organization, 2017). Because, Thai people usually prefer to eat white rice, the rice grain accordingly is subjected to be milled or polished to remove its bran and germ. Rice bran is a byproduct of the rice milling process, and it constitutes approximately 8-11% of the total weight of rough rice. The rice bran is one of the valuable by-products of the rice processing industry because it is a good health-promoting source of oil, protein, dietary fiber and valuable substances such as vitamin, phytosterols and γ-oryzanol. Almost 70% of rice bran used as animal feed, while only about 20% of rice bran is extracted into rice bran oil (Patel, 2005).

The oil extraction of human consumption is one alternative to use the rice bran. There are two methods can be extracted rice bran oil by pressing or solvent extraction. Pressing extraction is not commonly used because the large quantity of oil (20-30%) still remains in the rice bran after pressing (Orthoefer and Eastmen, 2004). Solvent extraction is more commonly used because the process leaves less than 1% of the residual oil content. After extraction solvent is removed to obtain crude rice bran oil and is further purified. After oil extraction, crude rice bran oil (CRBO) contains 3-20% free fatty acid (FFA), 4.8% wax, 5 to 8% unsponification pigments (Narayan, 2006). There are two methods for purification of rice bran oil consisting of chemical refining and physical refining to be edible grade vegetable oil. In physical refining we use the pressure and temperature gradients only to remove impurities such as odor, FFA and wax. The chemical refining process used sodium hydroxide to remove free

fatty acids and others impurity and these processes can produce rice bran oil soapstock (RBOS) after deacidification step. A phytochemical in RBO namely γ-oryzanol was lost during alkali refining in the range 83-95% (Krishna et al., 2001; Narayan et al., 2006). At present, most soapstock is used in the non-food application such as animal feed, soap and detergent industries. It could be used as a nutritional or functional ingredient in the food industry. The utilizations of RBOS as noted can help adding value to agricultural products and also reducing pollution from rice bran oil industrial waste.

The γ-oryzanol is a mixture of ferulic acid esters of triterpene alcohols (phytosterols) as cycloartenylferulate, 24-methylen-cycloartanylferulate, β-sitosteryl ferulate and campesterylferulate (Garcia et al., 2009). The γ-oryzanol is an antioxidant compound and is associated with decreasing plasma cholesterol, lowering serum cholesterol, decreasing platelet aggregation. However, at increased levels of γ-oryzanol of 200–2,000 mg, it has been shown to provide the desired health benefits (Seetharamaiah and Chandrasekhara, 1988; Latha et al., 2014).

Extraction of γ-oryzanol from RBO and RBOS such as solid-liquid extraction (leaching) with organic solvent and ultrasound-assisted aqueous extraction, which have been studied (Rodrigues et al., 2006; Zullaikah et al., 2009, Jesus et al., 2010; Kaewboonnum et al., 2010; Khoei and Chekin, 2016). Several studies reported methods for isolation or purification of γ-oryzanol from RBO such as Xu et al. (2000) was studied the convectional solvent extraction for the extraction of γ-oryzanol from rice bran oil that the yield was 11.8%. Moreover, the γ-oryzanol with a purity and recovery of 93-95% and 59%, respectively, was obtained from CRBO (Zullaikah et al., 2009). Furthermore, there are several works about the extraction and purification of the RBOS as, Indira et al. (2005) reported a process for obtaining γ-oryzanol of 43% (w/w) purity and 80% (w/w) recovery. Relate to Kaewboonnum et al. (2010) have been studying the use of solvent extraction to extract γ-oryzanol obtain 55.71% (w/w) purity and 74.6% recovery. In addition, Jesus et al. (2010) was found soxhlet extraction with ethyl acetate to obtain high γ-oryzanol content (3.3% w/w) and recovery rate of γ-oryzanol content (58.6% w/w). Although there are many studies extraction process, but there were no reports that any suitable method to be used on an

industrial and several methods can be made only in the laboratory. Therefore requires additional study the suitable methods and conditions of extraction and purification to be used on an industrial

Interest in the production and application of γ-oryzanol has remarkably increased. A novel interfacial engineering technology has been used in the preparation of emulsion and it can be shown to have better barrier against environmental conditions. Proteins and polysaccharides are wildly used in the preparation of multilayer interfacial membrane emulsions and serves as wall materials during the microencapsulation process (Gu et al., 2005; Klinkesorn et al., 2005; Klinkesorn et al., 2006). Microencapsulation of γ-oryzanol has been studied by some researchers in order to protect γ-oryzanol from the heat-induced loss of its antioxidant effect (Suh et al., 2007). Furthermore, encapsulation rice bran oil and spray-dried in order to protect phytosterol in rice bran oil was investigated (Ratchanee et al., 2012). However, there are no published reports regarding the encapsulation and spray-dried γ-oryzanol extracted. In addition, the degradation of encapsulated γ-oryzanol upon storage is the main problem of the quality loss, which also determine their shelf-life. The kinetic order and rate constant are the basic requirement to define shelf-life (Zhang et al., 2016). Latha et al. (2014) reported that the Indian traditional food added γ-oryzanol, which packed in metallized polyester pouches stored at 27 °C and 65% RH, it had a shelf-life of 4 months.

Therefore, the objective of this research was to study the extraction and purification of γ-oryzanol from RBOS using soxhlet extraction and ultrasonic-assisted extraction. The γ-oryzanol extracted subsequently was processed to encapsulated γ-oryzanol powder of which its, characteristics, antioxidant activity, shelf-life and application as a food ingredient was investigated.

1.2 Objectives

The overall aim of this work was to utilize soapstock from rice bran oil. The knowledge were gained from research that can lead to help adding value to agricultural products and also reducing pollution from rice bran oil industrial waste. Specific objectives were to:

1.2.1 To study the optimization of extraction and purification of γ-oryzanol from RBOS using soxhlet extraction and ultrasonic-assisted extraction.

1.2.2 To study production of γ-oryzanol powder using encapsulation technique and spray dry.

1.2.3 To investigate the characteristics and antioxidant activity of γ-oryzanol extract and encapsulated γ-oryzanol powder.

1.2.4 To evaluate shelf-life of γ-oryzanol powder using accelerated shelf-life testing (ASLT) technique.

1.2.5 To evaluate the potential use of γ-oryzanol powder as food ingredient.

1.3 Scope of work

This research work was contained 4 parts;

Part1 Optimization of extraction and purification of γ-oryzanol from RBOS

The suitable extraction process of soxhlet extraction and ultrasonic assisted extraction were studied. For conventional extraction methods, process parameters that need to be considered are solid to solvent ratio (1:5, 1:10 and 1:15 w/v), temperature (60, 70 and 80 °C), and times (4, 6, and 8 h). Another, the suitable conditions of ultrasonic assisted extraction as ultrasonic frequency (10, 12, and 14 kHz), temperature (60, 70 and 80 °C), and times (30, 45, and 60 min) were analyzed. Moreover, the optimum conditions of ultrasonic assisted extraction were investigated, after that the solid phase was extracted by optimum condition of soxhlet extraction, which were combined between optimized ultrasonic assisted extraction condition and optimized soxhlet extraction condition. The optimal condition for an extraction yield of γ-oryzanol was analyzed using the response surface methodology. After the suitable extraction process γ-oryzanol enriched fraction was obtained, and then purified further though two step crystallization. The influence of process variable such

as the solvent mixture (ethyl acetate: methanol) ratio (3:7, 4:6 and 5:5) and time (6, 12, 24, 48, and 72 h) for crystal growth was investigated.

Part 2 Production of γ-oryzanol extract powder and antioxidant activity of γ-oryzanol extract powder

The research work is to produce spray-dried γ-oryzanol/rice bran oil emulsion encapsulated with interfacial membranes using maltodextrin and whey protein as wall materials. Yield, encapsulation efficiency and characterization of powder were measured.

The antioxidant activity of γ-oryzanol extract and encapsulated γ-oryzanol extract powder were measured in comparison with pure γ-oryzanol, commercial γ-oryzanol, synthetics antioxidant (BHA) and tocopherol.

Part 3 Shelf-life of the encapsulated γ-oryzanol extract powder.

This study has focused on quality change during storage of encapsulated γ-oryzanol extract powder was investigated. There is method for evaluation shelf-life of encapsulated γ-oryzanol extract powder indirect method (accelerated method).

Part 4 Application γ-oryzanol powder as food ingredient.

This part has concentrated on the preparation of encapsulated γ-oryzanol powder fortified salad dressing with the utilization of γ-oryzanol as encapsulated γ-oryzanol extract powder. The effect of incorporating γ-oryzanol extract powder to salad dressing on physicochemical properties, DPPH scavenging antioxidant activity, sensory quality and stability during storage were investigated.

CHAPTER 2
LITERATURE REVIEW

2.1 Rice and rice grain structure

Rice cultivation is almost 7,000 years old. Rice is grown over vast areas of land around the world and is a major staple for food for more than half of the world population. In 2016-2017, rice is the fourth largest cultivated crop produced worldwide with current annual world production approximately 740 million metric tons of paddies. The Asian continent accounts for approximately 90 percent of rice production and is also the major consumer (Food and Agriculture Organization, 2016).

The structure of the mature rice grain is shown in Figure 2.1. The principle components of the rice grain are the hull, pericarp, seed coat, nucellus, aleurone layer, endosperm and embryo. The components of rice comprise 20% husk, 1-2% of rice germ, 8-9% of rice bran and 70% of rice starchy endosperm. The rice caryopsis is made of the pericarp (1-2%), aleurone, seed coat and nucellus (combined 4 %), embryo (2%) and endosperm (89 %) (Orthoefer and Eastmen, 2004)

2.2 Chemical characteristics

Good eating quality relates to high stickiness, sweet flavor, gloss of cooked rice, and palatability as shown in Table 2.1. Eating quality of a particular lot of rice is evaluated objectively by a taste panel. Taste panel scores are then used to calibrate “taste analyzers” which determine taste scores based on physicochemical properties. Protein, amylose, and moisture are the primary determinates of taste in white rice. In contrast, brown rice taste scores are a function of protein, amylose, moisture, and fatty acid content. Most fatty acids are contained in the bran layer, which the milling process removes. A criterion for palpability evaluation used to further grade table rice consists of six sensory tests. These include: Appearance, Aroma, Taste, Stickiness, Hardness, and Overall evaluation.

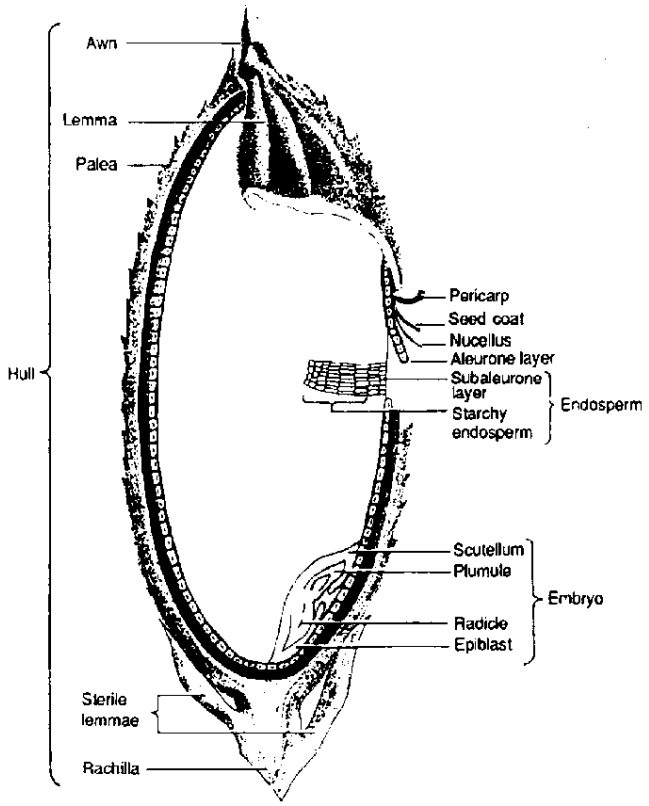


Figure 2.1 Structure of the Mature Rice gain

Source: Marshall and Wadsworth (1994)

Table 2.1 Range of Chemical and Physical Characteristics of Long, Medium and Short Grain Rice Types

Characteristic	Long grain	Medium grain	Short grain
Amylose (%)	23-26	15-20	18-20
Alkali spreading value	3-5	6-7	6-7
Gelatinization temperature (°C)	71-74	65-68	65-67
Gelatinization class	Intermediate	Low	Low
Water uptake (ml/100g)	121-136	300-340	310-360
Protein (%)	6-7.5	6-7	6-6.5
Parboiling stability, solid loss (%)	18-21	31-36	30-33

Table 2.1 Range of chemical and physical characteristics of long, medium and short grain rice types (Continued.)

Amylographic paste viscosity			
Characteristic	**Long grain**	**Medium grain**	**Short grain**
Peak	765-940	890-820	820-870
Cooked 10 min at 95° C	400-500	370-420	370-400
Cooled at 50° C	770-880	680-760	680-690
Brewing Cookability, sec	120	5-15	5-10

Source: Webb (1985)

2.3 Rice bran

Rice bran is one of the valuable by-products from rice processing industry. During the milling process to obtain white rice, rice bran (8-9 wt % of the rice paddy) and rice germ (1-2 %) are usually removed, and the removed by-products are generally called rice bran. At the present, production of rice is 740 million tons. In the process of rice production consisting of rice milling to remove rice hulls (164 million tons) and rice bran (59.2 million tons). Therefore, the amount of white rice production is 411 million tons (Hui Y.H.,1996).

Rice bran is a rich source of fiber, natural oil, and nutrients such as vitamin E and vitamin B (Peretti et al., 2002). Rice bran is composed of fractions from the pericarp, the aleurone, the sub-aleurone layers, the seed coat, the nucellus along with the germ, or embryo, and a small portion of endosperm (Houston, 1972; Juliano, 1985; Salunkhe et al., 1992; Hargrove, 1994).

The physical and chemical nature of bran depend on rice variety, treatment of grain before milling, type of milling, degree of milling, and fractionation process operative before milling.

Rice bran is rich in vitamins and minerals, including vitamin E, thiamin, niacin, aluminum, calcium, chlorine, iron, magnesium, manganese, phosphorus, potassium, silicon, sodium and zinc (Juliano, 1985; Sunders, 1990; Hu, 1995; Xu, 1998). The importance of rice bran as a processed product is mainly attributed to its lipid content. Interest in rice bran oil is growing due to its various beneficial effects on health.

2.4 Rice bran oil (RBO)

RBO is the oil extracted from the hard outer brown layer of rice after chaff (rice husk). Generally, the two most common methods for separating oils from raw materials are solvent extraction and hydraulic pressing or expeller pressing (Proctor and Bowen 1996). The composition of crude rice bran oil (CRBO) is given in Table 2.2 whereas Table 2.3 gives details of important characteristics of rice bran oil. Rice bran oil has oleic acid (38.4 %), linoleic acid (34.4%) and linolenic acid (2.2%) as unsaturated fatty acids, and palmetic (21.5%) and stearic acid (2.9%) as saturated fatty-acids (Rukmani and Raghuram, 1991; Xu, 1998). Three major fatty acids palmitic, oleic and linoleic make up 90 % of the total fatty acids of the rice bran oil. Rice bran is a good source of linoleic acid that is essential to human health (Ramezanzadeh et al., 2000).

Table 2.2 Composition of crude rice bran oil

Composition of CRBO	
Saponifiable Lipid	96%
Triacylglycerols	81-84%
Diacylglycerol	2-3%
Monoaclyglycerol	1-2%
Free fatty acid	2-6%
Wax	3-4%
Glycolipids	0.8%
Phospholipids	1-2%
Unsaponificable	4%

Source: Ghosh (2007)

Table 2.3 Fatty acid composition for rice bran oil (% of total lipid)

Fatty acid	Rice bran
Palmaitic (C 16:0)	21.5
Stearic (C 18:0)	2.9
Oleic (C 18:1)	38.4
Linoleic (C 18:2)	34.4
Linolenic (C 18:3)	2.2

Source: Xu (1998)

2.5 Extraction of rice bran oil

Rice bran oil is unique among edible oil due to be rich source of commercially and nutritionally important phytoceutical such as oryzanol, lecithin, tocopherols and tocotrienols. (Patel and Naik, 2004)

Extracting process starts with raw material preparation. Rice bran is the first screened and then heated by steam at temperature higher than 100 °C to stop lipase hydrolysis in rice bran prior to extraction. RBO is extracted from bran using solvent extraction (hexane) and pressing (screw press or hydraulic press). After extraction, the refining of rice bran oil is required. This is because of presence of very high amounts of by-products like gums, waxes and free fatty acids in crude RBO. The color of curd RBO is dark greenish brown to light yellow, depending on the condition of bran, extraction method and composition of the bran.

2.6 Refining

There are two methods for rice bran oil refining that chemical and physical refining process (Figure 2.2). The chemical refining process usually comprises the following steps as degumming, neutralization, bleaching and deodorization and the physical refining process has similar steps to chemical refining process except that for removal of free fatty acid. In chemical refining, free fatty acid is removed by using alkali treatment (neutralization) but in physical refining process, it is removed by steam distillation process similar to deodorization (Ghosh, 2007).

2.6.1 Chemical refining

Degumming: This step is designed to remove phospholipids (gums), both hydratable and nonhydratable gums in crude oil. Hydratable gums can be removed by adding water or acid into the oil. This process is called water degumming or acid degumming.

Deacidification or Neutralization: Sodium hydroxide is used in this procedure to remove free fatty acids and other impurities such as phosphatides, proteinaceous, and mucilaginous substances. Slight excess sodium hydroxide was mixed with the heated oil at the temperature about 60-80 °C and left for the aqueous phase to settle. The aqueous phase is known as soapstock which is removed from the neutral oil by washing with hot water, followed by settling or centrifugation. Even though the main purpose of the deacidification step is to remove of free fatty acid, the process also causes a reduction in phospholipid and coloring matter content (Belitz et al., 1987).

Winterization or Dewaxing: This step is designed to remove high melting point waxes in rice bran oil. Winterization is a process whereby the wax is crystallized and removed from the oil by filtration to avoid clouding of the liquid fraction at cooler temperature conditions by using temperature to control crystallization. In a conventional process, the oil is heated to around 90 °C to destroy any existing nuclei. After that, the oil is cooled with stirring to around 20 °C and then allowed to mature for a minimum of 4 h. Wax is separated by filtration in plate and frame filters.

Bleaching: Bleaching of rice bran oil is more difficult than other vegetable oils due to the high chlorophyll, and red pigment content, and also the oxidized products of tocopherols and metallic salts of fatty acids. Generally, bleaching of rice bran oil is done after deacidification step by using adsorbents called bleaching earths (Fuller’s earth, acid activated monmorillonite clays or activated carbon) to absorb coloring components. During bleaching, gums, soaps and some oxidation product are also adsorbed along with the pigments (Belitz et al., 1987). Bleaching rice bran oil prior to deacidification step is sometimes recommended because chlorophyll tends to be stabilized by alkali and heat, which then is more difficult to be removed (Cowan, 1976). In addition to removing pigments, bleaching

under high vacuum and at a temperature of around 110 °C helps reduce the amounts of oxidation products as the catalytic activity of activated earth at this temperature decomposes hydro-peroxides and the ion exchange properties of bleaching earth promote metal removal (Ghosh, 2007).

Deodorization: The aim of this step is to remove volatile compounds mainly aldehydes and ketones. These compounds have low threshold values for detection by taste or smell. Deodorization is essentially a steam distillation process carried out at temperature between 200 and 220 °C and pressure 6-10 mmHg. This treatment unfortunately also resulted in the removal of some of the natural protectants of oils such as tocopherols and sterols. As a result, the addition of citric acid is often made to chelate traces of pro-oxidant metals, hence diminish their activity that imparts to the oils, even at the diminished tocopherol content, thus increases the oil stability (Belitz et al., 1987).

The chemical refining process has major drawbacks in that it always incurs an oil loss of an average three times the free fatty acid value. Moreover, most nutritional components present in rice bran oil are destroyed or removed during deacidification step (Ghosh, 2007). Table 2.4 shows the loss of two nutritional components: tocopheral and oryzanol, during each step of the chemical refining process. High loss of tocopheral can be observed at deodorization step as it is removed into deodorizer distillate. The highest oryzanol loss is found during deacidification step as it is removed into soapstock (Krisnangkura et al., 2000).

Krishna et al. (2001) also report that alkali treatment removes considerable amount of γ-oryzanol (83-95 %) while degumming and dewaxing do not affect the content of γ-oryzanol in the oil considerably.

Table 2.4 Oryzanol content in rice bran oil after process of purifying CRBO

Process	Oryzanol content in refined oil (%)	Loss (%)
Control RBO (FFA 6.8%)	1.86	-
Degumming	1.84	1.10
Dewaxing	1.75	5.90
Control RBO + alkali treatment	1.10	94.60
Degummed RBO + alkali treatment	1.11	94.10
Dexaxing RBO + alkali treatment	1.13	93.00

Source: Krishna et al. (2001)

2.6.2 Physical refining

Physical refining process has similar steps to chemical refining except that for the removal of free fatty acid. In chemical refining, free fatty acid is removed by using alkali treatment but in physical refining, it is removed by steam distillation process similar to deodorization. In physical refining the removal low volatility of fatty acids (depending upon length) requires higher temperatures than those required for deodorization only. In practice, a maximum temperature of 240-250 °C is sufficient to reduce free fatty acids content to the levels of about 0.05-0.1 percent. A prerequisite for physical refining is that phosphatides be removed to a level below 5 ppm (FAO) as small amounts of phosphatides and iron are the probable cause of heat darkening during distillation (Ghosh, 2007). In chemical refining, this level is easily achieved during deacidification step. However in physical refining, a special degumming technique is needed to achieve the desired phosphorus level mentioned above. In addition to degumming, prebleaching is also necessary to remove color bodies and essentially all trace metals. The economy of deacidification by using physical refining versus chemical refining normally favor physical refining only when high free fatty acid like rice bran oil are processed (Sullivan et al., 1976). Moreover, refined rice bran oil obtained from physical refining can retain most of important constituent oryzanol compared with chemical refining process (Krishna et al., 2001).

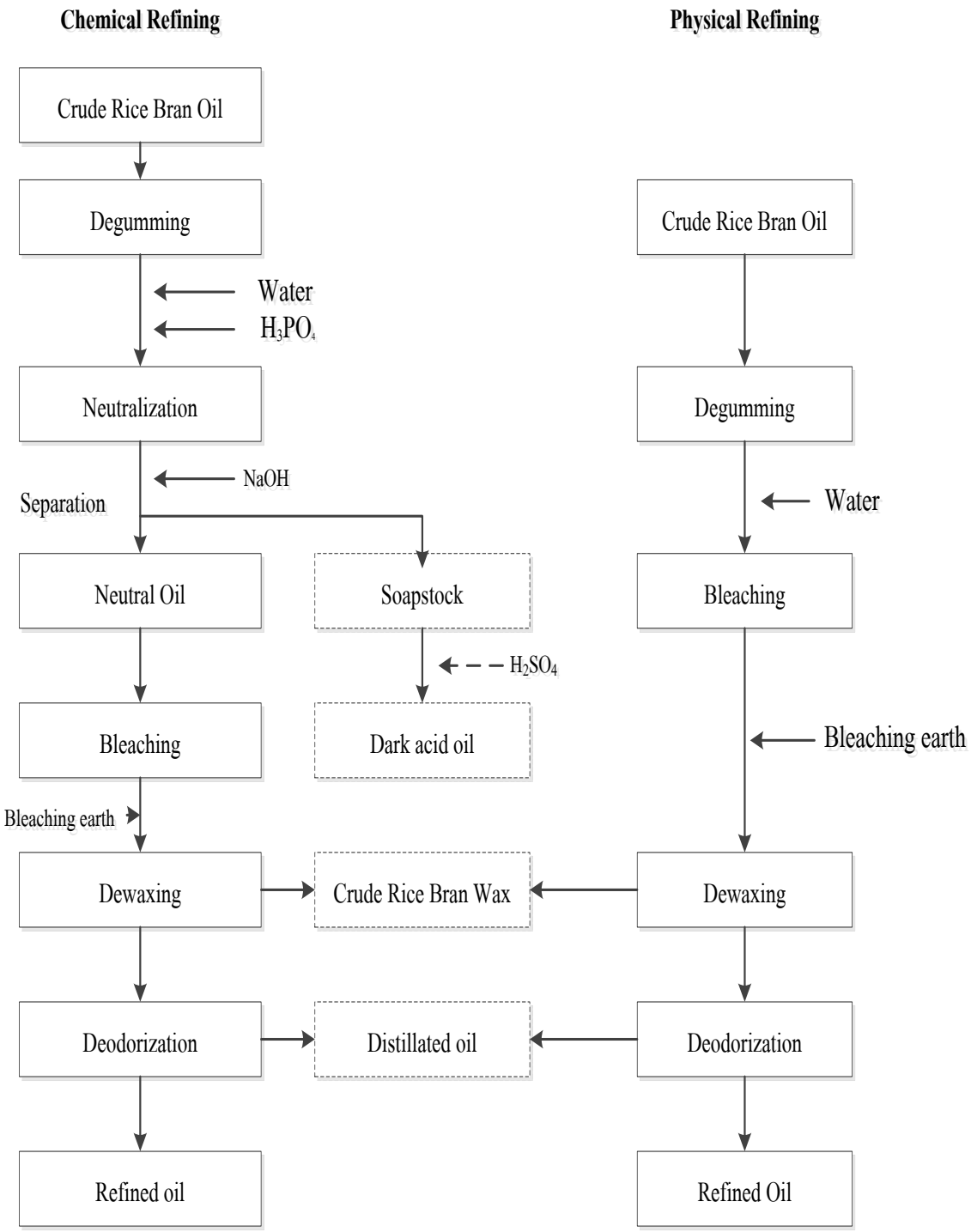


Figure 2.2 Chemical refining and physical refining of rice bran oil.

Source: Kasisuri Co., Ltd. Thailand (2015)

2.7 Soapstock

During chemical refining process, soap is formed in the deacidification step and significant amount of rice bran oil gets trapped within the soap. When the soap is removed by centrifugation, some oil is also removed with it. This by product is therefore called soapstock which is generally used as animal feed (Das et al., 1998). Often time, soapstock is reacidified by adding sulfuric acid to prepare acid oil which can be handled more easily and has longer shelf life.

2.7.1 Composition of soapstock

Soapstock consists of heavy alkali aqueous emulsion of lipids containing water as a major component. Conditions of refining and seasonal variation in oil seed composition dictate the type and quantity of impurities in the soapstock.

Table 2.5 Composition of soapstock

Component	Value
Water	65-70
Soap	20-22
Glycerides	2.2.5
Unsponified matter	7-7.5
Sterols	42
Fatty alcohol	24
Oryzanol	20
Hydrocarbon	10
Unidentified compound	2

Source: Ghosh (2007)

In addition to lipid loss during decidification step, a significant amounts of micronutrients especially oryzanol are also removed along with the oil into soapstock as its sodium salts (ranging 0.1-1.8 %) and is contained in unsaponifiable fraction whose composition is shown in Table 2.5 (Narayan et al., 2006).

2.7.2 Impurities in RBOS

During rice bran milling, impurities get into the rice bran and subsequently rice bran oil and then RBOS after deacidification step. Knowledge of the types of impurities is essential for separation of γ-oryzanol. These impurities include:

2.7.2.1 Soap is sodium salts of free fatty acid that is contained in soapstock at approximately 20-22 % wt. RBOS are highly to moderately soluble in water and methanol, but insoluble in acetone and ethyl acetate. This contrasting solubility behavior between soap and γ-oryzanol is the basis for separation of γ-oryzanol.

2.7.2.2 Glycerides present in soapstock in form of mono-glycerides, di-glycerides in minor amounts depend on the extent of hydrolysis of oil and mostly in form of triglycerides. The amount of tri-glyceride in soapstock depends on operating condition during deacidification step. Tri-glycerides are soluble in organic solvent such as hexane, isopropanol, chloroform and ethyl acetate but mono-glycerides and diglycerides have lower solubility in these solvent compare with tri-glycerides. A technique for removal of glycerides from soapstock is by converting them into soaps by saponification. The degree of saponification varies with conditions, but tri glycerides saponify rapidly in aqueous alkali compared with the hydrolysis of oryzanol (Das et al., 1998). Oryzanol is then separated from the soap by extraction.

2.7.2.3 Phospholipids (gums): Both hydratable and nonhydratable gums are present in soapstock and their proportions vary depending on the efficiency of the degumming step. The gums, especially the nonhydratable phospholipids, are the most interfering impurities affecting γ-oryzanol separation from soapstock, probably owing to their high surface activity. During solvent extraction of soapstock, the gums stabilize the soapstock solvent microemulsion and thereby decrease the rate of phase separation. To reduce their interference during the extraction step (solid-liquid and liquid-liquid) used for isolating oryzanol, efficient degumming of rice bran oil is desirable prior to the alkalirefining step.

2.7.2.4 Waxes are esters of saturated fatty acid (C_{16} to C_{26}) and saturated fatty alcohols (C_{24} to C_{30}) and present in rice bran oil depends on extraction conditions. The common range in rice bran oil is 2–4 % with a level of <0.5 % being desirable. Rice bran waxes can be classified into two classes: soft waxes (m.p.< 75°C)

and hard waxes (m.p. > 80°C). The majority of waxes (about two thirds) exist in polymeric form with the remainder being monomeric. During deacidification step, some wax gets into soapstock and tends to form stable emulsion. Waxes are insoluble in acetone, ethyl acetate, and isopropyl alcohol but soluble in hot hexane.

2.7.2.5 Sterols are a major portion of the unsaponified matter of RBOS. Sterols in rice bran oil are mainly present as neutral sterols (free sterols and steryl esters), with some polar sterols (steryl glycosides and acylated steryl glycosides). The major sterols in rice bran oil are β-sitosterol, campesterol, and stigmasterol. During alkali refining, considerable amounts of steryl esters and steryl glycosides are extracted into the soapstock from the crude oil. The content of individual types of sterols in soapstock varies depending on processing conditions during alkali refining. Harsh processing conditions, such as high temperature and high pH during isolation of oryzanol, promote several chemical reactions of sterols such as hydrolysis, oxidation, dehydrogenation, and isomerization. Temperature, pH, and time of processing also contribute to the rate of sterol degradation. Alkaline hydrolysis cleaves the ester bond of steryl esters and releases its fatty acid into the reaction mixture along with the sterol. Acid hydrolysis cleaves the acetal (glycosidic) bond of steryl glycosides and releases the carbohydrate from the sterol or steryl ester. Neutral sterols (free sterols and steryl esters) are soluble in organic solvents such as acetone, chloroform, and ethyl acetate. Steryl glycosides contain carbohydrate moieties (sugar units) attached to sterol or steryl esters and therefore require relatively more polar solvents to solubilize them. This difference in solubility of esters and steryl glycosides forms the basis for the separation of sterol glycosides from steryl esters during the isolation process.

2.7.2.6 Resinous material in soapstock are thought to be formed by polymerization of wax components. The resinous materials can be saponified to triacontanol and soaps. The wax-like components are significant interfering impurities during the purification of oryzanol. These impurities interfere with oryzanol separation during chromatography because they strongly adsorb onto the support along with oryzanol, and interfere in crystallization of oryzanol by disrupting crystal growth. During crystallization of oryzanol from the unsaponified fraction of soapstock, the wax-like (mucilaginous) impurities precipitate out first when the

temperature of solventmiscella is reduced from about 60–70 °C (that is, the reflux temperature) to 25–30 °C. At this temperature (25–30°C), oryzanol also crystallizes, thus making the separation of wax and oryzanol difficult. Hence, the separation of supernatant miscella from mucilaginous impurities should be carried out judiciously.

2.7.2.7 Pigments: CRBO contains pigments such as chlorophyll, carotenoids (lutein, xanthophylls), and protein degradation products. Among these pigments, concentrations of chlorophyll compounds are very high in CRBO. The content of peptides in rice bran oil varies depending on the degree of heating during stabilization of the rice bran (Gingras, 2000). These impurities are removed by bleaching using adsorbents such as activated clay after beaching step. If these impurities are not removed during bleaching step, they are carried into the soapstock during alkali refining.

2.7.2.8 Glycolipids (phosphoglycolipids) are critical interfering impurities affecting the purification of oryzanol. They interfere with the degumming step of rice bran oil refining as a result of their very high surface activity, which leads to high oil losses into soapstock during the deacidification step. It is recommended that glycolipids be kept to as low content as possible in CRBO (Narayan et al., 2006).

2.8 Extraction and purification of γ-oryzanol

2.8.1 Method of extraction

2.8.1.1 Solvent extraction

Since γ-oryzanol is readily dissolved in organic solvents, hexane was typically used in extraction of γ-oryzanol from rice bran (Diack and Saska, 1994; Rogers et al. 1993; Seitz, 1989; Norton, 1994). However, all components of γ-oryzanol contain an alcohol group in the ferulate portion, which brings about a relatively low polarity of these components. They may also be soluble in low polar solvents, such as isopropanol and ethyl acetate, in addition to the non-polar solvents, hexane or heptane and the maximum wavelengths (λ_{max}) for γ-oryzanol analysis shown in Table 2.6. The solvent strength of extraction may affect the extractability of γ-oryzanol from rice bran. The effects of various solvents on the yield of γ-oryzanol in extraction have not been reported. Another unclear factor in extraction is the effect of

saponification on the efficiency of extraction. In previous studies, saponfication was performed prior to the solvent extraction (Diack and Saska, 1994; Rogers et al. 1993; Seitz, 1989; Norton, 1994). Saponification, which is important for reducing interfering lipids and for breaking down the matrix of rice bran for improved recovery of extraction, may have a negative effect in the extraction of γ-oryzanol. It is possible that the ester bond between the ferulate and triterpene components of γ-oryzanol also is broken down under alkali condition. This could result in decomposition of γ-oryzanol and decrease the yield of extraction. The effect of saponification on yield of γ-oryzanol in solvent extraction has not been reported.

Table 2.6 Maximum wavelengths (λ_{max}) for γ-oryzanol analysis

Solvent	λ_{max} (nm)		Calibration curves	
	Ref	Experimental	Regression Equation*	R^2
Hexane	314	315	Y= 0.0359x	0.997
Ethyl Acetate	320	320	Y= 0.0386x	0.999
Isopropanol	326	327.5	Y= 0.0393x	0.996
Butanol	326	328	Y= 0.0350x	0.995

*y = absorbance, x = concentration (ppm)

Source: Srisaipet and Nuddagul (2014)

2.8.1.2 Ultrasonic assisted extraction (UAE)

Ultrasound-assisted extraction uses acoustic cavitation to cause molecular movement of solvent and samples, which offers advantages of improved efficiency, reduced extraction time, low solvent consumption and higher level of automation compared with conventional extraction techniques. Consequently, the increasing use of ultrasound extraction has been investigated in the pharmaceutical, chemical and food industries. This is a well-established method in the processing of plant material, especially in extraction of low molecular weight substances, and of bioactive substances from plants.

2.8.2 Source of γ-oryzanol

2.8.2.1 Separation of γ-oryzanol from rice bran

Commonly, hexane is used as a solvent to commercially extract oil from rice bran but it poses potential fire, health and environmental hazards. Hu et al. (1996) proposed to compare isopropanol with hexane as extraction solvents for the enrichment of vitamin E and γ-oryzanol in oil from stabilized rice bran. The researchers suggested that isopropanol is a promising alternative solvent to hexane for extraction. Beside organic solvent extraction, supercritical fluids have received attention for extraction of lipid. Xu et al. (2000) reported that the yield of γ-oryzanol by supercritical fluid extraction was higher than that obtained by solvent extraction. Limitation of this method is however fluctuations in the flow rate and pressure which cause the variation in the results. Moreover, the equipment and installation are also expensive (Narayan et al., 2006).

2.8.2.2 Separation of γ-oryzanol from rice bran oil

Many researchers have employed chromatographic methods for separation of γ-oryzanol from rice bran oil with the main objective to separate individual component of γ-oryzanol. Rogers et al. (1993) used semi preparative HPLC to fractionate rice bran oil γ-oryzanol components into individual component analysis by chemical ionization-mass spectrometry. γ-oryzanol were isolated into five components (cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campestanyl ferulate, cycloartanyl ferulate, and β- sitosteryl ferulate) in C:18 reverse phase column with a mixture of acetonitrile/ methanol/isopropanol/water as the mobile phase. Later, Xu and Godber (1999) achieved to separate ten components of γ-oryzanol (Figure 2.3) that had not been previously reported. The researchers used normal phase HPLC for purification of γ-oryzanol, then separation of individual components of γ-oryzanol in reverse phase HPLC, and identification was conducted by a GC/MS. Although chromatographic method is an effective method to achieve high purity and recovery of γ-oryzanol, it is difficult to recover solvent and scale up.

2.8.2.3 Separation of γ-oryzanol from RBOS and acid oil

During rice bran oil refining, γ-oryzanol is one of the micronutrients that is removed in high quantity from rice bran oil after deacidification step of the chemical refining process. The by-product from this step, soapstock, was

found to have high γoryzanol content therefore it is worth separating of γ-oryzanol from the soapstock. Seetharamajah et al. (1986) used four steps process to recover γ-oryzanol from RBOS. The process consists of liquid-liquid extraction, column chromatography, crystallization and recrystallization to obtain γ-oryzanol as show the following diagram (Figure 2.3). However, this process involves several steps of extraction result the decrease in the yield of γ-oryzanol, and therefore is unsuitable for scale up. Later, Mingzhi and Yanyan (1999) proposed to recovery γ-oryzanol using second soapstock as a starting material. Second soapstock was the soapstock fraction formed from the soapstock that once again underwent alkali refining by adding excess alkali. This material was subjected to multiphase fractional crystallization to obtain 98 % (w/w) pure γ-oryzanol, however the yield was only 1.9 % (w/w). γ-oryzanol recovered from this process has high purity but unsatisfied yield thus it cannot be used in commercial scale.

In rice bran oil industry, soapstock are usually converted into acid oil immediately in order to make it easy to handle and to extend shelf life. Das et al. (1998) proposed to recovery γ-oryzanol from rice bran acid oil. The acid oil was subjected to conventional vacuum distillation for free fatty acid removal to obtain material called pitch which is used as a starting material for the process. After purification by charcoal treatment, 96 % purity and 76 % yield of γ-oryzanol was obtained. Although this method obtained high yield and purity of γ-oryzanol, it is predominantly laboratory procedure and therefore was difficult to scale up to commercial process.

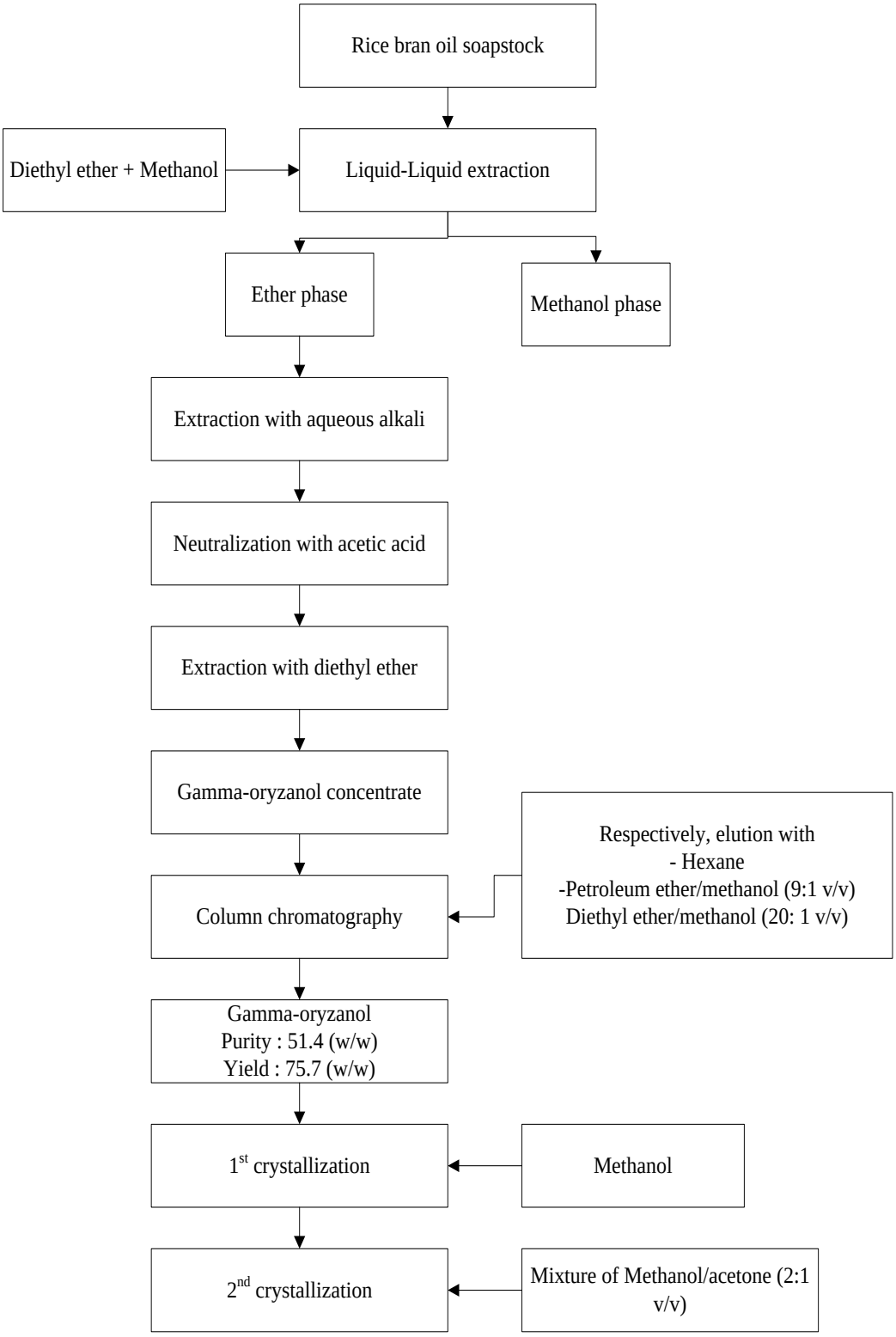


Figure 2.3 Seetharamajah et al.'s process

Source: Seetharamajah et al. (2003)

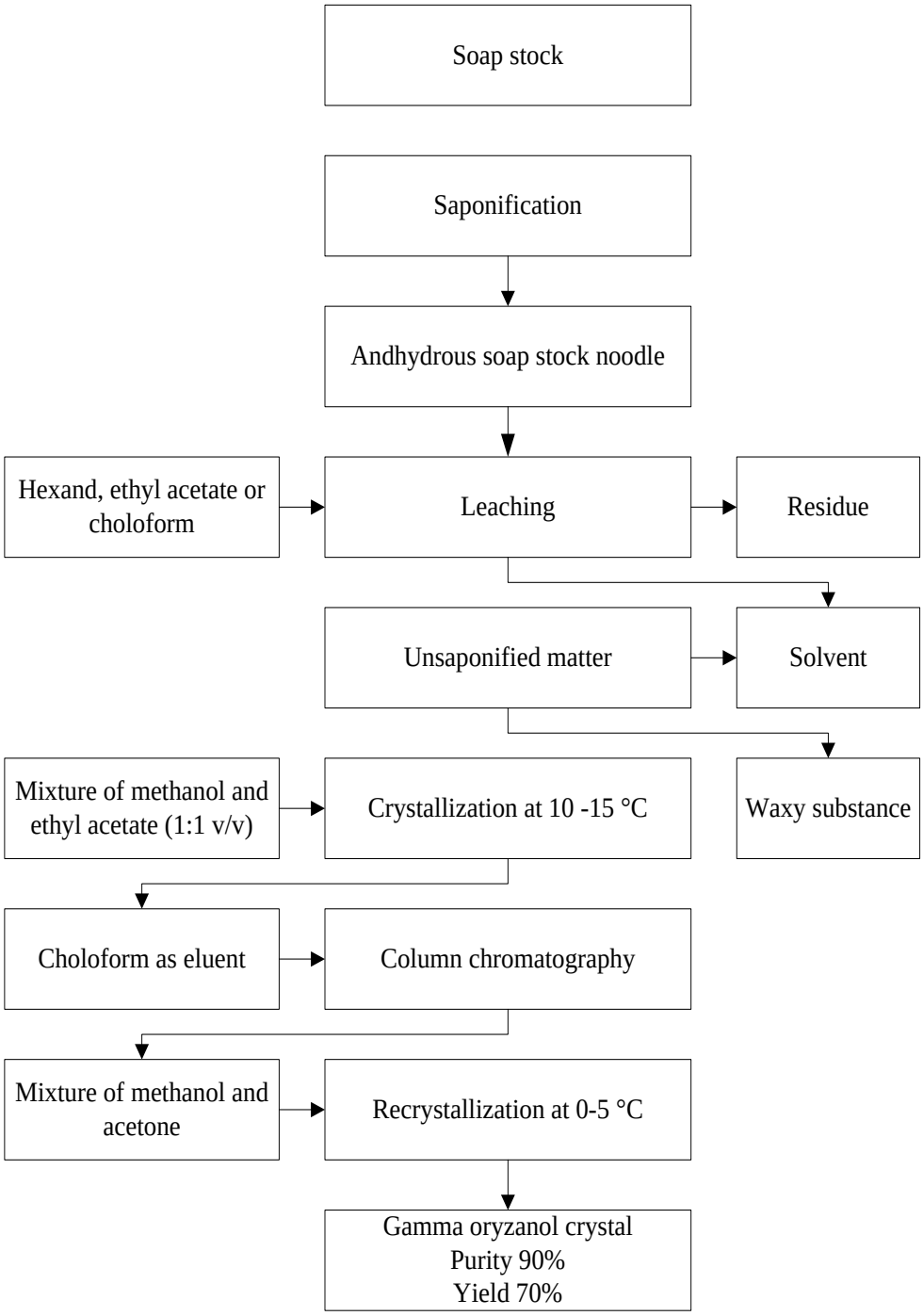


Figure 2.4 Rao et al.'s process

Source: Rao et al. (2002)

When developing into large scale process, several factors such as productivity, environment and health problems, process investment, and separation efficiency (purity and yield) must be considered (Narayan et al., 2006). Processes for isolation of γ-oryzanol have been continuously developed in order to improve the purity and yield. Rao et al. (2002) proposed a method that was easy to handle, store and extract soapstock by this converting the soapstock into soap noodle. After saponification, anhydrous soap noodle was conducted and was then subjected to process as shown in diagram (Figure 2.5). Although this process provided high purity and yield of γ-oryzanol, it has some disadvantages such as requiring tedious pretreatment and extrusion to form noodle, involving a large number of unit operations. Furthermore column chromatography is obstruction for commercial scale.

Indira et al. (2005) propose an improved technique for saponification, dehydration, and leaching to obtain enriched fraction of γ-oryzanol. Saponification step is achieved by using mild operating condition LTST (low temperature and short time) to avoid degradation of γ-oryzanol. Dehydration was performed by using HTST (high temperature and short time) process such as drum drying. In addition, Indira et al., 2005 proposed a technique for improving selectivity during leaching by using pack bed mode. This process is summarized as shown in Figure 2.5. They obtained higher yield and purity of γ-oryzanol than extraction of porous anhydrous noodle soapstock proposed by Rao et al. (purity 30 % and yield 54 %). Related to Indira et al.'s process, Narayan et al. (2004) proposed a simple process for crystallization of γ-oryzanol from γ-oryzanol enriched fraction. This process comprises of nonselective temperature assisted dissolution of oryzanol enriched unsaponifiable fraction in a suitable mixture, fractional precipitation of mucilaginous interfering impurities, and crystallization of γ-oryzanol from supernatant as summarized in Figure 2.5. Due to rather high purity and yield and uncomplexity of both processes, it can be scaled up to commercial process.

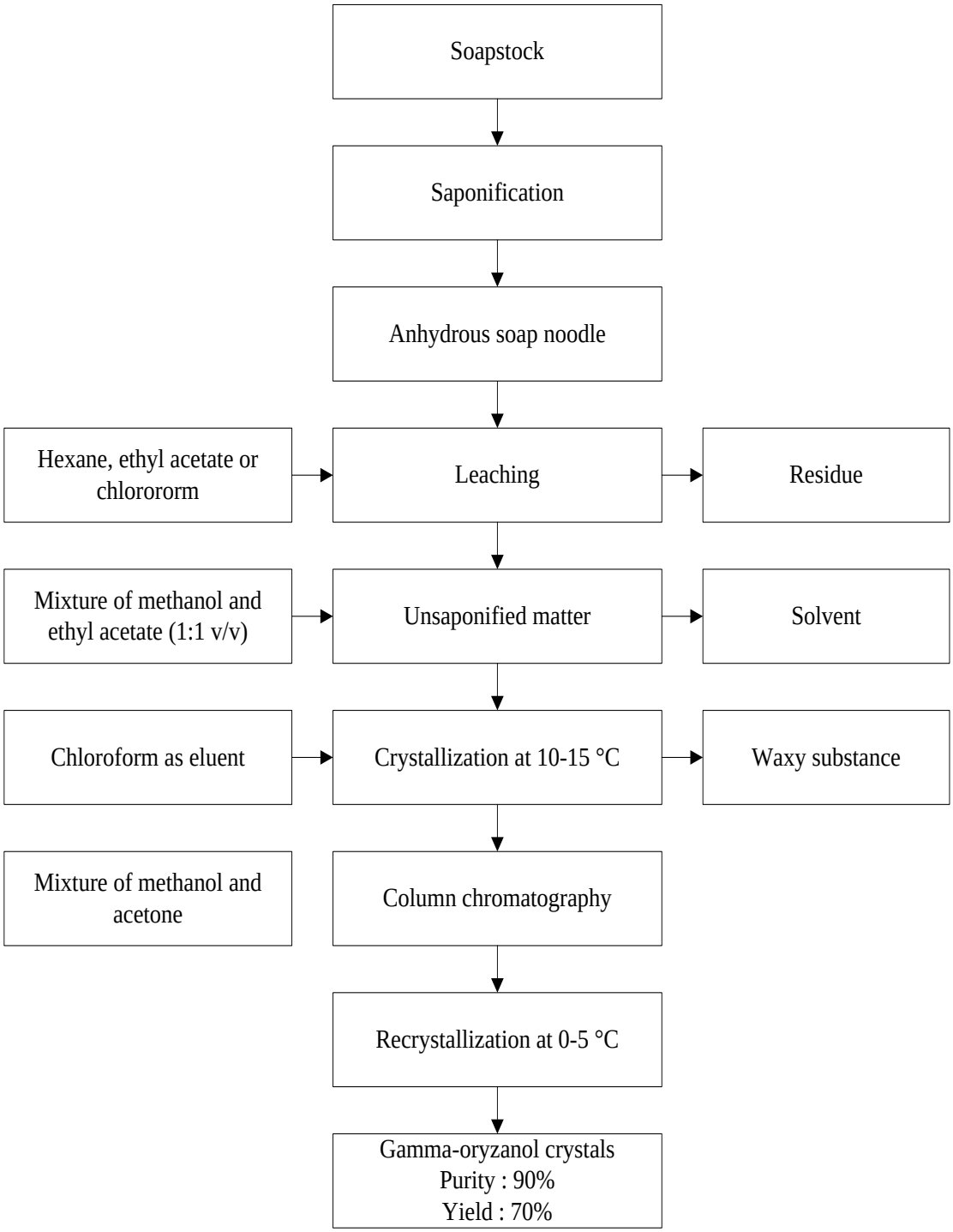


Figure 2.5 Indira et al.'s process

Source: Indira et al. (2005)

2.8.2.4 Response surface method

Response surface methodology (RSM) is a group of mathematical and statistical techniques. They are employed for modeling and analysis of dates which affected by several variables. The purpose of analysis is to optimize the response.

Box–Behnken design is a rotatable independent quadratic design. It is based on three-level incomplete factorial designs. The treatments are at the midpoints of edges of the process space and at the center viewed as a cube (Figure 2.6). In addition, Box–Behnken design can be viewed as consisting of three interlocking 2^2 factorial designs and a central point.

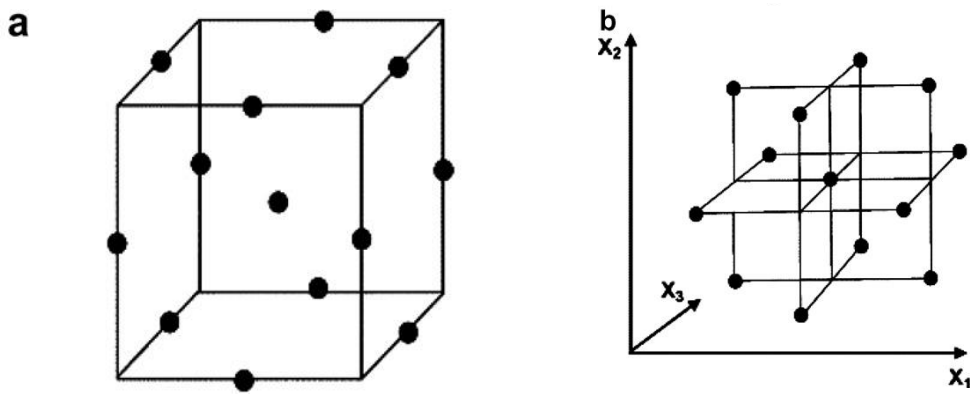


Figure 2.6 Box–Behnken design (a) a cube form; (b) interlocking 2^2 factorial experiment

Source: Aslan and Cebeci (2006)

For the three factors and three levels, 15 experiments are required in the Box–Behnken design. For the model form, it is as followed: (Aslan and Cebeci, 2006)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1 X^2_1 + \beta_2 X^2_2 + \beta_3 X^2_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon$$

Where, Y is the estimated response.

β_0 is the constant term.

β_i is the linear coefficient. (i = 1-3)

β_{ij} is the quadratic coefficient. (i = 1-3 and j = 1-3)

ε is the random error.

Yoo et al. (2006) employed RSM to optimize microencapsulation yield of alpha tocopherol (MY). The ratio of coating material to core material (X_1), the emulsifier concentration (X_2), and the $CaCl_2$ concentration (X_3) were independent variables. They showed that the regression model was MY (%) = $56.02 + 3.64X_2 + 3.18X_1X_2 - 3.74X_2^2$

Ahn et al. (2007) used RSM to optimize the microencapsulation condition of sunflower oil using four independent variables: sunflower oil concentration (X_1), proportion of milk protein isolates to coating wall (X_2), soy lecithin concentration (X_3), and homogenizing pressure (X_4). The regression equation was fitted as follow: MEE = $4.137772 + 3.524183X_1 + 3.475205X_2 + 2.914167X_3 - 0.074532X_1^2 - 0.067482X_2^2$.

2.8.3 Process of γ-oryzanol purification

The problems encountered during extraction of oryzanol are mainly due to variations in the compositions of RBOS, which include surface-active impurities such as soaps, phospholipids, waxes, and glycolipids. The processing conditions used during oil refining and seasonal variations in oilseed composition dictate the type and quantity of the impurities extracted with the soapstocks. Thus, the amounts of individual impurities that are removed vary among soapstocks. Hence, an isolation procedure developed for one soapstock does not necessarily work well with another soapstock. The impurities that stabilize the soapstock dispersion (solid-liquid and liquid-liquid ratios) vary depending on the type and amount of impurities present. Impurities also affect processing conditions and hamper the equilibria governing the separation processing steps, such as extraction, isolation, and purification.

High-purity γ-oryzanol can be obtained by crystallisation (Du, Zhang, Feng, 2004; Zullaikah, Melwita, and Ju, 2008). Also, Lai, Hsieh, and Chang (2005) developed an efficient chromatographic separation method for the production of pure c-oryzanol. First, crude RBO was obtained by soxhlet extraction. Then, γ-oryzanol was purified by preparative NP-HPLC on a silica gel packed column. The three-step gradient elution was carried out with n-hexane/ethyl acetate mobile phases. A >90%

γ-oryzanol recovery, with a 90–98% purity, was achieved, but the productivity was only about 10 mg per injection. Hence, further scaling-up work was needed.

Yu et al. (2006) investigated the independent and sequential fractionations of RBO with acetone at low temperatures. The aim was to prepare oil fractions enriched in unsaturated fatty acids (UFAs). The liquid fraction from independent fractionation at 35 °C was the best (89.6% UFAs). However, when weight yield was considered, the largest amount of UFAs was obtained by sequential fractionation at 0 °C (87.5 % wt recovered in a fraction containing 71.5% UFAs). As the fractionation temperature was lowered, the content of γ-oryzanol in the liquid fractions gradually increased.

2.9 γ-oryzanol

γ-oryzanol is a naturally occurring component in rice bran and rice germ which consists of a mixture of ferulic acid esters of sterols and triterpene alcohols

2.9.1 Structures

γ-oryzanol was first recovered from rice bran oil by Kaneko and Tsuchiya in 1954. It was then thought to be a single compound. Subsequent studies reveal that oryzanol is not a single compound but instead comprises a variety of ferulic acid esters called α-, β- and γ-oryzanol. Of these, γ-oryzanol has been the best characterized (Graf, 1992). γ-oryzanol is a mixture of two molecules. The largest part is triterpene alcohols or plant sterols and the other part is ferulic acid.

Among these, cycloartenol, b-sitosterol, 24-methylenecycloartenol and campesterol predominate (Xu and Godber, 1999; Lloyd et al., 2000). The molecular structures of trans-ferulates of these four phytosterols are shown in Figure 2.7. γ-Oryzanol also contains lower concentrations of esters of the trans-ferulic acid with Δ7-stigmasterol, stigmasterol, Δ7-campesterol, Δ7-sitostenol, campestenol and sitostenol (Xu et al., 1999), as well as esters of cis-ferulic (Akihisa et al., 2000) and caffeic acids (Fang, Yu, and Badger, 2003).

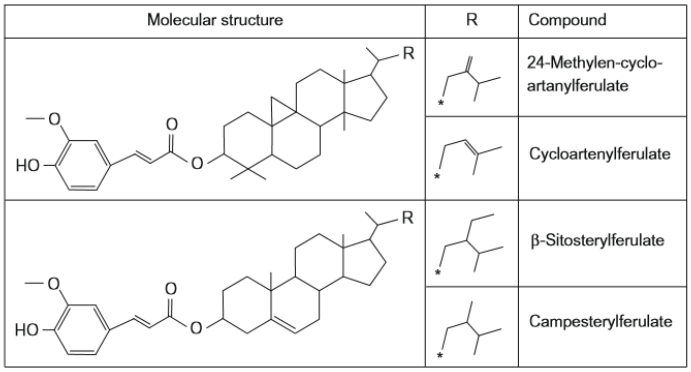


Figure 2.7 Chemical structures of the four main components of γ-oryzanol
Source: Lerma (2009)

2.9.2 Properties

γ-oryzanol ($C_{40}H_{58}O_4$) is a white or slightly yellow odorless crystalline powder and is stable at room temperature (Tamagawa et al.,1992). It has a melting point of 137.5-139.5 °C and shows absorption maxima at 231, 290 and 315 nm in heptane (Tsuchiya and kaneko, 1954). γ-oryzanol is insoluble in water, however, all of component of γ-oryzanol contains hydroxyl group in the ferlulate portion which gives rise to a relatively high polarity. Therefore, it may be soluble in more polar solvent such as isopropanol and ethyl acetate as well as nonpolar solvent such as n-heptane and hexane (Xu and Godber, 2000).

2.9.3 Physiological and biological effects

Over the past decades, a number of investigations have shown the beneficial physiological and biological effects associated with γ-oryzanol intake. These include:

2.9.3.1 γ-oryzanol emulsions are used as antioxidants and preservatives for cosmetics, food and etc. Such emulsions are effective in preventing color change (Minami and Morito, 1982).

2.9.3.2 γ-oryzanol containing pharmaceutical formulation is used in preventing motion sickness and in the treatment of nervous system disorders (Sakada and Hideharu, 1982).

2.9.3.3 γ-oryzanol exhibits hypochoresterolemic activity, which is associated with platelet aggregation. When added to a high cholesterol diet, it can significantly inhibit the platelet aggregation in rat (Seetharamajah et al., 1990).

2.9.3.4 A plethora of γ-oryzanol containing transdermal pharmaceutical and moisturizing cosmetic preparations has been prepared for the treatment of skin disorders (Tokuda and Yasuaki, 1989).

2.9.3.5 Nail lacquers containing γ-oryzanol prevent discoloration of nails (Hayafune and Sato, 1990).

2.9.3.6 Lipid peroxidation can be prevented in the retina by γ-oryzanol due to its antioxidant property (Tadahisa et al., 1990).

2.9.3.7 γ-oryzanol has been shown to be highly effective against lipogenic liver cirrhosis in sapontaneously hypertensive rats (Ito et al., 1992).

2.9.3.8 Pharmaceutical preparations containing γ-oryzanol have been shown to reduce wrinkles in aged women (Sakai et al., 1993).

2.9.3.9 Melanin formation accelerating topical preparations containing γ-oryzanol (1 wt %) have been shown to convert gray hair into natural black hair (Nakahara, 1996).

γ-oryzanol not only has been known for the therapeutic benefits, its safety to use as a drug has been demonstrated. For example, Tsushimoto et al. (1991) showed that oryzanol is non-genotoxic and non-inhibitory of cellular communication. Summary of functional effect of γ-oryzanol is shown in Table 2.7.

Table 2.7 Summary of functional effect of γ-oryzanol

System	**Functional**
Brain	Post-menopausal syndrome
	Automatic nerve disturbance
	Anti-stress
Whole body	Anti-allergy
	Anti-inflammation
	Anti-oxidation

Table 2.7 Summary of functional effect of γ-oryzanol (Continued)

System	Functional
Skin	Elevation of surface temperature
	Activation of sebaceous glands
	Inhibition of melanin formation
Stomach	Anti-ulcer
	Centrally acting stomach movement
Metabolism/digestion	Lowering of cholesterol
	Accumulation of glycogen
	Centrally acting intestinal movement
	Anti-colon cancer
	Inhibition of IBS

Source: Prasad et al. (2011)

2.10 Applications of γ-oryzanol

γ-oryzanol has a focus on market potential and has been applied to a wide range of products. Cholesterol-lowering and antioxidant activity of oryzanol makes it suitable in food products such as cereal and margarine. It can also be put into frying oil for its stability and into food coating or packaging material for antioxidant effect to extend shelf life. Moreover, γ-oryzanol can be used in pharmaceutical products such as sun UV protection skin lotion and skin-care products for repairing dry and sensitive skin (Huang, 2003).

The stabilizing effects of RBO on fats and oils are mainly due to the antioxidant and radical scavenging properties of γ -oryzanol. These effects are important in relation to both the traditional use of RBO in many Asian countries as a cooking and salad oil (Ghosh, 2007), and the manufacturing of industrial products of improved stability. Normally, less stable liquid oils are hydrogenated to enhance their oxidative stability for deep-fat frying purposes; however, considerable amounts of nutritionally undesirable trans and positional isomer fatty acids are formed during hydrogenation. An alternative to hydrogenation is blending with polyunsaturated oils containing

natural antioxidants, such as virgin olive and sesame seed oils, and RBO; the stability of snacks fried in these oils substantially increased (Kochhar, 2000).

The antioxidant effects of RBO and partially hydrogenated soybean oil, when used in the preparation of French fries, were investigated (Abidi and Rennick, 2003). Polar fractions of the three oils were analyzed for non-volatile components by size-exclusion HPLC with ELSD. Thermal degradation via hydrolysis and the extent of polymer formation were much greater in partially hydrogenated soybean oil than in RBO. The amounts of various polymeric species, including trimers and higher polymers, were lower in high γ-oryzanol RBO than in RBO. The effect of γ -oryzanol microencapsulation on the stability against heat-induced lipid oxidation was studied (Suh, Yoo, and Lee, 2007). Using TBARS, lard treated with microencapsulated γ-oryzanol displayed greater oxidative stability than lard treated with γ-oryzanol. During heating, a substantially larger amount of γ-oryzanol remained in the treated lard as well. Apparently, microencapsulation could be used to protect γ-oryzanol from the heatinduced loss of its antioxidant effect.

The capability of rice bran powder in inhibiting lipid peroxidation of fried rice flour dough during storage was evaluated (Chotimarkorn and Silalai, 2008a). Rice flour dough containing rice bran powder was fried in soybean oil and stored at 60 °C. PUFAs decreased rapidly in fried dough without rice bran powder. Also, in fried dough containing rice bran powder the absorption of oxygen in vial headspace and the lipid hydroperoxide and TBARS values were lower, and tocopherol degradation was slower. During storage, the γ-oryzanol contents of fried dough remained unaltered.

Stabilization and development of other food products Brown rice, rice bran and normal and enriched RBO have been used to stabilize a variety of food products, being also attractive candidates to develop functional foods (Bergman and Xu, 2003). The oxidation of low-heat whole milk powder during storage was reduced by adding 0.1% RBO (Nanua, McGregor, and Godber, 2000). When compared with control milk powder, consumers could not detect any effect on the flavour of reconstituted whole milk powder containing 0.1% RBO. Also, beef patties containing γ-oryzanol had higher oxidative stability during storage than did beef patties with other antioxidants (Kim, Suh, Yang, and Lee, 2003). The cooked beef containing γ-oryzanol gave the

lowest TBARS values, warmed over flavor scores, and C_7-oxidised cholesterol, hydroperoxide and hexanal levels.

2.11 Microencapsulation

Encapsulation refers to the coating or entrapment of a pure material or mixture into another material. The coated material is known as the core material, actives, or internal phase whereas the coating material can be called the wall material, capsule, shell or carrier.

The encapsulation can be accomplished by various techniques. Some techniques are based on physical phenomena. Others are relied on chemical reaction. In some ways, the processes of encapsulation are related to both physical and chemical phenomena. Therefore, to make it easy many researchers classified the encapsulation techniques into 2 types: chemical processes and physical processes as shown in Table 2.8. (Vilstup, 2001)

Table 2.8 Encapsulation processes

Chemical processes	**physical processes**
Complex coacervation	Spray drying
Polymer/polymer incompatibility	Spray chilling
Interfacial polymerization in liquid media	Fluidised bed
	Electrostatic deposition
In situ polymerization	Centrifugal extrusion
In-liquid drying	Spinning disk or rotational suspension separation
Thermal and ionic gelation in liquid media	Polymerization at liquid/gas or solid/gas interface
Desolvation in liquid media	Pressure extrusion or spraying into solvent extraction bath
	Hot-melt extrusion

Source: Ré (2006)

The concept of microencapsulation stems from idealization of the cell model, in which the core is wrapped in a semi permeable membrane that protects it from the outer medium while also controlling the entry and exit of substances in the cell. Likewise, the microcapsule consists of a layer of encapsulating material that acts like a protective film, isolating the active substance and avoiding the effects of its improper exposure (Jizomoto et al., 1993; Ré, 2006).

Gadet et al. (2004) entrapped safou pulp oil in 6DE maltodextrins by using two different methods (spray drying and freeze drying). They found that both spray drying and freeze drying had the same ability of entrapment, but during storage, freeze dried particles can protect the oil from oxidation reaction much better than particles obtained from spray drying process.

Microencapsulation has been used successfully in the food industry to protect substances that are sensitive to temperature, light, oxygen and humidity, to reduce the transfer rate from the core to the medium in which the product is located and to modify the physical characteristics of the material, facilitating handling (Desai and Park, 2005).

Spray drying is a process widely used for microencapsulation of oils and flavors (Fuchs et al., 2006; Ahn et al., 2008; Bae and Lee, 2008; Partanen et al., 2008). It results in powders with good quality, low water activity, easier handling and storage and also protects the active material against undesirable reactions. Both wall material selection and emulsion properties (stability, viscosity and droplet size) can affect the process efficiency and the microencapsulated product stability. A successful microencapsulation must result in a powder with minimum surface oil and maximum retention of the active material.

2.11.1 Spray drying

Spray drying is an economical and effective method for protecting materials while specialized equipments are not required. The operating cost of spray dry is lower than that of freeze dry 30 – 50 times (Desobry et. al., 1997). Normally, spray drying's operation is to feed emulsion into the spray dryer in order to evaporate the solvent. The rapid evaporation from the surface produces a surface layer in which the selective diffusion mechanism occurs (Diziezak, 1988).

To begin with, an atomized droplet of feed exposes to the hot air. The droplet was dried on the outside. The moisture content at the surface of drying droplet was reduced very rapidly. Until the moisture content reaches 7 – 23 %, most flavor compounds were not allowed to diffuse out while water molecules still pass through the dried surface of a droplet. This shows that the dried surface of a droplet acted as the semipermeable membrane. As a result, the dried particles which contain the core material inside are obtained (Coumans et al., 1994 and King, 1995).

In order to achieve the most effective, the spray drying process should have the following characteristics: (Karel, 1988)

(1) Short residence time prior to droplet formation

(2) Rapid evaporation once droplets are formed

(3) High degree of selectivity of the relatively “dry” surface layers

(4) Absence of lipid circulation within the droplets

(5) Avoidance of surface “cracks”

Spray drying method has a number of advantages such as readily available equipment and be able to produce large amount of capsules. In contrast, the limited conditions of spray drying method are the concentration of coating material solution and the amount of core loading. (Vilstrup, 2001) However, spray dry is widely employed to entrapped food ingredients such as flavors and lipids. For the applications of spray drying in the field of microencapsulation in food industry, it had been review by Gharallaoui et al., (2007).

Turchiuli et al. (2004) studied the entrapment of oil by spray drying and fluidized bed agglomeration. First, they used the spray drying to synthesis particles. Next, spray dried powders were agglomerated by fluidized bed agglomeration. They found that the flowability and wettability of the spray dried powder were very poor, and the encapsulation properties of powders which pass agglomerating process did not changed but extremely improved the wettability.

2.11.2 General coating material

2.11.2.1 Maltodextrin

Maltodextrin is hydrolyzed starch which has the dextrose equivalent (DE) less than 20. It is often used as a coating material because of its high

water solubility, the low viscosity of solutions, bland flavour, and low cost. However, maltodextrin has the negative aspect which is a lack of emulsifying ability.

Anandaraman and Reiceccius (1986) studied the stability of orange peel oil encapsulation. The author reported that higher DE maltodexrins form more dense oxygen impermeable matrix which resulted in a longer shelf life for orange peel oil capsule.

Carneiro et al. (2013) reported evaluating the potential of maltodextrin combination with different wall materials in the microencapsulation of flaxseed oil by spray drying, in order to maximize encapsulation efficiency and minimize lipid oxidation. Maltodextrin (MD) was mixed with gum Arabic (GA), whey protein concentrate (WPC) or two types of modified starch (Hi-Cap 100^{TM} and Capsul TA$^{@}$) at a 25:75 ratio. The feed emulsions used for particle production were characterized for stability, viscosity and droplet size. The best encapsulation efficiency was obtained for MD:Hi-Cap followed by the MD:Capsul combination, while the lowest encapsulation efficiency was obtained for MD:WPC, which also showed poorer emulsion stability. Particles were hollow, with the active material embedded in the wall material matrix, and had no apparent cracks or fissures. During the oxidative stability study, MD: WPC combination was the wall material that best protected the active material against lipid oxidation.

2.11.2.2 Acacia gum or gum Arabic

Acacia gum or gum arabic is a natural hydrocolloid which is produced from natural exudation of acacia trees. For chemical structure, acacia gum consists of D-glucuronic acid, three different types of sugar (L-rhamnose, D galactose, and L-arabinose), with approximately 2% protein. The physical properties of this gum are high solubility in water (up to 50%), stability in an acid medium (up to pH 3), film forming and surface active features. Due to its excellent emulsification properties, acacia gum is frequently used as the encapsulant. This property of the acacia gum results from the presence of this protein portion (Dickinson, 2003).

Problems associated with the use of acacia gum for encapsulation are the expensive cost, limited supply, and the high viscosity of concentrated solutions. Hence, a mixture of gum arabic and other wall materials is widely used.

Turchiuli et al. (2004) studied oil encapsulation in a wall material which was a combination of maltodextrin and acacia gum. They suggested that a 3:2 mixture of maltodextrin DE12 and acacia gum could be used as the wall material, instead of pure acacia gum or maltodextrin

Shaikh et al. (2004) reported the black pepper oleoresin entrapped with gum arabic by spray drying method was greater protected as compared with modified starch encapsulation.

Krishnan et al. (2005) entrapped cardamom oleoresin by spray drying technique and acacia gum, maltodextrin, and modified starch were used as wall materials. They found that for encapsulation of cardamom oleoresin acacia gum was the best coating material compared with maltodextrin, and modified starch and the obtained particles exhibited a free flowing character. However, a mixture of acacia gum : maltodextrin : modified starch at the ratio of 4:1:1 provided more efficient than the other blends and also better than 100% of acacia gum.

Kanakdande et al. (2006) studied the efficiency of encapsulation and the stability of acacia gum, maltodextrin, and modified starch as coating materials for cumin oleoresin encapsulation by using spray drying method. They found that in general, acacia gum provied better protection than maltodextrin and modified starch, while a blends of acacia gum : maltodextrin : modified starch at a ratio of 4:1:1 provided a better protection than acacia gum.

2.11.2.3 Modified starch

The molecules of native starch are hydrophilic, thus they have slightly affinity for hydrophobic substances. However, their hydrophilic characteristic can be changed by etherifying them by hydrophobic groups or negative charged groups such as n-octenyl succinic anhydride (n-OSA). n-OSA starch contains hydrophobic octenyl side chain which conveys emulsifying property to the starch. (Qi and Xu, 1999)

The substitution of the hydrophobic group along the starch chins created the formation of emulsions with tight alignment of the polymer around an oil droplet. This greatly stabilizes the emulsion, a significant factor for encapsulation of oil. (Kenyon, 1995)

US FDA approved OSA starch for food use in 1972. The regulation limits OSA treatment to 3 percent of starch. This level can create a degree of substitution in the range of 0.02. The chemical structure of n-OSA starch is shown in Figure 2.8.

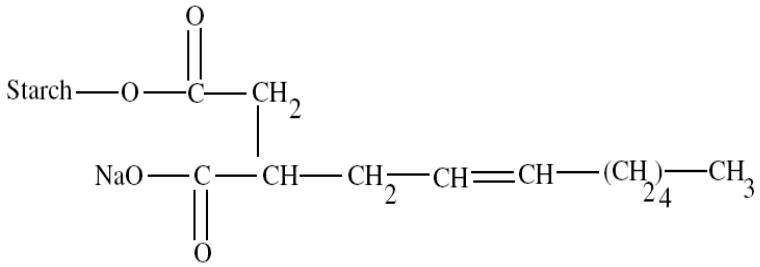


Figure 2.8 Structure of starch octenyl succinate

Source: Qi and Xu (1999)

Chattopadhyaya et al. (1998) used oxidized starches which was prepared from corn and waxy amaranth starch, acacia gum and amiogum 688 (a known gum arabic substitute) for encapsulation of vanillin by using spray drying. They reported encapsulation efficiency of oxidized corn and amaranth starch is better than gum arabic; amiogum 688 and both the oxidised starches and amiogum 688 were free flowing and using oxidised starch in encapsulated flavours with advantages such as freedom from hygroscopicity.

Loksuwan (2006) tested the ability of acid-modified tapioca starch, native tapioca starch, and maltodextrin for encapsulating β-carotene. The author found that the particle size distribution of the capsules produced from acid modified tapioca starch was broad range and its particle size was the smallest diameters. The acid modified tapioca starch capsules had the highest total β-carotene content, whereas the total β-carotene content of was maltodextrin particles was lowest. In addition, the surface β-carotene content of acid modified tapioca starch capsules was the lowest, while the capsule produced native tapioca starch has highest surface β-carotene content. The author concluded that the acid modified tapioca starch was higher efficient than its native starch in field of β-carotene retention and

the acid modified tapioca starch can be considered as potential coating material for β-carotene encapsulation.

Jafari et al. (2007) used maltodextrin mixed with a surface-active biopolymer (modified starch or whey protein concentrate) for coating fish oil. They observed that modified starch was better than whey protein concentrate since it resulted in fish oil capsule with less surface oil.

Pagola et al. (2009) used the three different modified starches (acetylated, n-octenylsuccinylated (n-OSA) and phosphorylated waxy maize) for encapsulation of flavoring agents by spray drying. They found that n-OSA starch was the good emulsifying capabilities and was able to retain the 94.75% of oil. For starch phosphates and acetates, they had oil content of 55.75% and 61.31%, respectively.

2.11.3 The ratio of core material to coating material

The amount of wall material used in synthesis of particles also has an effect on the characteristic of particles. Many studies investigated the ratio coating material to core material.

Gadet et al. (2004) encapsulated safou oil in 6DE maltodextrins at different ratios (oil:maltodextrins; 30:70; 40:60; 50:50; and 60:40) and storied them for 2 months in at four temperatures (4, 20, 30, and 50^{O}C) to evaluate the stability. They observed that the optimum ratio was 50:50 and a 6DE maltodextrin matrix provide the good protection against oxidation, at 50 ^{O}C.

Kaushik and Roos, (2006) studied encapsulation of limonene in various kinds of coating material and two different ratio of total solid to limonene (9:1 and 8.5:1.5 w/w) by using freeze-drying. They found that highest limonene content (75.3%) was retained in the particle which was prepared by the mixture of acacia gum and limonene the ratio of 9:1 (w/w). For a mixture of gelatin–sucrose–acacia gum (0.66:0.17:0.17), the highest amount of entrapped limonene (71.8%) is in the proportion of 8.5:1.5 (TS: limonene).

2.12 Properties of microcapsule

2.12.1 Size is an important characteristic of particle because it influences the core material loading and release. The smaller particles have greater risk of aggregation of particles during storage. The particle size have effected on wall

material deterioration. The larger particles tend to faster degradation and the core material release. The methods for determining particle size are photon-correlation spectroscopy or dynamic light scattering and scanning or transmission electron microscopy (SEM or TEM).

2.12.2 Morphology: To observe the morphology of particle, the scanning or transmission electron microscopy (SEM or TEM) are normally employed. The morphology of capsules is widely characterized because the morphology of particle relates to many capsule properties, especially the stability of particles, release behavior and powder handle properties.

The formation of dented surfaces of spray-dried particles results from the rapidly decreasing drying rate of materials during the drying process. A dented surface contributes to a good protection of active material against oxygen transfer and possible degradation. Formation of spherical particle with smooth surface can be achieved by using plasticizers. (Gharsallaoui et al, 2007)

Jafari et al. (2007) found that powder particle containing whey protein concentrate has a smooth surface and less shrinkage than modified starch (Hi-cap). It means that the wall matrix solidification of fish oil encapsulated powder containing whey protein concentrate was a slowly rate and it is a higher elasticity of these wall systems.

2.12.3 Total core material content: The perfect particle system should have a large core material-containing in particles. The entrapment efficiency is normally expressed as percentage of the core material entrapped into the particles referred to the initial amount of core material used for synthesis particles.

2.12.4 Surface core material content: For encapsulating process, the presence of core material on the surface of the particles is the most undesirable property of encapsulated powders since the surface core material easily contact to the environment. This leads to the deterioration of core material and the loss of core material.

For entrapment of oil, the oil on the surface easily exposes to oxidation; therefore, the rancidity and an off flavor are developed. Furthermore, the wettability and dispersability of the powder are reduced by the surface oil (Jaferi et al, 2007).

2.13 Lipid oxidation

Lipid oxidation is a problem in food because it produces compound that degrade product quality, alter textural properties, and adversely affect the color and nutrition of food. In foods, unsaturated fatty acids; typically those once esterified to the glycerol backbone of a triacylglycerol (TAG) or phospholipid; decompose into volatile compounds with low molecular weights that produce off-aroma associated with rancidity. Ultimately, lipid oxidation reduces shelf-life and therefore causes food spoilage, an important factor in food security as understood to be the availability to, and accessibility of, high quality food.

Susceptibility of fatty acids to lipid oxidation increases with the degree of unsaturation due to increasingly lower bond dissociation energies of methylene-interrupted carbons (McClements and Decker, 2008) Polyunsaturated fatty acids (PUFAs) are generally viewed as healthier for consumers, but if manufacturers abide by the latest Dietary Guidelines (WHO, 2003; EFSA, 2007; U.S. Dept. of Health and human Services, 2010) to reduce solid fats by simple subtitling PUFAs for saturated fats, they risk severely decreasing food acceptability and shelf-life.

Although rancidity has been studied in many type of foods, little systematic research has been conducted on low-moisture food; those with water activity (a_w) below 0.5 and whose shelf-life is primary limited by lipid oxidation and non-enzymatic browning (Labuza et al., 1970). The process by which lipid oxidation proceeds has been extensively reviewed elsewhere (Labuza and Dugan, 1971; Kamal-Eldin, 2003; Frankel, 2005; McClements and Decker, 2008). Briefly, lipid oxidation is characterized by three phases: initiation, propagation, and termination. Initiation begins when hydrogen is abstracted from a fatty acid, thereby generating an alkyl radical ($R^{\bullet}$). Typically, abstraction occurs at a methylene-interrupted carbon of a PUFA, where the covalent bond strength between hydrogen and its methylene carbon is reduced. As more double bonds are added to the fatty acids, oxidation susceptibility increases due to the addition of more methylene-interrupted carbon reaction sites. Research has shown that the addition of one double bond to a PUFA at least doubles the rate at which the fatty acid oxidizes (Holman and Elmer, 1947; Bolland, 1948; Buttery et al., 1961), with the cis form often oxidizing more readily than the trans form (Sargis and Subbaiah, 2003). Therefore, the likelihood of lipid oxidation and

formation of deleterious sensory products increases with increasing unsaturation. Enzymes, metals, metalloproteins, light, high processing temperatures, and irradiation can all promote lipid oxidation in the initiation stage as they primarily accelerate oxidation via reactions that produce free radicals and/or reactive oxygen species. After initial hydrogen abstraction from the fatty acid, the energy of the resulting alkyl radical is decreased by isomerization to form conjugated double bonds. This is followed by propagation where the alkyl radical undergoes addition with atmospheric oxygen to form a peroxyl radical ($ROO^{\bullet}$). This peroxyl radical has sufficient energy to promote the abstraction of hydrogen from another unsaturated fatty acid, thus forming another alkyl radical and a lipid hydroperoxide (ROOH). Thus, propagation involves the transfer of a free radical from one fatty acid to another. Lipid hydroperoxides themselves, however, are not volatile and therefore do not contribute to rancid aromas. ß-scission reactions promoted by heat, light, and transition metals-cause decomposition of the lipid hydroperoxides into alkoxyl radicals ($RO^{\bullet}$). This step represents the formation of a second free radical that can attack additional fatty acids and causes an exponential increase in oxidation rates. In addition, alkoxyl radicals are high energy allowing them to break the aliphatic chain of the fatty acid and generate the low molecular weight volatiles that are associated with the characteristic rancid smell of oxidized fats.

Many researchers measure both hydroperoxide content and aldehyde content in final products when determining the extent of lipid oxidation to monitor both propagation and ß-scission reactions. During the termination phase, two radicals react to form one, non-radical molecule, such as fatty acid dimers, trimers, and oligomers. Consequently, these polymers often either precipitate or lead to increases in oil viscosity. Polymeric products are common in frying oils, where the high temperatures reduce oxygen solubility and thus minimize hydroperoxide formation and therefore b-scission reactions. Termination reactions are not as important in other foods because higher oxygen allows b-scission reactions to predominate and because most foods are deemed rancid before termination reactions are significant. When monitoring lipid oxidation over time, one typically observes a lag phase where accumulation of lipid oxidation products is slow. This lag phase is the result of the slow formation of free radicals prior to hydroperoxide accumulation and b-scission reactions and also the

presence of antioxidants that are preferentially oxidized and thus prevent free radicals from attacking fatty acids. Food manufacturers strive to maximize the duration of the lag phase where the concentration of the products responsible for rancidity are below sensory threshold levels. Therefore, the lag phase is the best representation of the oxidative shelf life of a food product.

2.14 Accelerated shelf-life tests (ASLT)

Traditionally, deterioration rates at elevated temperatures are extrapolated to lower ones assuming that their temperature dependence obeys the Arrhenius equation, or a similar model. For such methods to succeed, the spoilage kinetics must be known in advance and be completely defined by a single rate constant that must be independent of the food's thermal history. These assumptions may hold for certain foods and model systems, but they cannot be universally applicable. In a proposed alternative approach, no kinetics is assumed at all and it is taken for granted that the food's thermal history can and does affect the deterioration pattern. Consequently, the isothermal chemical degradation or microbial growth data are described in terms of empirical models that have the needed number of temperature dependent parameters, usually two or three parameter. ASLT is applicable to any deterioration process using a kinetic model. ASLT The shelf-life of edible fats and oils at ambient conditions has been estimated by plotting the logarithm of oxidative stability results versus high temperatures and extrapolating to room temperature. It has been shown that extrapolation from the Rancimat values (oxidative stability index, OSI) to ambient conditions lead to either over-prediction or under-prediction of the actual shelflife depending on the fatty acid composition of the oils (Kaya, Tekin, & Oner, 1993). Mizrahi (2004) explains the principles underlying the method, presents several mathematical models to estimate the deterioration rate of stored foods and alerts the reader to potential sources of error in the use of accelerated storage data.

Man (2002) defines shelf life as the period of time, under defined conditions of storage, for which a food product will retain its desired sensory, chemical, physical, functional, or microbiological characteristics. When studying the shelf life of oil, it is important to take into consideration all the factors that will contribute to degradation. Real-time storage studies are difficult to perform due to the time required for high-

stability oil to deteriorate. Accelerated storage tests have been developed to increase the rate of deterioration and shorten the time required to achieve significant changes in quality, thus enabling extrapolation of deteriorative rates to expected storage temperature.

During storage tests, indicatory measurements are taken to determine the rate of deterioration during storage. Labuza and Schmidl (1985) report that analytical tests during an accelerated shelf-life test must be based on the mode of deterioration of the test product in order to be valid indicators of degradation. Choice of test should be established based on what is reported in the literature. Testing includes specific chemical analyses and sensory evaluation to determine the end of shelf life. Oxidative rancidity is the primary mechanism that affects stability of properly processed and packaged vegetable oils. Shelf life of vegetable oil is dependent on many factors: fatty acid content, temperature of storage, exposure to light, presence of free-fatty acids, oxygen concentration, presence of antioxidants, and presence of pro-oxidants (McClements and Decker 2008). To understand what tests would be best to perform over the course of the study, some background on oxidation of vegetable oils, specifically oils with a high content of oleic acid, is required.

CHAPTER 3
MATERIAL AND METHOD

3.1 Raw material

3.1.1 Rice bran oil soap stock (RBOS)

Rice bran oil soap stock (RBOS) used as the starting material for preparation of RBOS in part 1 was obtained from Kasisuri Co., Ltd, Thailand.

3.1.2 Biopolymer and food grade ingredients

3.1.2.1 Whey protein concentrate (WPC) was purchased from Siam Whey Co., Ltd. Thailand. The powdered WPC had a composition of 82.03% protein, 2.70% ash, and 6.75% fat (dry weight basis) and 4.91% moisture (wet weight basis).

3.1.2.2 Maltodextrin (MD) with a DE of 10 was purchased from Nutrition SC Co., Ltd. Thailand.

3.2 Chemicals

3.2.1 γ-oryzanol, cumene hydroperoxide, 3-hexanol, and tocopherol purchased from Sigma (Sigma-Aldrich, USA)

3.2.2 Sodium hydroxide, hexane, i-propanol, acetone, ethyl acetate, petroleum ether, potassium iodine, methanol, ethanol, hydrochloric acid, butanol and isooctane purchased from Merck (Merck, Germany), Fluka (Fluka, Switzerland), APS AJAX FINECHEM (NSW, Australia) and Fisherbrand (Fisher Scientific, USA)

3.2.3 Ferrous sulfate, Barium chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH)

3.2.4 Ethyl acetate commercial grade (Etulmar.,LTD, Thailand)

3.3 Instruments

3.3.1 Hot air oven (Memmert, Modell 100-800, Germany)

3.3.2 pH meter (FiveEasy^{TM} Plus FEP20, Switzerland)

3.3.3 Spectrophotometer (T60, PG Instrument limited, Canada)

3.3.4 Mini-spray dryer (Buchi Mini spray dryer B-191, Switzerland)

3.3.5 Solvent extraction unit (Soxhlet apparatus, VELP Scientifica, SER 148 Series, Italy)

3.3.6 Ultrasonic extractor (Transsonic Digitals, Elma, Mascot, NSW)

3.3.8 High Performance Liquid Chromatography (HPLC) (1200 series Agilent Technologies, USA)

3.3.9 High speed homogenizer (SPX Lab Homogenizer APV-2000, UK)

3.3.10 Hot plate stirrer (MS7-H550-S, SCILOGEX, USA)

3.3.11 Microscope (Nikon microscope Eclipse E-400, Nikon Corporation, Japan)

3.3.12 Scanning electron microscopy (SEM) (Magellan 400, FEI Company, USA)

3.3.13 Colorimeter (Hunter Lab, Color Flex, USA)

3.3.14 Water activity analyzer (PS200 S/N 9809020, Novasina, USA)

3.3.15 Rotary evaporator (Buchi rotary evaporator R-300, Switzerland)

3.3.16 Gas Chromatography (Shimadzu AOC-6000, Japan)

3.3.17 Water bath (Memmert, Germany)

3.3.18 Zetasizer (ZEN3600, Malvern Instruments, and Worcestershire, UK)

3.3.19 Viscometer (DV2T Viscometer, Brookfield, Germany)

3.3.20 Centrifugal (CL10, Thermo, USA)

3.3.21 4-Decimal electronic balance (BSA2245-cw, Sartorius, Germany)

3.3.22 Vortex mixer (model G 560E, vortex-2-Genie, scientific industries, USA.)

3.4 Methods

This research work was contained 4 parts;

Part 1 Optimization of extraction and purification of γ-oryzanol from Rice Bran Oil Soapstock (RBOS)

3.4.1 Preparation of RBOS

The RBOS and saponified dehydrated rice bran oil soapstock (RBOS-SD) are shown in Figure 3.1. RBOS-SD was prepared using a modified method of Kaewboonnum (2010). RBOS sample (100g) were added 2 N NaOH solution (100 mL) whose extract quantity require for the reaction can be calculated based on the

saponification value determine using AOCS official method (1989). The reaction was carried out at 80 °C and constant stirring over the period of 2 h. After reaction was completed, the saponified RBOS samples were adjusted to pH 9.5 using 0.1N HCl and then dehydrated using hot air oven at 90 °C for 8 h. The RBOS was passed through a sieve (60 mesh) and stored in a refrigerator (4 °C) for use as required.

RBOS and RBOS-SD were characterized by the following analyses: moisture content (AOAC, 2000), fat content (AOAC, 2000), pH (AOAC, 2000), saponification value (AOCS, 1989) and γ-oryzanol content (Kaewboonnum, 2010)

Figure 3.1 Rice bran oil soapstock (a) and saponified dehydrated soapstock (RBOS-SD) (b)

3.4.2 Extraction Methods

Diagram for the extraction process of γ-oryzanol from RBOS is shown in Figure 3.2. There are three extraction methods involved in the study: (3.4.2.1) soxhlet extraction, (3.4.2.2) ultrasonic assisted extraction and (3.4.2.3) ultrasonic combination with soxhlet extraction

3.4.2.1 Soxhlet extraction

The soxhlet extraction was performed according to Jesus et al. (2010) with modifications. The extraction process were performed at different sample to solvent ratio (1:5, 1:10 and 1:15 w/v), extraction temperature (60, 70, and 80 °C), and extraction time (4, 6, and 8 h). The RBOS-SD sample and solvent (ethyl acetate) were placed into a soxhlet apparatus and kept under extraction times.

Box–Behnken design for three factors and three levels required 16 experiments corresponding to the mid-point of edge and the four replications at the center points of the cube. All of 16 experiments with three independent factors and their three different levels and the responses were shown in Table 3.1.

Table 3.1 Coded and uncoded levels of independent variables (soxhlet extraction conditions) in Box-Behnken experimental design for 16 treatment runs

Treatment runs	Sample to solvent (w/v)		Extraction temperature (°C)		Extraction time (h)	
	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded
1	-1	1:5	-1	60	0	6
2	1	1:15	-1	60	0	6
3	-1	1:5	1	80	0	6
4	1	1:15	1	80	0	6
5	-1	1:5	0	70	-1	4
6	1	1:15	0	70	-1	4
7	-1	1:5	0	70	1	8
8	1	1:15	0	70	1	8
9	0	1:10	-1	60	-1	4
10	0	1:10	1	80	-1	4
11	0	1:10	-1	60	1	8
12	0	1:10	1	80	1	8
13	0	1:10	0	70	0	6
14	0	1:10	0	70	0	6
15	0	1:10	0	70	0	6
16	0	1:10	0	70	0	6

Experimental design and statistical analysis: Box-Behnken design (BBD) with three independent variables was used for optimization. The parameters sample to solvent ratio, extraction temperature and extraction time were chosen as key variable based on the results of preliminary experiments and designed as X_1, X_2, and X_3, respectively, as show in Table 3.1. The complete quadratic equation was used is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1 X^2_1 + \beta_2 X^2_2 + \beta_3 X^2_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \quad (3.1)$$

Where, Y is the estimated response (% yield of γ-oryzanol),

β_0 is the constant term,

β_i is the linear coefficient (i = 1-3),

β_{ij} is the quadratic coefficient (i = 1-3 and j = 1-3),

ε is the random error.

3.4.2.2 Ultrasonic assisted extraction (UAE)

Ultrasonic assisted extraction was done based on Khoei and Chekin (2016) with some modifications. The γ-oryzanol was extracted from RBOS-SD using ethyl acetate with the suitable sample to solvent ratio (w/v). The mixture was mixed by hand homogenizer for 1 min and then placed into ultrasonic bath at different ultrasonic frequency, extraction temperature (60, 70 and, 80 °C), and extraction time (30, 45, and 60 min).

Experimental design and statistical analysis: Box-Behnken design (BBD) with three independent variables was used for optimization. The parameters types of solvent, extraction temperature and extraction time were chosen as key variable based on the results of preliminary experiments and designed as X_1, X_2, and X_3, respectively, as show in Table 3.2. The complete quadratic equation was used is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1 X^2_1 + \beta_2 X^2_2 + \beta_3 X^2_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \quad (3.2)$$

Where, Y is the estimated response (% yield of γ-oryzanol),

β_0 is the constant term,

β_i is the linear coefficient (i = 1-3),

β_{ij} is the quadratic coefficient (i = 1-3 and j = 1-3),

ε is the random error

Box–Behnken design for three factors and three levels required 16 experiments corresponding to the mid-point of edge and the four replications at the center points of the cube. All of 16 experiments with three independent factors and their three different levels and the responses were shown in Table 3.2.

Table 3.2 Coded and uncoded levels of independent variables (Ultrasonic-assisted extraction conditions) in Box-Behnken experimental design for 16 treatment runs

Treatment runs	Ultrasonic frequency (kHz)		Extraction temperature (°C)		Extraction time (min)	
	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded
1	-1	10	-1	60	0	45
2	1	14	-1	60	0	45
3	-1	10	1	80	0	45
4	1	14	1	80	0	45
5	-1	10	0	70	-1	30
6	1	14	0	70	-1	30
7	-1	10	0	70	1	60
8	1	14	0	70	1	60
9	0	12	-1	60	-1	30
10	0	12	1	80	-1	30
11	0	12	-1	60	1	60
12	0	12	1	80	1	60
13	0	12	0	70	0	45
14	0	12	0	70	0	45
15	0	12	0	70	0	45
16	0	12	0	70	0	45

For SE and UAE, after the extraction process the solvent was evaporated from the extract using a rotary vacuum evaporator before determine of γ-oryzanol extracted.

After the multifactor analysis of variance and the 2^{nd} order model prediction determinations, the optimal extraction conditions were obtained by the desirability function approach using Minitab statistical software v.16 (Minitab Inc., USA). The response surface plots were developed using the Statistica program (Statistica version 7.0, Statsoft Inc., Tulsa, OK, USA) and represented a function of two independent variables while keeping the other two independent variables at optimal values.

3.4.2.3 Ultrasonic and soxhlet extraction (combination)

For the ultrasonic and soxhlet extraction experiment was carried out ultrasonic and soxhlet extraction apparatus. RBOS-SD sample was extracted using optimal condition of SE and UAE as chosen from section 1 and 2. Briefly, five gram of RBOS-SD sample was soaked in ethyl acetate and then extraction was carried out using optimal condition of UAE as chosen from section 2. After extraction process, the sample was filtered using rotary evaporator under vacuum (300 mmHg) and the filtrate was collected at 4 °C. The residue was extracted with ethyl acetate using optimal conditions of SE as chosen from section 1 and the extract was filtered. The obtained filtrates were pooled together before evaporation.

In addition, analysis of variance (ANOVA) at 0.05 significant levels were used to analyze the experimental data by using SPSS program (version 22) and Duncan's multiple range test (DMRT) at 0.05. P-value was used to test the differences between means.

3.4.2.4 Determination purity of γ-oryzanol content

Two methods were used to determine the quantification of γ-oryzanol content in the samples.

UV-spectrophotometric analysis: The γ-oryzanol content was performed by UV-spectrophotometric according to Zullaikah et al. (2009). Absorbance of the sample (0.1 g) solution in ethyl acetate (10 ml) was measured at the wavelength of 320 nm in 1-cm cell using UV-Visible spectrophotometer. The γ-oryzanol (pure, Sigma Chemical Co, USA) was used as a standard.

High Performance liquid chromatography (HPLC): The γ-oryzanol content was determined according to Tuncel and Yilmaz (2010) using Agilent 1200 Series HPLC, diode-array detector (DAD) and autosampler. Data analysis was performed using Open LABCDS EZChrom software. Separation was achieved at 25 ºC on a Poroshell120 EC-C18, 3.0×45 mm, 2.7μm (Agilent Technologies, USA). The mobile phase consisted of isopropanol-methanol (30:70v/v) and was pumped at a flow rate of 1.0 mL/min. The injection volume of 20 μL was injected into the system. The quantitation wavelength was set at 325 nm.

The yield, purity, and recovery of γ-oryzanol were calculated from the following equations:

$$\text{Yield of } \gamma\text{-oryzanol (\%)} = \left(\text{Amount of } \gamma\text{-oryzanol in extract}\right)\times 100 \qquad (3.3)$$

$$\text{Purity of } \gamma\text{-oryzanol (\%)} = \left(\frac{\text{Amount of } \gamma\text{-oryzanol in extract}}{\text{Weight of sample}}\right)\times 100 \qquad (3.4)$$

$$\text{Recovery (\%)} = \left(\frac{\text{Amount of } \gamma\text{-oryzanol in extract}}{\text{Amount of } \gamma\text{-oryzanol in initial RBOS}}\right)\times 100 \qquad (3.5)$$

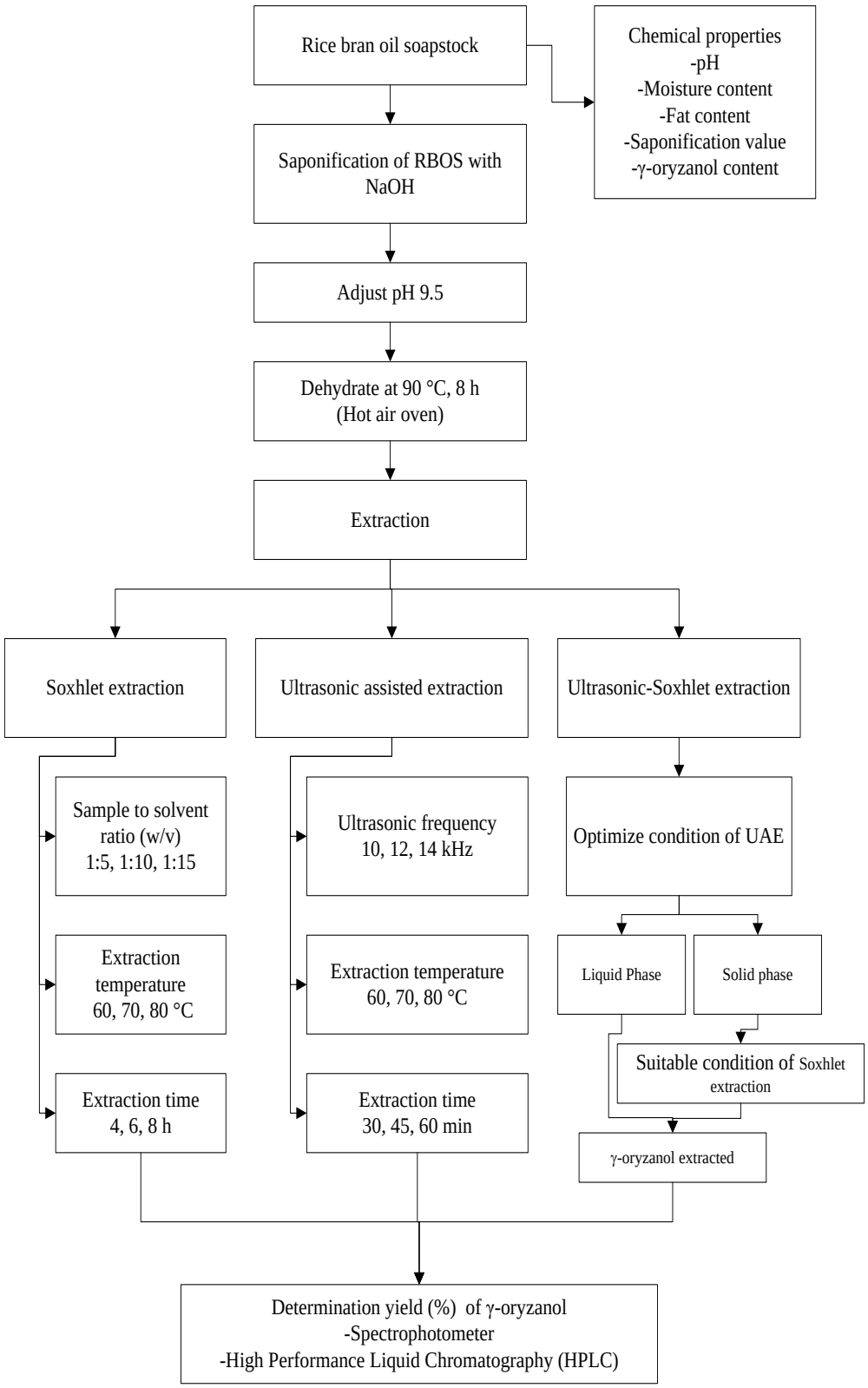


Figure 3.2 Flow chart of procedures for extraction process γ-oryzanol from RBOS.

3.4.3 Purification

γ-oryzanol enriched fraction was obtained, which was then purified further through two-step crystallization (Figure 3.3) with some modifications with Zullaikah et al. (2009). The first crystallization step was to precipitate the mucilaginous impurities including waxes and gums that would otherwise, by disrupting the crystal growth, interfere with the crystallization of γ-oryzanol in the second step. The supernatant was crystallized again in the second step to obtain γ-oryzanol crystals with increased purity.

3.4.3.1 First Crystallization

In the first crystallization, γ-oryzanol enriched fraction (10g) with 50 ml ethyl acetate: methanol (3:7, 4:6 and 5:5) in a 250 ml glass beaker with the magnetic stirring 1,000 g. for 1 h at ambient temperature. The solution was stored at -22 °C for 24 h. Liquid phase and solid phase were separated by filtering though filter paper under vacuum. The liquid phase was used to the second step, while the solid phase was used to determine the percentage of γ-oryzanol loss.

3.4.3.2 Second Crystallization

The liquid phase obtained from the first step was kept at 5 °C for 16-72 h to allow the growth of γ-oryzanol crystal. The γ-oryzanol rich product and white γ-oryzanol crystal were separated by filtering though filter paper (No. 1) at ambient temperature. All white γ-oryzanol crystal was stored at -20 °C before quantified for γ-oryzanol content using HPLC. The γ-oryzanol content quantification was performed by HPLC/UV according to Tuncel and Yilmaz (2010) as those described above.

3.4.3.3 Statistical Analysis

Analysis of variance (ANOVA) was performed in a completely randomized design, using Duncan's Multiple Range Test. All determinations were done at least in triplicate and all were averaged. The confidence limits use in this study was based on 95 % ($p<0.05$) using SPSS program (version 22).

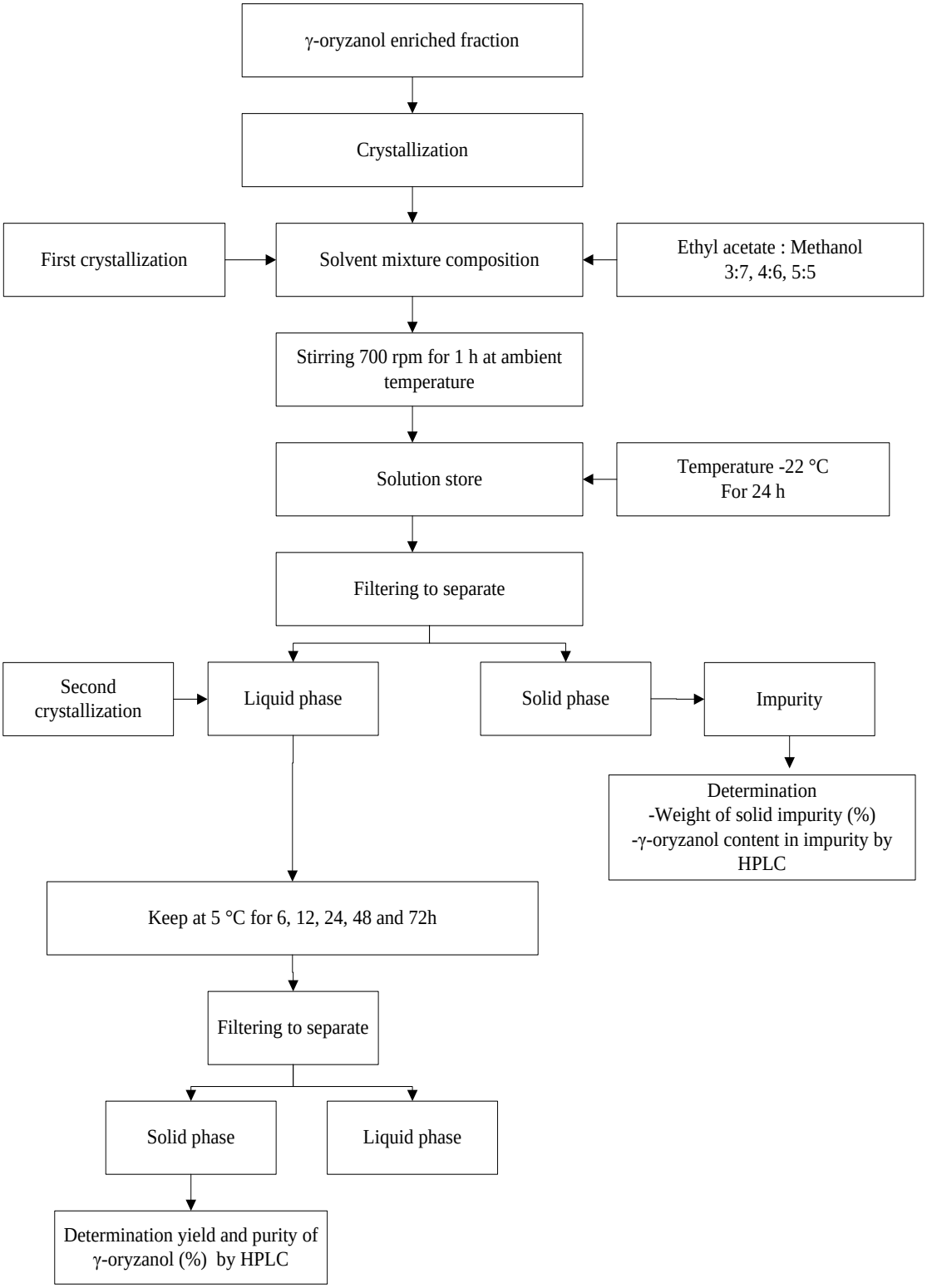


Figure 3.3 Flowchart of purified γ-oryzanol extract from dehydrated saponified rice bran oil soapstock

Part 2 Production of of γ-oryzanol extract powder and antioxidant activity of γ-oryzanol extract powder

3.4.4 Production of γ-oryzanol extract powder

The research work is to produce spray-dried γ-oryzanol/rice bran oil emulsion encapsulated with interfacial membranes using maltodextrin and whey protein as wall materials. Yield, encapsulation efficiency and characterization of powder were measured.

3.4.4.1 Preparation of encapsulated γ-oryzanol extract powder

Aqueous phase were prepare by dissolving 0.2-1.0% wt of WPC in aqueous phase into a 5 mM phosphate buffer, pH 7, containing 10% w/v maltodextrin (MD) followed by stirring for 30 min at ambient temperature and then adjusted to pH 7 using either 0.1 N HCl or NaOH. The γ-oryzanol/rice bran oil (oil-in-water emulsions) were prepared by blending 20% rice bran oil (with 2% γ-oryzanol extracted in rice bran oil) and 80% aqueous phase , that was made by blending the oil phase and aqueous phase for 2 min (T25 digital ultra turrax, IKA, Germany) and passing through a two stage high-pressure homogenizer (SPX Lab Homogenizer APV-2000, UK) at 3,000 psi for 2 times.

The emulsion were feed into spray drier (Buchi Mini spray dryer B-191, Switzerland) at a rate of 17-18 mL/min. The drying conditions were had inlet air temperature of 180 ± 2 °C and the outlet air temperature of 90 °C. The dried powders were collected in laminated pouches and stored at room temperature until analysis.

3.4.4.2 Determination of emulsion characterization

Creaming index: emulsion samples (10 ml) were place in the glass test tube and then were stored at ambient temperature for 24 h before analysis. The susceptibility of the emulsion for creaming was ascertained by measuring the high of boundary layer between the opaque droplet-rich layer at the top and the transparent or turbid droplet-depleted layer at the bottom of the test tubes (Demetriades and McClements, 2000). Creaming results were reported as the following equation

$$\text{Creaming index} = \left(\frac{\text{Height of interface}}{\text{Height of total emulsion}} \right) \times 100 \qquad (3.7)$$

Particle size determination and ζ-potential measurement: Mean droplet diameter, particle size distribution and electrical charge (ζ -potential) of the reconstituted emulsions were measured using a dynamic light scattering instrument (Zetasizer Nano, Malvern Instruments Ltd., UK). The measured particle size and ζ -potential were reported as the Z-average diameter. The ζ -potential was determined by measuring the direction and velocity of droplet movement in the applied electric field. To prevent multiple scattering effects, the emulsions were diluted to a droplet concentration of approximately 0.005% using buffer solutions. For ζ -potential measurements, the ζ -potential of each individual sample was calculated from the average of five measurements on the diluted emulsion, and the results were reported as the mean and standard deviation.

3.4.4.3 Characterization of encapsulated γ-oryzanol extract powder.

1) Encapsulation yield (EY): The percent of production yield was calculated from the weight of the encapsulated powders with the total amount of solid mass to be spray dried (Wu et al., 2014).

2) Encapsulation Efficiency (EE): The percent of the encapsulation efficiency was calculated form the ratio of total oil and free oil contents.

The total oil and free oil contents of the powder were determined according to the A/S Nitro Atomizer (2003). This is a standard method which may use for all milk powder and other dry daily products. The 1.5 g of powder was weighted and load into the shaking cylinder. Then, 10 ml of water and 1.5 mL of NH_3-solution were added to mixture. After that the mixture were shacked occasionally and heated in a water bath for 15 minutes to 65 °C.

Ethyl ether (25 ml) was added into the solution, which was mixed by turning the cylinder up and down for 1 minute. The 25 ml of petroleum ether was then added and the mixture was allowed to stand at least 2 h. After that, the ether phase was transferred to an Erlenmeyer flask. This total oil extract was repeated

for 2 times. An Erlenmeyer flask was dried at 102±2 °C until constant weight was reached and the total fat was calculated using the equation shown below.

For free fat content, 5g of powder was weighted and loaded into 250 ml Erlenmeyer flask. The petroleum ether (50 ml) was added to the mixture. The mixture was then shaking by the shaking device for 15 minutes. After the solution was filtered, the filtrate was collected. The 25 ml of the filtrate was transferred into the Erlenmeyer flask and dried in the drying oven for 1 h at 105±2 °C. Then, it was cooled in the desiccator and weighted. The free fat was calculated using the equation shown below.

The % of total fat and free fat was calculated using the equation

$$\text{Total Fat (\%)} = \frac{W_1}{W_2} \times 100 \tag{3.8}$$

$$\text{Free Fat (\%)} = \frac{a \times 25 \times 2 \times 100}{25 - \frac{a}{0.91} \times b} \tag{3.9}$$

Where, W_1 is weight in g of the evaporation residue,

W_2 is weight in the powder used,

a is evaporation residue from 25 mL of solvent,

b is g of powder used.

Then, the Encapsulation Efficiency (EE) was calculated as follows:

$$\text{EE (\%)} = \left(\frac{\text{Total Fat (\%) - Free Fat (\%)}}{\text{Total Fat (\%)}} \right) \times 100 \tag{3.10}$$

3) Moisture content and water activity (a_w): Moisture content of the encapsulated powders was determined using according to A.O.A.C. (2000) and water activity of the powder was measured using a water activity analyzer (PS200 S/N 9809020, Novasina, USA) (A.O.A.C., 2000).

4) Color: Color of the powders was measured using a colorimeter (Hunter Lab, Color Flex, USA) and it was reported as L*, a* and b* values. Each sample was measured 3 times and find average value of color parameter. In this color system, L* value is a measure of lightness to darkness (0 = black and 100 = white); a* is a measure of redness (+) to greenness (-), with a higher positive a* value indicating more redness; and b* is a measure of yellowness (+) to blueness (-), with a higher positive b* value indicating more yellowness.

5) Powder morphology: The overall morphology of the powders was observed by Scanning electron microscope (SEM). The micrographs of the sample were obtained with SEM at 5 kV (Magellan 400, FEI Company, USA)

6) Solubility index (SOL): SOL was determined using a modification of the method of Mandala and Bayas (2004). The encapsulated powders (5 g) were dispersed in 50 mL of 5 mM phosphate buffer pH 7. The reconstituted emulsion was centrifuge at 5000 g for 10 min. Supernatant was separated from precipitated past. The supernatant phase was dried at 105 °C for 24 h. After cooling, the dried solid in supernatant (WS) was weighted. The SOL is the percentage of dry mass of soluble in supernatant to the dry mass of whole sample (WO).

$$\text{SOL (\%)} = \left[\frac{\text{Soluble solid}\left(WS\right)}{\text{Dry mass of whole sample }\left(\text{WO}\right)} \right] \times 100 \qquad (3.11)$$

7) The γ-oryzanol content: For quantification of γ-oryzanol content in the sample, UV-spectrophotometric analysis was used according to Zullaikah et al. (2009) with some modifications. The sample (5g) dissolved in ethyl acetate (15ml) was mixed, then separated only ethyl acetate fraction and evaporation before analyzed. Absorbance of the extract (0.025g) dissolve in ethyl acetate (10 ml) measured at the wavelength of 320 nm in 1-cm cell using UV-Visible spectrophotometer. γ-oryzanol (Pure, Spectrum, USA) was used as a standard.

3.4.4.4 Statistical Analysis

Analysis of variance (ANOVA) was performed in a completely randomized design, using Duncan's Multiple Range Test. All determinations were done at least in triplicate and all were averaged. The confidence limits use in this study was based on 95 % ($p<0.05$) using SPSS program (version 22).

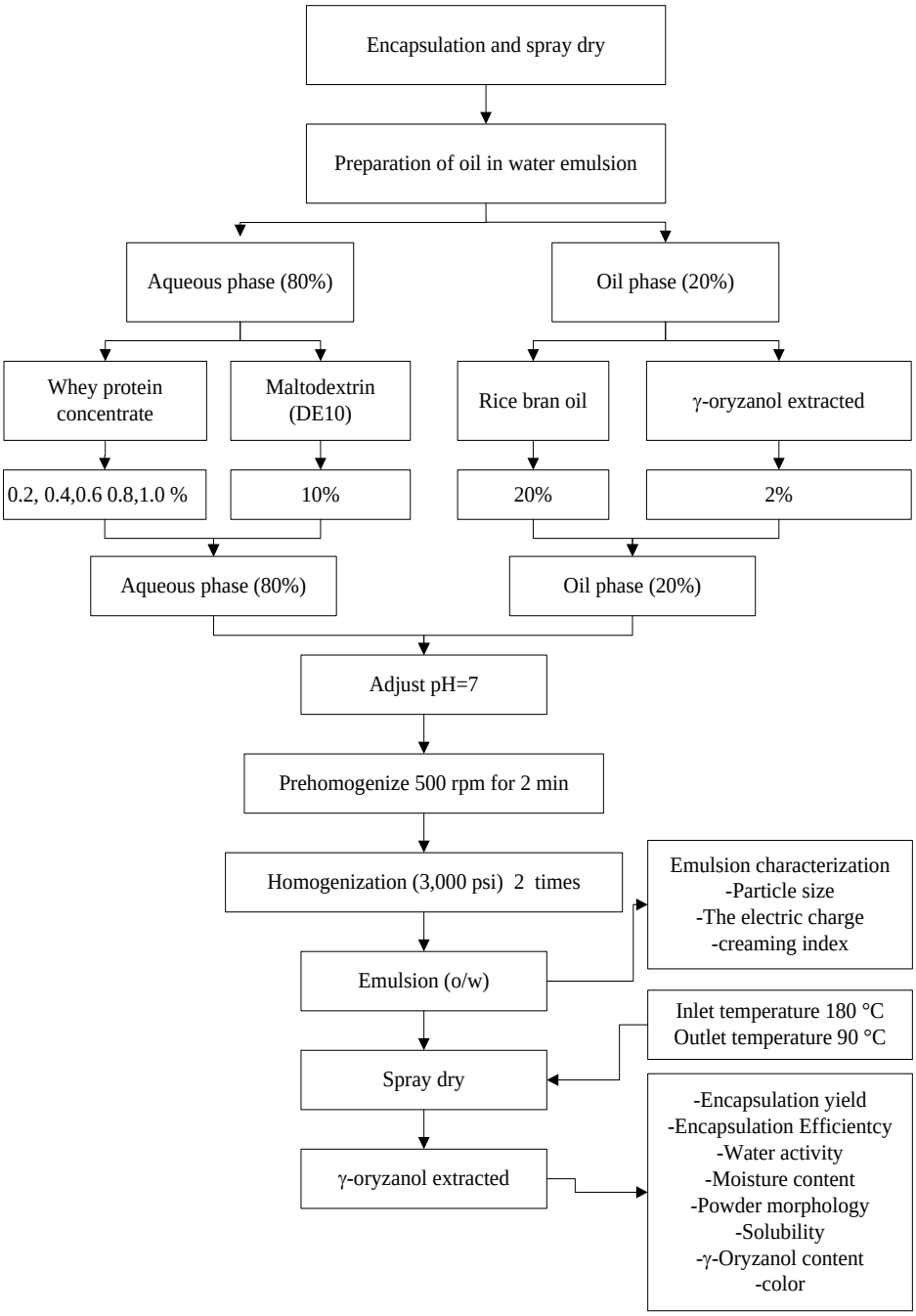


Figure 3.4 Flowchart of procedure of encapsulated γ-oryzanol extract powder

3.4.5 Antioxidant activity of γ-oryzanol extract and the encapsulated γ-oryzanol extract powder

The aim of this section was to determination antioxidant activity of enriched γ-oryzanol extract (part 1) and the encapsulated γ-oryzanol extract powder (part 2). They were measured in comparison with γ-oryzanol pure standard, γ-oryzanol commercial, synthetic antioxidant as BHA and α-tocopherol.

3.4.5.1 DPPH radical scavenging assay

The DPPH radical scavenging activity of the γ-oryzanol extracted powder was determined as described by Xu et al., 2010. Briefly, 0.5 ml extract was mixed with 4.5 ml of 60 μm methanolic solution of DPPH. The mixture was vortexed for 1 min and absorbance of the sample solution was recorded at 517 nm after incubation in the dark for 30 min. DPPH radical scavenging activity was calculated as μg Trolox/g dry sample.

3.4.5.2 ABTS radical cation decolorization assay

For the ABTS radical cation decolorization assay, the procedure was followed the method Wang et al. (2015) with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Sample (1 ml) was allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. ABTS radical scavenging activity was calculated as μg Trolox/g dry sample.

3.4.5.3 Ferric ion reducing antioxidant power (FRAP) assay

This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4- diene chloride (TPTZ) to the ferrous form at low pH. This reduction is monitored by measuring the change in absorption at 593 nm, using a diode-array spectrophotometer. Antioxidant assay can be conducted by the

method developed by Benzie and Strain (1999) with slightly modification. An amount of 200 µl samples were mixed with 3 mL FRAP reagent in test tubes and undergoes vortex. Blank samples were prepared for both methanol and deionized water extracted samples. Both samples and blank were incubated in water bath for 30 minutes at 37°C and the absorbance of the samples was determined against blank at 593 nm. The content of Fe^{2+} was evaluated as expressed as µg Trolox/g dry sample.

3.4.5.4 Statistical Analysis

Analysis of variance (ANOVA) was performed in a completely randomized design, using Duncan's Multiple Range Test. All determinations were done at least in triplicate and all were averaged. The confidence limits use in this study was based on 95 % ($p<0.05$) using SPSS program (version 22).

Part 3 Shelf-life of γ-oryzanol extract powder

This study has focused on quality change during storage of encapsulated γ-oryzanol extract powder was investigated. There is method for evaluation shelf-life of encapsulated γ-oryzanol extract powder indirect method (accelerated method).

Accelerated storage has been a widely used method to assess the shelf-life of foods, pharmaceuticals, cosmetics and many other industrial products of limited durability. Since such products are especially designed to undergo minimal changes during their normal distribution and storage, accelerated storage is perhaps the only practical way to predict when such products have to be withdrawn from the market, if not already consumed. Accelerated storage can also be helpful in predicting the shelf-life of perishable commodities, such as refrigerated dairy or meat products.

3.4.5 Evaluate shelf-life of the encapsulated γ-oryzanol extract powder

There is a method to evaluate shelf-life of γ-oryzanol extract powder accelerated storage shown in Figure 3.5. Samples 20 g were weighted into aluminum foil bag and sealed. The powders were stored at various temperatures; 30°C, 40°C and 50°C for 3 months. Evaluation of shelf-life was conducted at 10 day intervals for color, moisture content, water activity, hydroperoxide, hexanal, γ-oryzanol content and antioxidant activity as described below.

3.4.5.1 Color

Color of encapsulated γ-oryzanol extract powder was measured, using a colorimeter (Hunter Lab) and record by using the CIE color system profile of L*, a*, b*. Changing of color from start sample encapsulated γ-oryzanol extract power can be calculated by finding ΔE value.

$$\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \quad (3.13)$$

Where L = lightness of the sample

a = Greenness (-a) – Redness (+a) of the sample

b = Blueness (-b) – Yellowness (+b) of the sample

L_0, a_0 and b_0 = Color value of initial sample

3.4.5.2 Moisture content and water activity

Moisture content and water activity (a_w): Moisture content of the microencapsulated powders were determined using according to AOAC (2000) and water activity of the powders were measured using a water activity analyzer (PS200 S/N 9809020, Novasina, USA) (AOAC, 2000).

3.4.5.3 Lipid oxidation

Spray-dried γ-oryzanol rice bran oil powders each storage temperature were reconstituted by dissolving 1g of powders in 4 ml of 5mM phosphate buffer pH 7.0. After reconstituted, the emulsion was analyzed for lipid hydroperoxide and hexanal.

1) Hydroperoxide: For lipid hydroperoxide, reconstituted emulsions (5 ml) were placed in lightly sealed screw-cap test tube and allowed to oxidize at 20 °C. Lipid hydroperoxides were measured according to Shantha and Decker (1994) by mixing 300 µl of emulsion with 1.5 ml of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, three times) and isolation of organic solvent phase and centrifugation at 5000 g for 2 min. The organic solvent phase (200 µl) was added to 2.8 ml of methanol/1-butanol (2:1, v/v), followed by 1.5 µl of 3.94 M ammonium thiocyanate and 15 µl of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). The absorbance of the solution was measured at 510 nm, 20 min

after the addition of the iron. Hydroperoxide concentrations were determined using a standard curve made form Cumene hydroperoxide (Sigma Chemical Co.).

2) Hexanal: For headspace analysis, reconstituted emulsion sample (1 ml) were placed into 10 ml headspace vials and sealed with poly (tetrafluoroethylene)/butyl rubber septa. Headspace hexanal was determined (Min et al., 2003) using a Shimadzu AOC-6000 gas chromatograph equipped with a Hewlett-Packard 19395A Headspace sampler. The headspace conditions were as follows: sample temperature, 55 °C; sample loop and transfer line temperature, 110 °C; pressurization, 10 s; venting, 10 s; injection, 1.0 min. The aldehydes were separated isothermally at 70 °C on a HP methyl silicone (DB-1) fused silica capillary column (50 m, 0.31 mm i.d., 1.03 μm film thickness). The splitless injector temperature was 250 °C, and the eluted compounds were detected with a flame ionization detector at 250 °C. Concentrations were determined from peak areas using a standard curve made from authentic hexanal.

3.4.5.4 DPPH radical scavenging activity

The DPPH radical scavenging activity of the encapsulated γ-oryzanol extract powder was determined as described by Xu et al., 2010. Briefly, 0.5 ml extract was mixed with 4.5 ml of 60 μm methanolic solution of DPPH. The mixture was vortexed for 1 min and absorbance of the sample solution was recorded at 517 nm after incubation in the dark for 30 min. DPPH radical scavenging activity (%) was calculated according to the following equation

$$\text{DPPH radical scavenging activity}(\%) = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right] \times 100 \qquad (3.14)$$

3.4.5.5 The γ-oryzanol content

UV-spectrophotometric analysis was used according to Zullaikah et al. (2009) with some modifications. Briefly, 0.5 ml extract was mixed with 4.5 ml of ethyl acetate. Absorbance of the sample solution in ethyl acetate was measured at the wavelength of 320 nm in 1-cm cell using UV-Visible spectrophotometer (T60, PG

Instrument limited, Canada). The γ-oryzanol (Pure, Sigma Chemical Co.) was used as a standard.

3.4.5.6 Accelerated shelf life testing (ASLT)

(1) Kinetic reaction order

The characterization of dried powder was changed during storage, increased or decreased with time and temperature. Food quality was loss may be described by zero or first order (Pua et al., 2008). The data was best fit by zero or first order below;

$$\text{Zero order} \qquad C = C_0 + kt \tag{3.15}$$

$$\text{First order} \qquad \ln(C) = \ln C_0 - kt \tag{3.16}$$

Where C_0 = the initial quality at day 0 after spray drying,

C = the quality after t (time) of stability,

k = degradation rate constant,

t = storage time (days)

(2) Accelerated shelf life analysis

Accelerated shelf life analysis of the product was calculated from the Kinetics reaction with using the Arrhenius equation to calculate shelf life at a temperature of 30, 40 and 50 °C. Arrhenius law:

$$k = k_0 e^{-Ea/RT} \tag{3.17}$$

Where k = rate constant for deteriorative reaction,

k_0 = constant, independent of temperature,

Ea = activation energy (J/ mole),

R = ideal gas constant ($8.314 JK^{-1} mol^{-1}$), and

T = absolute temperature (K).

3.4.5.7 Statistical Analysis

Analysis of variance (ANOVA) was performing in a completely randomized design, using Duncan's Multiple Range Test. All determinations were done at least in triplicate and all were averaged. The confidence limits use in this study was based on 95 % ($p<0.05$) using SPSS program (version 22).

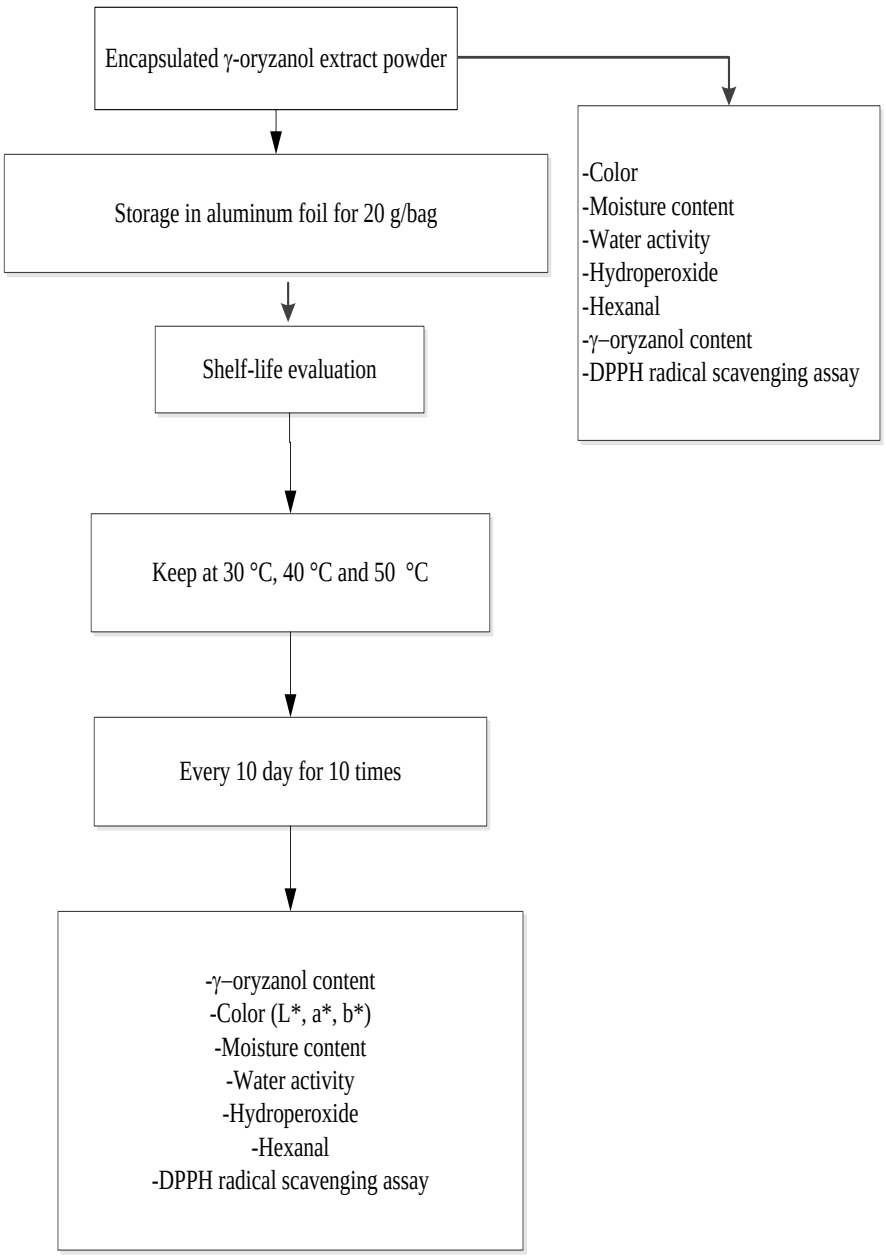


Figure 3.5 Flowchart of procedure for study on shelf-life of γ-oryzanol extracts powder

Part 4 Application γ-oryzanol powder as food ingredient

This part has concentrated on preparation of encapsulated γ-oryzanol powder fortified salad dressing with the utilization of γ-oryzanol as encapsulated γ-oryzanol extract powder. The effect of incorporating γ-oryzanol extract powder to salad dressing on physical properties, chemical properties, antioxidant activity (DPPH assay), sensory quality and stability during storage were investigated.

The aim of this study was evaluated the potential use of the encapsulated γ-oryzanol powder (part 2) as food ingredient. Salad dressing is an important type of water-in-oil food emulsion. This is widely consumed in many other countries around the world.

3.4.6 Preparation of salad dressing with γ-oryzanol powder

The salad dressings contained the following ingredients expressed as a percentage (w/w): rice bran oil 60%, egg yolk 10 %, water 10%, γ-oryzanol powder 5%, sugar 5%, vinegar 5% mustard 1%, salt 1% and enough citric buffer (pH 3) 3% to complete the formulation as shown in Figure 3.6. Preparation was processed as follows: All other ingredients except oil are then add and mix homogeneously. The oil is finally add and emulsification was achieved using an high frequency blender at 13,500 rpm for 3 min. Pasteurization was carried out at 72 °C for 20 s after the emulsions was made. Samples were stored in tightly capped glass bottles at 25°C until further analysis. The physical properties, chemical properties, sensory quality and physicochemical stability of salad dressing were determined.

3.4.6.1 Determination properties

1) Physical Properties

Color of salad dressing was measured, using a colorimeter (Hunter Lab) and record by using the CIE color system profile of L*, a*, b*. Viscosity of salad dressing was measured, using viscometer.

2) Chemical composition analysis of salad dressing with γ-oryzanol powder

Analysis of chemical composition according to AOAC (2000) such moisture, fat, protein and ash was determined.

3) Antioxidant activity

The DPPH radical-scavenging activity of the γ-oryzanol extracted powder was determined as described by Xu et al., 2010. Briefly, 0.5 ml extract was mixed with 4.5 ml of 60 μm methanolic solution of DPPH. The mixture was vortexed for 1 min and absorbance of the sample solution was recorded at 517 nm after incubation in the dark for 30 min. DPPH radical scavenging activity (%) was calculated according to the following equation.

$$\text{DPPH radical scavenging activity}(\%) = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right] \times 100 \qquad (3.18)$$

4) Sensory quality

The sensory analysis of salad dressing was carried out by a consumers (30 persons) by assigning score (9-point hedonic scale) for each quality attributes such as color, viscosity, odor, texture, taste, mouth feel and overall quality. The higher sensory scores indicate the better quality of the salad dressing.

5) Physicochemical stability of salad dressing

Physicochemical stability of salad dressing was evaluated periodically at and interval of 30 day during the storage of 120 days. Moisture content was measured by salad dressing sample (3 g) in hot air oven and dry at 105 °C for 16 h (AOAC, 2000). After it was cooled to room temperature in desiccator, it was weight and dry again. Color of salad dressing was using a color difference meter based on CIE system (L* a* and b*).

The pH was performed according to García-Martínez et al. (2002). Salad dressing (5 g) was dissolved with distilled water (50 ml) and measured pH by pH meter that was calibrated with buffer solutions of pH 4.0 and 7.0 before use. Viscosity of salad dressing (500 ml) was measured using viscometer (DV2T Viscometer, Brookfield), γ-oryzanol content and DPPH radical scavenging activity were determined using Zullaikah et al. (2009) and Xu et al., 2010, respectively. Which was the same procedure described from above. Each measurement was repeated three time, averaged and then statistic analyzed.

6) Statistical Analysis

T-test was performed in an experimental design. All determinations were done at least in triplicate and all was averaged. The confidence limits use in this study was based on 95 % ($p<0.05$) using SPSS program (version 22).

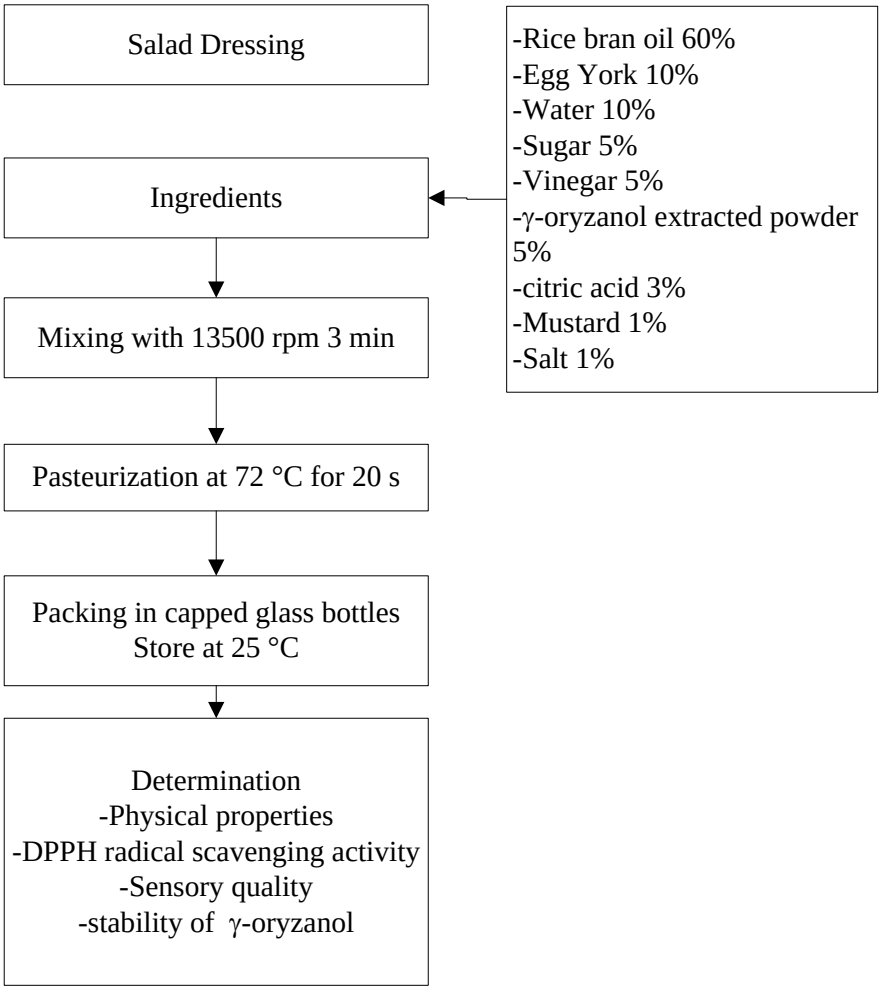


Figure 3.6 Flowchart of procedure for study on the application of γ-oryzanol extracted powder as food ingredient.

CHAPTER 4
RESULTS AND DISCUSSION

Part1 Optimization of extraction and purification of γ-oryzanol from Rice Bran Oil Soapstock (RBOS)

4.1 Raw material characterization

The rice bran oil soapstock (RBOS) used in this study was provided by Kasisuri Co., Ltd. The RBOS sample was yellow-green viscous fluid. In this study, the pH, the moisture content, saponification number, as well as the γ-oryzanol content of the soapstock sample was determined and some characteristics of RBOS are shown in Table 4.1.

The pH of the RBOS sample used in this study was found to be approximately 9.1 because of a chemical refining process by NaOH to remove free fatty acid (FFA). The saponification number was found to be approximately 30.24 (mg KOH/1g sample), which is the number of milligrams of potassium hydroxide required to neutralize the fatty acids resulting from the complete hydrolysis of 1g of fat. It gives information concerning the character of the fatty acids of the fat. The moisture content of the soapstock sample used in this study was found to be approximately 50.01 wt%, thus the other 49.99 wt% was therefore soap, glycerides, and unsaponifiable matters. Of particular interest, the γ-oryzanol content in the RBOS was measured to be approximately 4.9 wt % based on the wet basis, or 7.7 wt % based on the dry basis, while the literature values vary from 1.5-6.5 wt % on the wet basis (Rao et al., 2002; Indira et al., 2005; Narayan et al., 2006). Variation of starting rice bran and different extraction method could lead to difference γ-oryzanol concentration, which plays an important role in its antioxidant activity.

Table 4.1 Physical and chemical properties of rice bran oil soapstock

Properties	Value[a]
pH	9.10±0.00
Saponification Number (mg KOH/1g sample)	30.24±3.11
Moisture (%)	50.01±0.09
Fat (%)	16.03±0.81
γ-oryzanol content (%wet basis)	4.95±0.12
γ-oryzanol content (%dry basis)	9.91±0.18

[a] Mean±standard deviation of triplicate analysis

Raw material pretreatment: Saponified dehydrated soapstock was prepared using NaOH solution and dehydrate by hot air oven. To make the extraction process of γ-oryzanol easier, any remaining glycerides in the soapstock was converted into insoluble soap by saponification. Narayan et al. (2006) reported soapstock contains some amount of glyceride, which if remained in the soapstock, would be extracted into ethyl acetate along with γ-oryzanol, making the following separation step difficult. Therefore, the suitable quantity of NaOH could be estimated based on the saponification value, using a standard method (AOCS Cd 3b-76, 1989), which was found to be 30.24 mg NaOH/g soapstock (or 3.02 wt%).

However, the compound could undergo saponification and could be converted into ferulic acid and sterols (Krishna et al., 2003). Kaewboonnum et al., (2010) also reported after saponification can be found 40.5% loss of γ-oryzanol. There are two reasons for loss of γ-oryzanol could occur during the transfer of viscous material in the saponification process and could be converted into ferulic acid and sterols. The saponification process causes the pH to rise higher than the pka of the γ-oryzanol (pka = 10.8) could be converted into ferulic acid and sterols. Therefore, after the saponification should adjust the pH of the sample to 9.5 to increase the solubility of γ-oryzanol and decrease the loss of γ-oryzanol.

4.2 Optimization of soxhlet extraction

The optimization of the extraction condition represents a critical step in the highest performance to extraction since various parameters affect the soxhlet extraction process. The experiment was performed using Box-Behnken design. Independent variable including solid to solvent ratio (X_1), extraction temperature (X_2) and extraction time (X_3) were selected by prelimination experiment.

The Box–Behnken design for three factors and three levels required 16 experiments corresponding to the mid-point of edge and the three replications at the center points of the cube. All of the 16 experiments with three independent factors and their three different levels and the responses were shown in Table 4.2.

The regression analysis was implemented to fit a full response surface model consisted of all liner term (X_1, X_2, X_3), the quadratic term (X_1^2, X_2^2, X_3^2), and interaction term (X_1X_2, X_1X_3, X_2X_3). The coefficients for the quadratic equations in term of coded units were shown in Table 4.3. The predicted values calculated by these models are presented in Table 4.2.

In order to select the equation model for optimized the extraction, the estimation of several statistical parameters such as the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicted residual sum of squares (PRESS), and lack of fit of each model were considered.

Table 4.2 Box-Behnken design for optimizing conditions of amount of γ-oryzanol coded units together with experimental data (Exp.) and their predicted values (Pred.)

standard order	Code level of variables			γ-oryzanol (% dry basis)	
	X_1	X_2	X_3	Exp.	Pred.
1	0 (1:10)	-1 (60 °C)	-1 (4 h)	7.310±0.05	7.11
2	-1(1: 5)	1 (80 °C)	0 (6 h)	7.074±0.06	7.14
3	-1 (1: 5)	0 (70 °C)	1 (8 h)	7.589±0.11	7.32
4	-1 (1: 5)	-1 (60 °C)	0 (6 h)	6.375±0.22	6.52
5	1(1:15)	0 (70 °C)	1(8 h)	8.392±0.10	8.34
6	1(1:15)	1 (80 °C)	0 (6 h)	7.598±0.22	7.45
7	0 (1:10)	0 (70 °C)	0 (6 h)	8.593±0.08	8.60
8	0 (1:10)	0 (70 °C)	0 (6 h)	8.583±0.17	8.60
9	0 (1:10)	1 (80 °C)	-1 (4 h)	7.990±0.06	7.87
10	0 (1:10)	0 (70 °C)	0 (6 h)	8.636±0.11	8.60
11	0 (1:10)	1 (80 °C)	1 (8 h)	7.180±0.07	7.38
12	1 (1:15)	0 (70 °C)	-1 (4 h)	7.721±0.11	7.99
13	0 (1:10)	0 (70 °C)	0 (6 h)	8.566±0.12	8.60
14	1 (1:15)	-1 (60 °C)	0 (6 h)	8.195±0.06	8.13
15	-1 (1: 5)	0 (70 °C)	-1 (4 h)	7.046±0.15	7.10
16	0 (1:10)	-1 (60 °C)	1 (8 h)	8.075±0.17	8.19

*X_1(Sample to solvent ratio), X_2 (Extraction temperature, °C), X_3 (Extraction time, h)

The influence of three independent variables towards γ-oryzanol content (%dry basis) was reported through the significant ($p \leq 0.05$) coefficient of the second-order polynomial regression equation. The effect of solid to solvent ratio (X_1), extraction temperature (X_2) and extraction time (X_3) was significant ($p \leq 0.05$) in first-order linear effect (X_1), second-order quadratic effect (X_1^2,X_2^2, and X_3^2) and interactive effect (X_1X_2 and X_2X_3). In order to achieve the proper equations, all significant terms

were included and the insignificant terms was eliminated. The regression equation for the γ-oryzanol content (Y) was followed:

$$Y = 8.954 + 0.478X_1 - 0.618\,X_1^2 - 0.666\,X_2^2 - 0.290\,X_3^2 - 0.323\,X_1X_2 - 0.393\,X_2X_3 \quad (4.1)$$

$$R^2 = 0.956$$

Table 4.3 Regression coefficients for the 2nd order response surface models in terms of γ-oryzanol (% dry basis)

Parameter	Term	γ-oryzanol (% dry basis)	
		Coefficient	p-value
β_0	Intercept	8.594	0.000
β_1	X_1	0.478	0.001
β_2	X_2	-0.014	0.863
β_3	X_3	0.146	0.118
β_{11}	X_1^2	-0.618	0.002
β_{22}	X_2^2	-0.666	0.001
β_{33}	X_3^2	-0.290	0.043
β_{12}	X_1X_2	-0.323	0.029
β_{13}	X_1X_3	0.032	0.788
β_{23}	X_2X_3	-0.393	0.013

*X_1(Sample to solvent ratio), X_2 (Extraction temperature, °C), X_3 (Extraction time, h)

Moreover, the analysis of variance (ANOVA) was employed to test the property of the second order response surface models. The ANOVA was used to evaluate the significance of the quadratic polynomial model equation. Any terms in the models with a large *F*-value and small *P*-value would indicate a more significant effect on the respective response variables. The result of analysis of variance (ANOVA) of the quadratic model of amount of γ-oryzanol was shown in Table 4.4. The quadratic regression model was highly significant ($p<0.01$) at the 99% probability level. The regression coefficient (R^2 = 0.956) and adjusted multiple correlation coefficient

(adjusted R^2= 0.890) indicated a good fit between the experimental and predicted yield data. An R^2 value closer to one denotes a better correlation. Thus, the analysis of variance showed that the predicted 2^{nd} order models were statistically suitable. However, the lack of fits model was significant ($p<0.05$) which indicated the lack of models for this experiment. The linear and quadratic variables among solid to solvent ratio, extraction temperature and extraction time showed a significant difference ($p<0.05$), indicating the effect on γ-oryzanol content (%yield).

Table 4.4 Analysis of variance (ANOVA) for the 2^{nd} order response surface model of γ-oryzanol content.

Responses	Source	df	SS	MS	F	p value
Amount of γ-oryzanol (%dry basis)	Regression	9	6.681	0.742	14.510	0.002
	Linear	3	1.998	0.666	13.020	0.005
	Square	3	3.639	1.213	23.710	0.001
	Interaction	3	1.043	0.348	6.800	0.023
	Residual error	6	0.306	0.051		
	Lack of fit	3	0.304	0.101	115.750	0.001
	Pure error	3	0.003	0.001		
	Total	15	6.988			

The optimum conditions for γ-oryzanol content (% dry basis) were provided from the calculation by the response optimizer in Minitab program. The goal of the total the γ-oryzanol content (%) was maximized. For target values, they were 9.04% dry basis of the γ-oryzanol content. Each parameter had the different level of important which were 0.35, 0.01, and 0.13 for sample to solvent ratio, extraction temperature, and extraction time, respectively. The results of the optimization and the predicted responses were shown in Table 4.5.

Table 4.5 Optimization solutions obtained using the response optimizer

	Optimal solution			Predicted response	Experimented response (validation)
	X_1	X_2	X_3	γ-oryzanol content (% dry basis)	
Coded	0.35	0.01	0.13	9.04	8.74±0.05[a]
Uncoded	1:12	70.10 °C	7.26 h		

X_1(Sample to solvent ratio), X_2 (Extraction temperature, °C), X_3 (Extraction time, h)

[a] = Mean±standard deviation of triplicate analysis.

The predicted values of extraction yield were 9.04% (90.52% recovery) and experimental values of extraction yield repeated three times determined by spectrophotometer was 8.74% (87.52% recovery). The results indicated that soxhlet extraction was an effective method for leaching of γ-oryzanol from the dehydrated saponified soapstock as a portion of fresh solvent was repeatedly brought into contact with the sample, thereby mass transfer was enhanced. Kaewboonnum et al., 2010 reported the yield of γ-oryzanol extracted from rice bran oil soapstock using ethyl acetate as solvent that found to be approximately 39.6% recovery. Furthermore, there are several works about extraction RBOS as, Indira et al. (2005) reported a process of dehydration and leaching by ethylacetate and acetone for obtaining γ-oryzanol of 80% (w/w) recovery from rice bran oil soapstock. Moreover, Narayan et al. (2006) also reported a process of crystallization for obtaining 70% recovery from rice bran oil soapstock. Relate to Jesus et al. (2010) propose an improved technique for extraction byproduct from rice bran oil refining. The soxhlet technique was conducted in order to compare results with SFE. The highest yield (3.2% wt) of γ-oryzanol was obtained for soxhlet technique with hexane.

Many factors of extraction is important such as type of solvent, solid to solvent ratio, extraction temperature and extraction time. Moreover, Jesus et al., 2010 reported the suitable of solvent were non-polarity such as hexane and intermediate

polarity such as ethyl acetate (polarity=4.4) that also promoted a high yield of γ-oryzanol content from rice bran oil refining byproduct. In addition, the high temperature of the system (at the boiling temperature of the solvent) could increase the extractability. This loss of γ-oryzanol could be due to the fact that, even though γ-oryzanol is often classified as an unsaponifiable matter, the compound could undergo saponification and could be converted into ferulic acid and sterols (Narayan et al., 2006). Moreover, some γ-oryzanol loss could occur during the transfer of viscous material in the saponification process (Kaewboonnum et al., 2010). The problems encountered during extraction of γ-oryzanol are mainly due to variations in the compositions of RBOS, which include surface-active impurities such as soaps, phospholipids, waxes, and glycolipids (Narayan et al., 2006).

Figure 4.1 shows the estimated response function and effects of the independent variables (X_1, X_2, and X_3) on the dependent variable (extraction yield). The response surface curves were plot to analyze the interaction of the variables and to determine the optimal condition of the responses of the independent variables with maximized γ-oryzanol extract yield. On the basis of equation 4.1, the three dimensional surface values were plotted to represent the effects of the soxhlet extraction variables on γ-oryzanol extract yield. Percentage of γ-oryzanol extract yield increased with increasing solid to solvent ratio and extraction temperature. However, when the solid to solvent ratio was increased to nearly 1:13 and the extraction temperature increased to 72 °C, the γ-oryzanol extract yield slowly decreased as shown in Figure 4.1A. The yield of γ-oryzanol also increased with an increase of solid to solvent ratio and extraction time as shown in Figure 4.1B. Using the interaction effect of extraction temperature and extraction time together was an increase in the amount of γ-oryzanol content. However, at nearly 72 °C and 8 h a small decreased in γ-oryzanol content was observed (Figure 4.1C). The reason being that the γ-oryzanol is degraded by the higher extraction temperature (80 °C) than the lower extraction temperature (70-72 °C). The solvent used in soxhlet extraction can be evaporated and condensed in the same way as the steam distillation, so the efficiency of the soxhlet extraction is higher than the solvent extraction (Imsanguan et al., 2008).

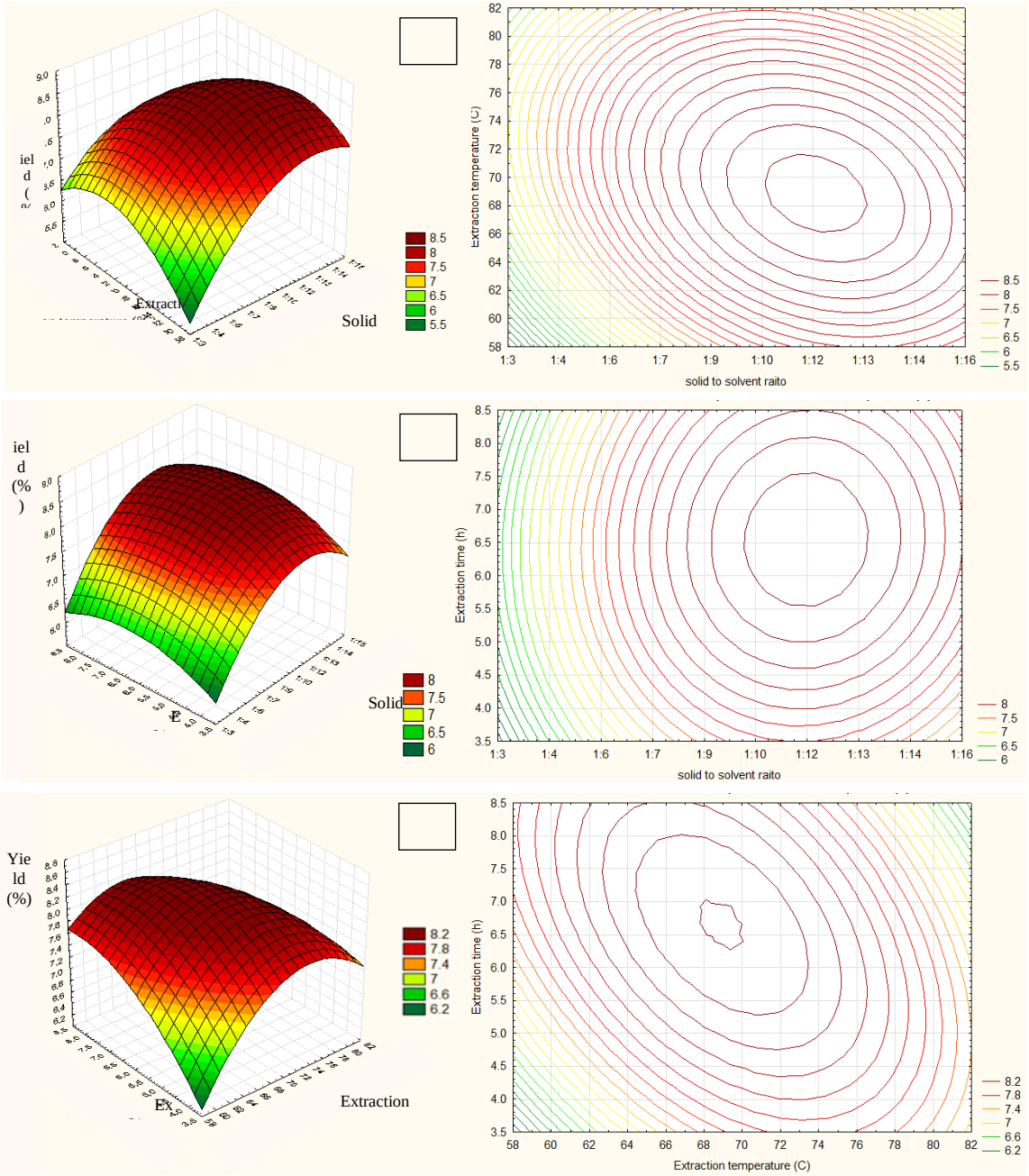


Figure 4.1 Response surface plots of showing the interaction effects of extraction variables on the extracted yield; A = solid to solvent ratio (X_1)-extraction temperature (X_2) effect and constant extraction time at 6h, B = solid to solvent ratio (X_1) – extraction time (X_3) effect and constant extraction temperature at 70 °C, and C = extraction temperature (X_2)-extraction time (X_3) effect and solid to solvent ratio 1:12

4.3 Optimization of ultrasonic assisted extraction

The ultrasonic-assisted extraction was applied to optimize the extraction of γ-oryzanol from RBOS-SD by response surface methodology (RSM). The ethyl acetate was chosen for ultrasonic-assisted extraction and solid to solvent ratio at 1:12.

Table 4.6 Box-Behnken designs for optimizing conditions of γ-oryzanol (% dry basis) coded units together with experimental data (Exp.) and their predicted values (Pred.)

standard order	Code level of variables			γ-oryzanol (% dry basis)	
	X_1	X_2	X_3	Exp.	Pred.
1	0 (12 kHz)	-1 (60 °C)	-1 (30 min)	4.792±0.03	4.81
2	-1 (10 kHz)	1 (80 °C)	0 (45 min)	4.552±0.04	4.64
3	1(14 kHz)	0 (70 °C)	1 (60 min)	4.569±0.03	4.70
4	1 (14 kHz)	-1 (60 °C)	0 (45 min)	4.533±0.04	4.44
5	0 (12 kHz)	0 (70 °C)	0 (45 min)	5.203±0.03	5.21
6	1 (14 kHz)	1 (80 °C)	0 (45 min)	4.185±0.05	4.07
7	1 (14 kHz)	0 (70 °C)	-1 (30 min)	4.322±0.03	4.40
8	0 (12 kHz)	1 (80 °C)	-1 (30 min)	5.036±0.04	5.08
9	0 (12 kHz)	-1 (60 °C)	1 (60 min)	5.372±0.06	5.33
10	-1 (10 kHz)	-1 (60 °C)	0 (45 min)	4.331±0.06	4.49
11	0 (12 kHz)	1 (80 °C)	1 (60 min)	4.893±0.03	4.88
12	0 (12 kHz)	0 (70 °C)	0 (45 min)	5.214±0.01	5.21
13	0 (12 kHz)	0 (70 °C)	0 (45 min)	5.217±0.06	5.21
14	0 (12 kHz)	0 (70 °C)	0 (45 min)	5.217±0.06	5.21
15	-1 (10 kHz)	0 (70 °C)	1 (60 min)	4.932±0.03	4.86
16	-1 (10 kHz)	0 (70 °C)	-1 (30 min)	4.961±0.03	4.83

X_1(Frequency), X_2 (Extraction temperature, °C), X_3 (Extraction time, min)

The Box-Behnken design matrix comprised 16 experiments. The independent variable were frequency, extraction temperature and extraction time. The Box-Behnken experiment is designated as X_1, X_2 X_3 and prescribed into three levels (+1, 0, -1 for high, intermediate and low values, respectively). The Box-Behnken design matrix along with the experiment values of the response are shown in Table 4.6.

In order to select the equation model for optimized the extraction, the estimation of several statistical parameters such as the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicted residual sum of squares (PRESS), and lack of fit of each model were considered.

The regression analysis was implemented to fit a full response surface model consisted of all liner term (X_1, X_2, X_3), the quadratic term (X_1^2, X_2^2, X_3^2), and interaction term (X_1X_2, X_1X_3, X_2X_3). The coefficients for the quadratic equations in term of coded units were shown in Table 4.7.

Table 4.7 Regression coefficients for the 2nd order response surface models in terms of amount of γ-oryzanol

Parameter	Term	γ-oryzanol (% dry basis)	
		Coefficient	p-value
β_0	Intercept	5.213	0.000
β_1	X_1	-0.146	0.017
β_2	X_2	-0.045	0.348
β_3	X_3	0.082	0.115
β_{11}	X_1^2	-0.570	0.000
β_{22}	X_2^2	-0.243	0.008
β_{33}	X_3^2	0.053	0.429
β_{12}	X_1X_2	-0.142	0.064
β_{13}	X_1X_3	0.069	0.313
β_{23}	X_2X_3	-0.180	0.028

* X_1(Frequency), X_2 (Extraction temperature, °C), X_3 (Extraction time, min)

The influence of three independent variables towards γ-oryzanol content (%dry basis) was reported through the significant ($p \leq 0.05$) coefficient of the second-order polynomial regression equation. The effect of frequency of ultrasonic (X_1), extraction temperature (X_2) and extraction time (X_3) was significant ($p \leq 0.05$) in first-order linear effect (X_1), second-order quadratic effect (X_1^2 and X_2^2) and interactive effect (X_1X_2 and X_2X_3). In order to achieve the proper equations, all significant terms were included and the insignificant terms was eliminated. The regression equation for the surface core material (Y) was followed:

$$Y = 5.213 - 0.146\,X_1 - 0.570X_1^2 - 0.243X_2^2 - 0.142\,X_1X_2 - 0.180\,X_2X_3 \qquad (4.2)$$

$$R^2 = 0.955$$

Moreover, the analysis of variance (ANOVA) was employed to test the property of the second order response surface models. The ANOVA was used to evaluate the significance the quadratic polynomial model equation. Any terms in the models with a large F-value and small P-value would indicate a more significant effect on the respective response variables. The result of analysis of variance (ANOVA) of the quadratic model of amount of γ-oryzanol was shown in Table 4.8. The quadratic regression model was highly significant ($p<0.01$) at the 99% probability level. The regression coefficient (R^2 = 0.955) and adjusted multiple correlation coefficient (adjusted R^2 = 0.887) indicated a good fit between the experimental and predicted yield data. An R^2 value closer to one denotes a better correlation. Thus, the analysis of variance showed that the predicted 2nd order models were statistically suitable. However, the lack of fits model were significant ($p<0.05$) which indicated the lack of models for this experiment. The linear and quadratic variables among solid to solvent ratio, extraction temperature and extraction time showed significant difference ($p<0.05$), indicating the effect on γ-oryzanol content (%yield).

Table 4.8 Analysis of variance (ANOVA) for the 2nd order response surface model of γ-oryzanol content

Responses	Source	df	SS	MS	F	p value
Amount of γ-oryzanol (%dry basis)	Regression	9	2.018	0.224	14.170	0.002
	Linear	3	0.240	0.080	5.060	0.044
	Square	3	1.547	0.516	32.590	0.000
	Interaction	3	0.231	0.077	4.860	0.048
	Residual error	6	0.095	0.016		
	Lack of fit	3	0.095	0.032	727.090	0.000
	Pure error	3	0.000	0.000		
	Total	15	2.113			

The optimum conditions for γ-oryzanol content (% dry basis) were provided from the calculation by the response optimizer in Minitab program. The goal of the total the γ-oryzanol content (% dry basis) was maximized. For target values, they were 5.14% of the γ-oryzanol content (% dry basis). Each parameter had the different level of important which were 0, 0.41, and 1 for Frequency, extraction temperature, and extraction time respectively. The results of the optimization and the predicted responses were shown in Table 4.9.

Table 4.9 Optimization solutions obtained using the response optimizer

	Optimal solution			Predicted response	Experimental response (validation)
	X_1	X_2	X_3	γ-oryzanol content (%dry basis)	γ-oryzanol content (%dry basis)[a]
Coded	0	0.41	1	5.14	5.15± 0.08
Uncoded	12 kHz	74.1 °C	60 min		

X_1 (Frequency, kHz), X_2 (Extraction temperature, °C), X_3 (Extraction time, min)

The results showed the optimum conditions for the highest yield of γ-oryzanol content (%) when an ultrasonic frequency at 12 kHz, an extraction temperature at 74.1 °C, and an extraction time at 60 minutes. The predicted response was 5.14% and the experimental value was 5.15±0.08% (51.57 %recovery).

A similar result of ultrasonic assisted extraction (UAE) has been reported by previous works. Zhang and Liu, 2007 reported the optimization condition for extracted lycopene from tomatoes. The results showed that the optimal conditions for 82.4 °C extracting temperature and 29.1 min for extracting time. Yun-liang et al., 2011 also reported the optimum condition were a ratio of water to raw material of 34 mL/g and extraction time of 30 min at 50 °C. Under these conditions, the experimental yield was 8.26%, of extracting polysaccharide from *Tremella mesenterica (TMP).* When compared with soxhlet extraction, the yield of γ-oryzanol extraction by ultrasonic assisted extraction (UAE) had lower than those of soxhlet extraction. The results indicated that UAE could not effectively swell the crude, while the sample to be extract are placed inside a thimble separated from heating element (Khoei and Chekin, 2016). Although soxhlet extraction method had higher yield of γ-oryzanol than UAE it has taken long time and the large volume of solvent released into the atmosphere for the extraction process (7.26 h). The advantage of UAE is requires less extraction time (60 min) and provide higher extraction efficiency due to its ultrasonic cavitation. The cavitation is phenomenon attributed by ultrasonic wave. The increase in the pressure and temperature caused by the compression leads to destruction of the bubble (Khoei and Chekin, 2016)

The interaction of the three dependent variables of ultrasonic frequency, extraction temperature, and extraction time was used to plot the response surface curves for the γ-oryzanol extracted using the three-dimensional equation 4.2 as shown in Figure 4.2. Increasing the frequency of ultrasonic and extraction temperature at constant extraction time at 45 min increase γ-oryzanol content. γ-oryzanol content from the high extraction temperature combined with moderate ultrasonic frequency was greater than that of low extraction temperature and higher or lower than that of ultrasonic frequency (Figure 4.2A). At 74 °C, γ-oryzanol content increased when ultrasonic frequency was increased from 10-12 kHz and extraction time increased

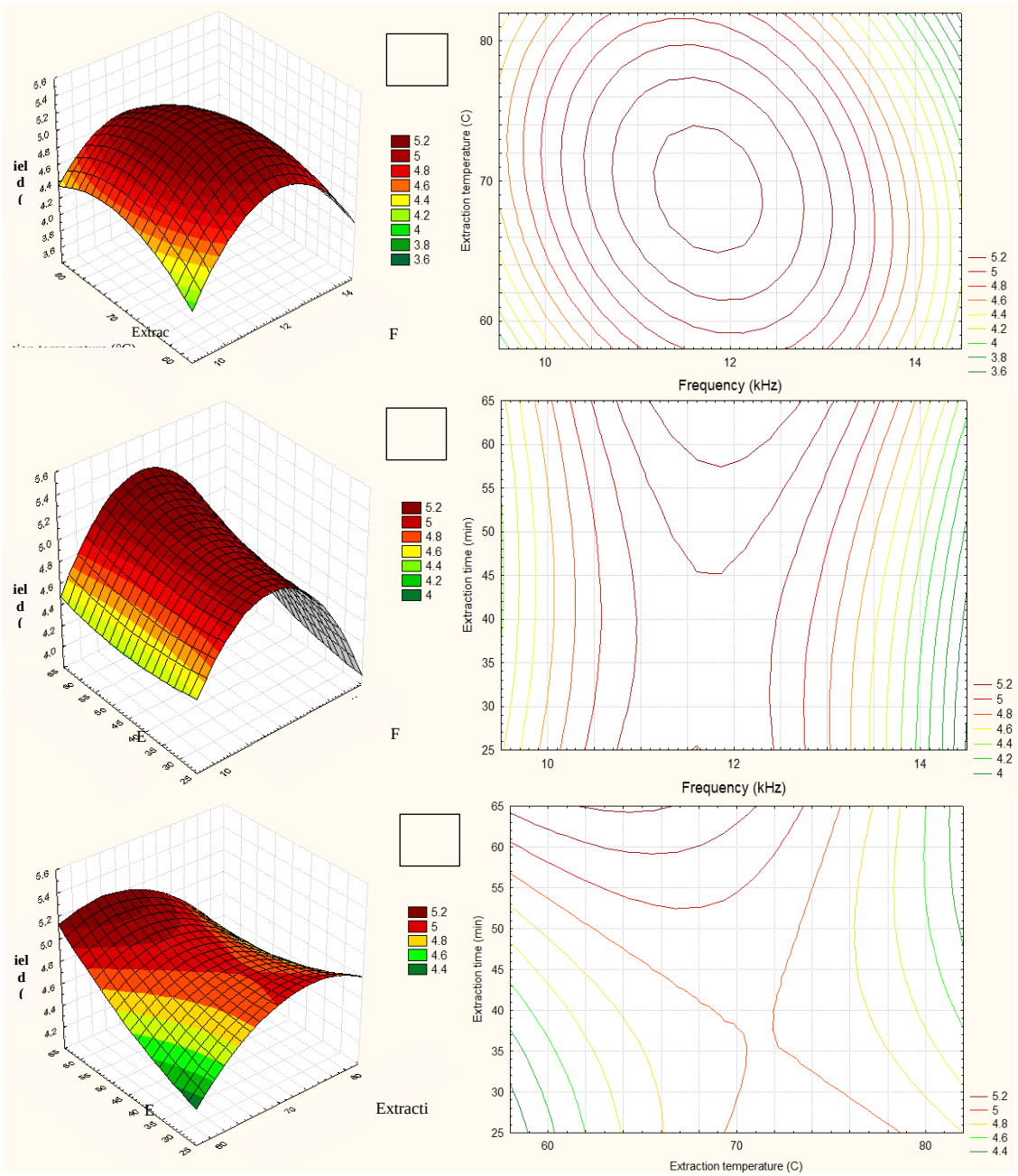


Figure 4.2. Response surface plots of showing the interaction effects of extraction variables on the extracted yield; A = frequency (X_1)-extraction temperature (X_2) effect using constant extraction time, B = frequency(X_1)–extraction time (X_3) effect using constant extraction temperature, and C = extraction temperature (X_2) -extraction time (X_3) effect using constant ultrasonic frequency.

from 45 to 60 min. However, a decrease in γ-oryzanol content was observed after increasing the extraction time to lower than 60 min and increasing the frequency ultrasonic to greater than 12 kHz. The results were in good agreement with other reports which also showed that increasing temperature and ultrasonic frequency causes a decrease in the concentration of phenolic content (Nipornarm et al., 2018). This indicates that some γ-oryzanol is degraded by the higher temperature and power in the UAE process where the collapse of cavitation bubbles destroys the compounds in the compounds in the solvent by the higher pressure created (Ayyidiz et al., 2018)

4.4 Combination Experiment (Ultrasonic assisted extraction and Soxhlet extraction)

For the ultrasonic and soxhlet extraction experiment were carried out ultrasonic and soxhlet extraction apparatus. In brief, 5g of RBOS-SD with suitable organic solvent from UAE and suitable extraction condition from section soxhlet extraction. After extraction process, liquid phase and solid phase were separated by filtering through filter paper under vacuum (300 mmHg) and liquid phase was kept at 4 °C. After UAE process, the solid phase was extracted by soxhlet extractor with suitable condition section soxhlet extraction. After soxhlet extraction process, liquid phase was mix with the γ-oryzanol enrich fraction from ultrasonic extraction and solvent were removed from the liquid phase by rotary evaporator to obtain the γ-oryzanol extracted. The γ-oryzanol content was investigated by spectrophotometer method. The results of the Combination Experiment were shown in Table 4.10.

The results of the combination experiment were shown in Table 4.10, which obtained yield of γ-oryzanol 9.07 % (90.85% recovery). These results showed that the highest yield of γ-oryzanol, when compared between soxhlet extraction (8.74%) and ultrasonic-assisted extraction (5.15%). Although, combination extraction can be extracted the highest γ-oryzanol content that used two instruments and take longer time than other method. Nevertheless, increasing the extraction time from 3 to 7.26 h resulted in an increase in the amount of γ-oryzanol content. Therefore, the extractions of γ-oryzanol from RBOS-SD can combination between UAE and SOX for the highest yield of γ-oryzanol.

Table 4.10 The results of the Combination extraction γ-oryzanol from RBOS using optimization condition of Ultrasonic assisted extraction and soxhlet extraction

Method	condition			%γ-oryzanol (db)[a]	% Recovery[a]
	Frequency	Extraction temperature	Extraction time		
UAE	12 kHz	74.10 °C	60 min	5.19±0.06	52.018
Soxhlet	1:12	70.10 °C	3.00 h	2.15±0.21	21.52
			5.00 h	3.64±0.09	36.45
			7.26 h	3.88±0.04	38.84
combine	Highest yield of γ-oryzanol			9.07	90.85

[a] = Mean±standard deviation of triplicate analysis

4.5 Purification

The results of extraction for γ-oryzanol content in extracted or purity with difference method were shown in Table 4.11. The purity of γ-oryzanol in extracted was determined HPLC to be about 24.30%, 14.30% and 25.17% for soxhlet, ultrasonic and combination between soxhlet and ultrasonic extraction, respectively. The problems encountered during extraction of γ-oryzanol are mainly due to variations in the compositions of RBOS, which include surface-active impurities such as soaps (20-22%), phospholipids (< 1%), waxes (2-4%), and triglyceride (2-2.5%); it was non-suitable to use in foods (Narayan et al., 2006). Therefore, the purification is necessary to remove the impurity from extracts.

Crystallization is based on the principles of solubility; compounds (solutes) tend to be more soluble in hot liquids (solvents) than they are in cold liquids. If a saturated hot solution is allowed to cool, the solute is no longer soluble in the solvent and forms the crystals. The solvent systems for crystallization should be such that the solubility could be adjusted during the process either by changing temperature or by adjusting the composition of the solvent mixture, as in the crystallization with anti-solvent. In this study, the means which the solubility was adjusted was by using temperature

reduction. Two systems of solvent mixture at different composition were selected for the crystallization study.

Table 4.11 Effect of extraction methods to γ-oryzanol content (%db) and purity (%) from rice bran oil soapstock (RBOS)

Method of extraction	γ-oryzanol content (%db)	Purity (%)
Soxhlet extraction	8.65±0.04[b]	24.30 ± 0.11[c]
Ultrasonic	5.15± 0.08[a]	14.30 ± 0.22[a]
Ultrasonic and soxhlet	9.06±0.03[c]	25.17±0.09[b]

* Each data point represents the mean±standard deviation of triplicate analysis
* γ-oryzanol content determination by HPLC
* The multiple comparisons were determined by Duncan at $p \leq 0.05$
* Bars with the different letter are significantly different ($p \leq 0.05$)

From the previous step, after solvent evaporation, γ-oryzanol enriched fraction was obtained, which was then purified further though two-step crystallization. The first crystallization step was to precipitate the mucilaginous impurities including waxes and gums from the extract mixture. The supernatant was crystallized again in the second step to obtain γ-oryzanol crystals with increased purity.

4.5.1 Effect of solvent mixture composition on first crystallization

The objective of the first crystallization was to obtain γ-oryzanol-rich liquid phase as the product. The best condition for this study was selected the lowest %loss of γ-oryzanol. In first crystallization step, the γ-oryzanol enriched fraction was dissolved in the crystallization solvent (ethyl acetate: methanol) with different ratio as 3:7, 4:6, and 5:5. The temperature of the solution was keep at -22 °C, to allow the precipitation of the mucilaginous impurities that would otherwise, by disrupting the crystal growth, interfere with the crystallization of γ-oryzanol in the second step. At this first step however, some γ-oryzanol also crystallized and was lost during the process. For this reason, it was crucial to determine the suitable polarity of crystallization solvent to reduce γ-oryzanol loss, and to obtain the product with high yield and purity. The effects of solvent mixture composition on first crystallization were showed in Table 4.12.

Table 4.12 Effect of solvent mixture composition on first crystallization in term of weight of solid (g), γ-oryzanol content in solid phase (%) and %Loss of γ-oryzanol content in solid phase (impurity) from first crystallization at -22 °C for 24 h

Extraction method	Mixture solvent (Ethyl acetate :methanol)	Weight of solid (g) ^{ns}	γ-oryzanol content (%) in solid phase	Loss of γ-oryzanol in solid phase (%)
Soxhlet extraction	3 : 7	1.13±0.05	2.24±0.02^{c}	36.91±0.44^{c}
	4 : 6	1.07±0.04	2.36±0.03^{b}	38.82±0.40^{b}
	5 : 5	1.01±0.01	2.65±0.01^{a}	43.65±0.21^{a}
Ultrasonic assisted extraction	3 : 7	0.93±0.01^{b}	1.14±0.02^{b}	31.50±1.11^{b}
	4 : 6	0.99±0.03^{a}	1.11±0.04^{b}	31.32±0.48^{b}
	5 : 5	0.99±0.01^{a}	1.26±0.01^{a}	34.78±0.49^{a}
Combination	3 : 7	1.05±0.05^{b}	2.31±0.03^{c}	36.40±0.64^{c}
	4 : 6	1.16±0.01^{a}	2.40±0.01^{b}	37.76±0.03^{b}
	5 : 5	1.13±0.09^{a}	2.61±0.06^{a}	41.12±0.97^{a}

* Each data point represents the mean±standard deviation of triplicate analysis
* The multiple comparisons were determined by Duncan at $p \leq 0.05$.
* Bars with the different letter are significantly different ($p \leq 0.05$) for each method of extraction.

From Table 4.12 the results showed a mixture of ethyl acetate to methanol (3:7) gave the best result because this condition had lowest %loss of γ-oryzanol in solid phase. At higher ratio ethyl acetate and methanol, the poor solubility of γ-oryzanol in these solvent mixtures caused it to crystallize along with other impurities, resulting in high γ-oryzanol loss. The mixture of 20% v/v of ethyl acetate in methanol was found to be the most suitable reported by Kaewboonnum et al. (2010). Moreover, the suitable mixture solvent ratio reported by Zullaikh et al. (2009) and Narayan et al. (2005) were 25% v/v of acetone in methanol that gave the lowest γ-oryzanol loss. The

results suggest, the polarity of solvent is an important parameter in the operation of solvent crystallization of the enrichment of γ-oryzanol.

4.5.2 Effect of storage time for second crystallization

The objective of the second crystallization is to produce pure oryzanol crystal. The supernatant from the first step was then separated and allowed to crystallize in the second step at 5 °C for 6, 12, 24, 48 and 72 h, the purity and yield of γ-oryzanol are shown in Table 4.13, 4.14 and 4.15 obtained by HPLC analysis, respectively.

The results shown that Table 4.13, 4.14 and 4.15 indicated that crystallization time in the range of this study did affect the yield and purity of γ-oryzanol. Therefore, crystallization time for 48 h was suitable to separate mucilaginous impurities and this was used for the subsequent study for the second crystallization step.

For the γ-oryzanol extract from UAE method, the yield (weight of solid) was increased with time increasing, while %purity was increasing insignificantly different ($p>0.05$) for the range of crystallization time. We also investigated the effect of storage time (6, 12, 24, 48, and 72 h) on the γ-oryzanol crystal and %purity, it was found that there are significant different among results obtained from the storage of 6-24and 48 h. Therefore, crystallization time for 48 h was suitable for produce γ-oryzanol crystal. In addition, the γ-oryzanol extract from combination method had more weight of γ-oryzanol crystal than ultrasonic assisted extraction. The γ-oryzanol crystal and %purity were increasing significantly different ($p\leq0.05$) for the range of crystallization time from 6 to 48 h. The suitable of crystallization time was also 48 h that can produce the highest γ-oryzanol crystal (1.7g) and %purity (87%).

Those result agrees with results reported that isolation γ-oryzanol from crude rice bran oil was achieved by two-step crystallization process. In first step, the mixture solvent of acetone and methanol (3:7, v/v) at -60 °C for 15 h gave highest yield and then the second step, liquid phase were added hexane and kept at room temperature for 72 h, which gave highest yield of γ-oryzanol (59% recovery, 93-95% purity) (Zullaikh et al., 2009). Kaewboonnum et al. (2010) also found suitable two-step crystallization. At the most suitable conditions using 20% v/v of ethyl acetate in methanol and at 30 °C and 1 h for the first-step, and 5 °C and 24 h for the second-

step, the yield and purity of γ-oryzanol were 55%wt and 74%, respectively. The results suggest, the factors of crystallization process of γ-oryzanol were polarity of mixture solvent, crystallization temperature, and crystallization time.

Table 4.13 Effect of storage time (6-72 h) for second crystallization at 5 °C on weight of solid (g), γ-oryzanol content and %Purity of γ-oryzanol in solid phase from second crystallization (enrich extract from Soxhlet extraction)

Crystallization Time (h)	Weight of solid (g) (Yield)	γ-oryzanol content (% db)	%Purity
6	0.33±0.03^{d}	20.34±0.53^{c}	80.61±1.56^{d}
12	0.85±0.05^{c}	21.45±0.35^{b}	85.57±1.22^{c}
24	1.39±0.06^{b}	21.84±0.25^{b}	87.83±0.81^{b}
48	1.69±0.03^{a}	23.14±0.25^{a}	90.98±0.70^{a}
72	1.73±0.05^{a}	23.90±0.21^{a}	91.61±1.43^{a}

* Each data point represents the mean±standard deviation of triplicate analysis

* The multiple comparisons were determined by Duncan at $p<0.05$

* Bars with the different letter are significantly different ($p<0.05$).

Table 4.14 Effect of storage time (6-72 h) for second crystallization at 5 °C on weight of solid (g), γ-oryzanol content and %Purity of γ-oryzanol in solid phase from second crystallization (enrich extract from Ultrasonic-assisted extraction)

Crystallization Time (h)	Weight of solid (g) (Yield)	γ-oryzanol content (%dry basis)	%Purity [ns]
6	0.16±0.01[d]	21.24±0.08[c]	85.65±1.16
12	0.39±0.01[c]	21.34±0.11[bc]	86.16±0.62
24	0.56±0.05[c]	21.53±0.01[ab]	87.16±0.56
48	0.97±0.04[a]	21.64±0.15[a]	87.72±1.11
72	0.95±0.02 [a]	21.63±0.03[a]	87.43±0.43

[a] = Mean±standard deviation of triplicate analysis

* The multiple comparisons were determined by Duncan at $p<0.05$

* Bars with the different letter are significantly different ($p<0.05$).

Table 4.15 Effect of storage time (6-72 h) for second crystallization at 5 °C on weight of solid (g), γ-oryzanol content and %Purity of γ-oryzanol in solid phase from second crystallization (enrich extract from combination method)

Crystallization Time (h)	Weight of solid (g) (Yield)	γ-oryzanol content (%dry basis)	%Purity
6	0.35±0.29[d]	21.21±0.39[b]	84.06±2.02[b]
12	0.87±0.04[c]	21.59±0.04[b]	86.14±0.08[ab]
24	1.46±0.06[b]	21.43±0.12[b]	86.17±0.67[ab]
48	1.76±0.11[a]	22.15±0.13[a]	87.09±0.68[a]
72	1.78±0.04[a]	23.91±0.21[a]	88.61±0.72[a]

[a] = Mean±standard deviation of triplicate analysis

* The multiple comparisons were determined by Duncan at $p<0.05$

* Bars with the different letter are significantly different ($p<0.05$)

Part 2 Production and antioxidant activity of encapsulated γ-oryzanol powder

4.6 Production of encapsulated γ-oryzanol powder

In this section, the purpose of this section we studied was to establish the influence of the minimum WPC amount of emulsifier that could be used to prepared the mean stable emulsions with low creaming index, small mean diameter of the droplets diameters, and suitable , zeta potential and creaming index produced by a high pressure homogenizer. Rice bran oil-in-water emulsions were prepared by homogenizing 20 % wt oil phase with 80% wt aqueous phase. Oil phase were prepared by dissolving 2%wt γ-oryzanol extracted from part 1. Aqueous phase were prepared by dispersing WPC (0.2-1.0%wt) and 10% of maltodextrin (DE10) in aqueous buffer solutions (pH 7). The mean particle diameter, zeta potential and creaming index of the emulsions were measured and the results are shown in Table 4.16.

The mean droplet size of oil-in-water emulsion prepared by WPC decreased when the protein concentration increased from 0.2% to 1.0% wt. There was a steep decrease in mean particle diameter when the protein concentration was increased from 0.2 to 0.6 % wt after which the mean particle diameter reached a constant value (~400 nm). This result may be explained that the amount of droplet surface area can be stabilized by protein increase and the rate at which the protein absorbs to the droplet surface increase therefore promoting droplet disruption and retarding droplet coalescence by forming protein membrane around the droplets. The ability of proteins to create repulsive interactions, such as steric and electrostatic, between the droplets and to form an interfacial membrane that is resistant to rupture plays a role stabilizing the droplets against flocculation during storage (McClements, 2004).

The zeta potential of the droplets in 0.2% WPC-stabilized emulsions was negative (-46.90 mV), became increasingly negative as the protein concentration was further increased until it reached a value of -77.76 mV at 1.0% wt WPC. A similar result of highly negative charges (~ -70 mV) of the droplets in the WPI stabilized emulsion has been reported by Onsaard et al., 2014. They reported that the zeta potential of the droplets in the WPI stabilized emulsion went from highly positive

(~ +60 mV) to highly negative charges (~ -70 mV) when the pH was increased from 3 to 8. This result suggested that increasing WPC concentration affected the surface charge of droplets because of the availability of more WPC to form the interfacial mono or multiple layers, cover droplet. Therefore, the surface charge of droplet provided to more negative charge around droplets (Sun, C. and Gunasekaran , 2009; Onsaard et al., 2014).

The creaming stability of the emulsions stabilized by WPC with different concentrations was stable to creaming at all concentrations (Table 4.16). Creaming can be attributed to the influence of the WPC on the colloidal interaction between droplets that the electrical charges on the emulsion droplets are sufficiently large to prevent flocculation. Sufficient protein enhanced to cover all of the droplet surface areas against flocculation leading to creaming. Therefore, the stability of the oil-in-water emulsion improved with increasing WPC concentration. Papalamprou et al., (2005) reported that creaming rate was consequently decreased with WPI concentration increasing. Because of higher WPI concentration may facilitate the adsorption of WPI to the interface of oil droplets, slightly increase the density of oil droplets, and decrease the creaming rate. Moreover, Sun and Gunasekaran (2009) reported that increasing WPI concentration slightly decreased the creaming index of 5% v/v oil-in-water emulsion. As for 20%v/v oil-in-water emulsion, increasing WPI concentration from 0.2 to 2.0 wt % lowered the creaming index, and delayed the lag period prior to visible creaming from 1 to 5 days. Increasing WPC concentration enhanced the surface coverage of oil droplets against flocculation and decreased creaming, leading to increased stability of the emulsion.

Table 4.16 Influence of emulsifier concentration on the droplets size, zeta potential and creaming index of the oil-in-water emulsion with 2% γ-oryzanol in 20% oil phase stabilized by whey protein concentrate (0.2-1.0% wt) and maltodextrin (10% wt)

WPC concentration	Droplets size (nm)	Zeta potential (mV)	Creaming index
0.2%	634.17±22.14^{d}	-46.90±1.57^{e}	0
0.4%	532.00±8.25^{c}	-55.96±0.93^{d}	0
0.6%	474.47±13.60^{b}	-68.66±1.65^{c}	0
0.8%	431.77±9.05^{a}	-73.56±1.96^{b}	0
1.0%	417.177±9.04^{a}	-77.76±1.51^{a}	0

* Each data point represents the mean±standard deviation of triplicate analysis

* The multiple comparisons were determined by Duncan at $p \leq 0.05$

* Bars with the different letter are significantly different ($p \leq 0.05$)

The encapsulated γ-oryzanol extract powder was found to have low moisture content (2.38-1.84 %) and water activity (0.49-0.41). Many studies have shown that the low moisture content of microencapsulated powders could be prepared by spray drying (Klinkesorn et al., 2005a; Klinkesorn et al., 2005b; Aghbashlo et al., 2012 ;Carneiro et al., 2013). Aghbashlo et al., 2012 reported that the moisture content of fish oil microcapsule within skim milk powder (SMP) by spray drying was in the range of 2.33–4.84%. Carneiro et al. (2013) also reported that moisture content values of microencapsulated flaxseed oil by spray drying using different combinations of wall materials was 1.11-1.65%. The maximum moisture specification of the most dried powders in the food industry is between 3% and 4% (Masters, 1991). Dehydrated foods have a_w value of about 0.3 in order to control, not only microbial growth but other physic-chemical and biochemical reactions deleterious to color, texture, flavor and nutritive value of foods (Alzamora et al., 2003). Therefore, the encapsulated γ-oryzanol extract powder is dehydrated food and also slightly physical, chemical, and microbiological change.

The color of the encapsulated γ-oryzanol extract powder with different WPC concentration (0.6, 0.8 and 1.0%) were evaluated. The lightness, greenness and yellowness of the powder samples were increased when the concentration increased, as shown in Table 4.17. The Lightness (L*) of the encapsulated γ-oryzanol extract powder with different WPC concentration (0.6, 0.8 and 1.0%) were 92.70, 93.64, and 94.12, respectively which suggest that the particles in the highest WPC concentration were more effective at scattering light. The yellowness (b*) of the powder was increased with the increasing of the protein concentrations, which can be attributed to the fact that the color intensity decreased as the lightness increased. These result related appearance characteristic of the powder samples. Similarly, Charoen et al. (2012) reported the production of rice bran oil encapsulated powder with whey protein isolate had white color (L*=91.45, a*=0.11 and b*=15.63) General drying, the color of the sample should be sustained to be the same as the sample before drying process as much as possible. Most of the drying process will often cause color changing of the product from the original. This is due to used heat in the drying process activate browning reaction in the product such as Maillard reaction.

Encapsulation yields (EY) and encapsulation efficiency (EE) of the encapsulated γ-oryzanol extract powder were increased when the WPC content increased from 0.6 to 1.0 %. The EY and EE encapsulated γ-oryzanol extract powder (1.0% WPC) using sprays drying were 92.84% and 92.80%, respectively (Table 4.17). The lower droplets size, the higher the encapsulation efficiency and lower the content of unencapsulated core material at the surface of the powder particles because the small oil droplets were enclosed more efficiently within the encapsulating agent of the microcapsules. Furthermore, emulsions with the droplets sizes were more stable during the spray drying process, which is also critical to achieving the optimum encapsulation efficiency (Jafari et al., 2008). Moreover, the encapsulation efficiency (EE) refers to the potential of the wall material to encapsulate or hold the core material inside the microencapsule. These results are consistent with the observation of (Klinkesorn et al., 2005a; Klinkesorn et al., 2005b; Aghbashlo et al., 2012 and Carneiro et al., 2013), who found that the encapsulation efficiency (EE) 84-96% of oil encapsulated powder. The EE value was depending on the type and composition of wall material, the drying process used, and the stability and physicochemical properties of the emulsions (Baik

et al., 2004). In addition, EE value is also related to the shelf-life of oil content in the powder (Zhang et al., 2009; Idham, Muhamad, & Sarmidi, 2012).

Table 4.17 Characteristics of the encapsulated γ-oryzanol extract powder with different WPC concentration (0.6-1.0%)

Powder Characteristic	WPC concentration (%)		
	0.6	**0.8**	**1.0**
Moisture content (%)	2.38±0.14[a]	2.15±0.08[b]	1.84±0.05[c]
a_w	0.49±.01[b]	0.48±.01[b]	0.41±.01[a]
Color L*	92.70±0.35[a]	93.64±0.35[b]	94.12±0.05[b]
a*	-1.12±1.30[a]	-0.93±0.02[b]	-0.78±0.03[c]
b*	11.83±0.12[a]	7.91±0.25[b]	7.38±0.16[c]
Total oil[ns](%)	42.28±1.10	43.08±0.23	43.24±0.37
Free oil (%)	3.57±0.12[a]	3.50±0.23[a]	2.92±0.28[b]
Oil Encapsulation Efficiency (%)	91.55±0.50[a]	91.87±0.53[a]	93.22±0.60[b]
Encapsulation Yield (%)[ns]	69.75±0.86	69.90±1.37	69.94±1.07
γ-oryzanol content (mg/g of encapsulated powder)	10.79±0.17[a]	11.13±0.27[b]	11.27±0.12[b]

* Each data point represents the mean±standard deviation of triplicate analysis

[* ns] = non-significant different ($p>0.05$)

* The multiple comparisons were determined by Duncan at $p\leq0.05$Rows with the different letter are significantly different ($p\leq0.05$).

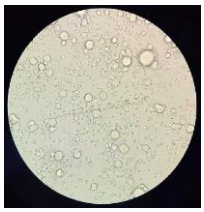
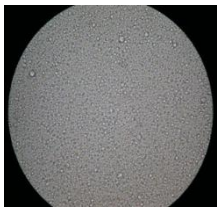
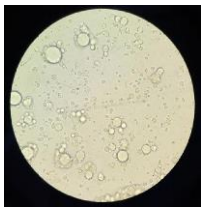
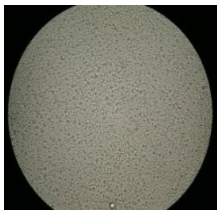
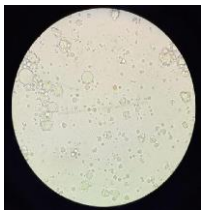
The γ-oryzanol content in the encapsulated γ-oryzanol extract powders were presented in Table 4.17. The γ-oryzanol content were 10.76, 11.13 and 11.27 mg/g of powder sample, which increasing with the WPC concentration (0.6-1.0%) increased, respectively. It was observed that the sample containing γ-oryzanol showed significantly ($p\leq0.05$) higher γ-oryzanol content throughout the oil encapsulation efficiency (%).

Stability of reconstituted encapsulated γ-oryzanol extract powder with different WPC concentration (0.6-1.0%) was investigated. The encapsulated samples were dissolved in phosphate buffer (pH 7) to reconstitute encapsulated emulsions. Droplets size, zeta potential, solubility index, reconstituted emulsion apparent and droplet dispersions were measured.

The droplets size of reconstituted encapsulated γ-oryzanol extract powder emulsion decreased when WPC concentration increased (Table 4.18). For the highest WPC concentration, the average droplets size of reconstituted encapsulated γ-oryzanol extract powder emulsion (after drying) increased from 417.18 nm to 1,078.17 nm. This finding also agrees with the result of the optical microscope in Table 4.18. The results showed various sizes of droplets because the temperature spray drying process was affected WPC characteristic. Moreover, the reconstituted emulsions had larger particles than the fresh emulsions. It was observed that droplet sizes increased after drying, indicating the possible occurrence of aggregation and coalescence. These reasons had an effect on increasing average particle size. Many studies have reported that emulsion droplets may coalesce during the intense shearing in the atomization device of spray drying, resulting in increased emulsion droplets and broader droplet size distribution (Klinkesorn et al., 2005b; Keogh et al., 2001; Aberkane, Roudaut, & Saurel, 2014).

The ζ-potential of the all reconstituted samples with different WPC concentration 0.6, 0.8 and 1.0% increased from -77.76 mV to -29.13 mV, -31.73 mV and -40.70 mV, respectively, when compared to those of the fresh emulsions (Table 4.18). These results suggest that the emulsion structure changed to bridging aggregation and coalescence during spray drying.

Table 4.18 Reconstituted emulsion properties of encapsulated γ-oryzanol extract powder with phosphate buffer (pH 7) in term of droplets size, zeta potential, solubility index, reconstituted emulsion apparent and droplet dispersions

WPC concentration	Droplets size (nm)	Zeta potential (mV)	Solubility index (%)	Reconstituted	Droplet dispersions	
					Before Drying	After Drying
0.6%	2123.67± 98.54[a]	-29.13± 0.75	61.94± 2.70[a]			
0.8%	1992.00± 328.77[a]	-31.73± 0.95[a]	75.47± 2.48[b]			
1.0%	1078.17± 334.70[b]	-40.70± 0.87[b]	76.51± 1.79[b]			

* Each data point represents the mean±standard deviation of triplicate analysis
* The multiple comparisons were determined by Duncan at $p \leq 0.05$
* Bars with the different letter are significantly different ($p \leq 0.05$).

The solubility index (SOI) of encapsulated γ-oryzanol extract powder with different WPC concentration (0.6-1.0%) (Table 4.18) was 61.94%, 75.47%, and 76.51%, respectively, which was found to have moderate SOI. The encapsulated γ-oryzanol extract powder produce by 1.0%WPC has the best SOI. These results suggest that the emulsion structure changed which was attributed to bridging aggregation and coalescence during spray drying. The reconstituted encapsulated

emulsion droplets may decrease in the surrounding water phase. This result indicates that spray drying process have an influence on SOI of the reconstituted samples.

The encapsulated γ-oryzanol extract powder and the scanning electron micrographs are shown in Figure 4.3. Observing the external morphology, particles showed a spherical shape and various sizes with no apparent cracks or fissures, which is an advantage, since it implies that capsules have a lower permeability to gases, increasing protection and retention of the active material. Moreover, the variety in size is a typical characteristic of particles produced by spray drying. Similar morphologies have been reported in powder formed during microencapsulation of rice bran oil, flaxseed oil, and sesame oil using whey protein and maltodextrin as a wall material, several investigators reported the powder produced with maltodextrin 20DE looked like smooth spheres, with hardly any surface cracks in the wall systems (Charoen, 2012; Murali et al., 2014; Lee & Choe, 2014; Onsaard et al., 2014). Moreover, Hee et al., (2015), the potential of gum Arabic (a thickening agent) and gelatin (gelling agent) as wall materials were evaluated in the coconut oil microcapsules with the combination of sodium alginate and maltodextrin by spray drying. Results indicated that both of gum Arabic and maltodextrin can produce the microcapsule. The similar results were also demonstrated in the canola oil (20-30%, w/w) microcapsule prepared with lentil protein isolate and maltodextrin (Chang et al., 2016).

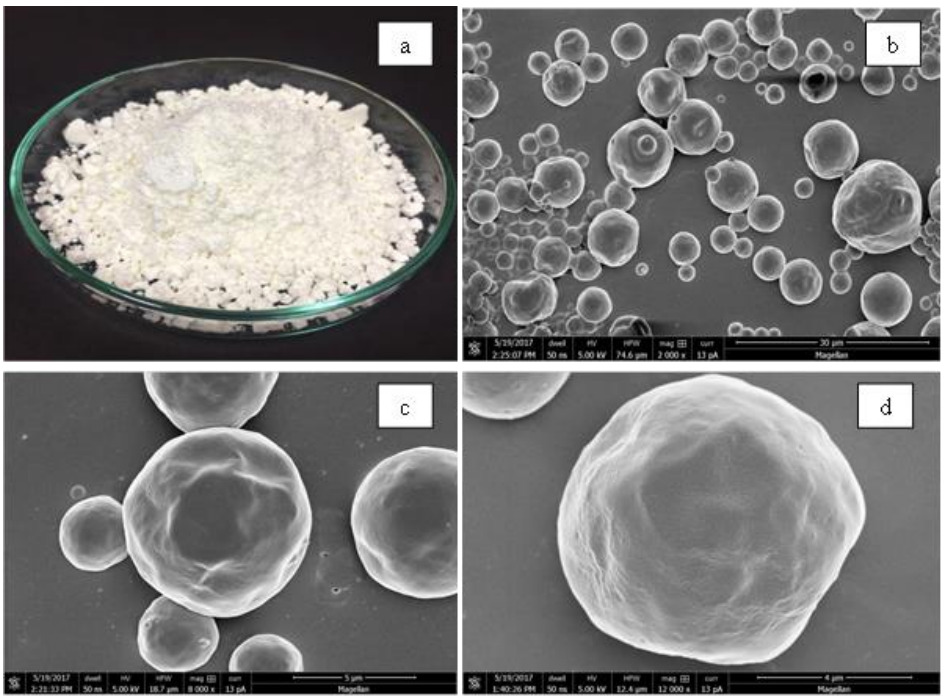


Figure 4.3. Encapsulated γ-oryzanol extract powder (a), scanning electron micrograph at 2,000X (b), scanning electron micrograph at 8,000X (c), and scanning electron micrograph at 12,000X (d)

4.7 Results of antioxidant activities in γ-oryzanol spray-dried powders

Antioxidant activities measured in the samples obtained using DPPH, ABTS, and FRAP assays. The antioxidant activity of γ-oryzanol pure standard, γ-oryzanol extract and purification, commercial γ-oryzanol, encapsulated γ-oryzanol extract powder, α-tocopherol and commercial BHT were measured in term of Trolox equivalents. The results of all experiments can be shown in Table 4.19.

The γ-oryzanol pure standard showed the highest DPPH scavenging activity followed by α-tocopherol, commercial γ-oryzanol, γ-oryzanol extract, commercial BHT and encapsulated γ-oryzanol extract powder. There was no significant difference ($p>0.05$) in DPPH scavenging activity (μg Trolox/g dry sample) between commercial γ-oryzanol and γ-oryzanol extract. Similarly, as shown in Table 4.19, all samples showed a dose-dependent ABTS+ scavenging activity and FRAP activity (μg Trolox/g dry sample), which calculated the activity base purity of the γ-oryzanol content for each sample. The results from this section indicated that the γ-oryzanol

extract was rather similar effective as an antioxidant activity similar with the commercial γ-oryzanol. Therefore, the γ-oryzanol extract can be claim the antioxidant activity. Furthermore, the encapsulated γ-oryzanol extract powder produced by encapsulate technique and spray dry, which can protect γ-oryzanol and they had antioxidant activity. It is well documented that ferulic acid and γ-oryzanol are the major antioxidants in rice bran, a substantial of work has been carried out to investigate their antioxidant activity in *vitro* and *vivo* base on different models. Xu and Godber (2001) investigated that γ-oryzanol components (cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate) possessed significantly higher antioxidant activity than that any of four vitamin E components (α-tocopherol, α-tocotrienol, γ-tocopherol, and γ-tocotrienol) in a cholesterol oxidation system accelerated by 2,2'-azobis (2-methylpropionamidine) dihydrochloride, of which 24-methylenecycloartanyl ferulate shows the highest antioxidant activity (Xu et al., 2001). The higher antioxidant activities of γ-oryzanol components might because the structure of γ-oryzanol components is similar to that of cholesterol, an important component in reducing oxidation stress and maintaining the functionality of cell, therefore, in accordance with the structural relationship theory, the γ-oryzanol components may have greater ability to associate with cholesterol in the small droplets of the emulsion and become more efficient in protecting cholesterol against free radical attack. However, they also found that ferulic acid showed the highest antioxidant activity, followed by vitamin E and γ-oryzanol at the three different ratios in a linoleic acid model (Xu and Godber, 2001). The antioxidant properties of ferulic acid could be attributed to its aromatic phenolic ring that stabilizes and delocalizes the unpaired electron within its aromatic ring, thereby acting as free-radical scavengers (Srinivasan, Sudheer, and Menon, 2007). Whereas the triterpene portion of γ-oryzanol may affect its antioxidant activity by lowering the mobility in the system due to its relatively larger molecular structure than free ferulic acid (Xu and Godber, 2001). Voraratn et al., 2010 concluded that rice bran oil and rice bran oil emulsion have high antioxidant activities tested by DPPH and FRAP assay. Rice bran oil showed a good amount of vitamin E and γ-oryzanol contents. Tuncel et al., 2011 studied antioxidant activity of rice milling fraction and observed high antioxidant activity in the rice

unpolihed rice compared with the white polished rice and also observed that 94% of γ-oryzanol content is reduced by whitening and polishing of the rice.

Table 4.19 Antioxidant activity of encapsulated γ-oryzanol extract powder determined by DPPH, ABTS, and FRAP assays in term of Trolox equivalents

Sample	DPPH	ABTS	FRAP value
	µg Trolox/g dry sample	µg Trolox/g dry sample DW	µg Trolox/g dry sample DW
γ-oryzanol Standard*	179.57±7.45[e]	209.56±1.26[e]	189.08±7.99[e]
Commercial γ-oryzanol*	119.28±8.73[c]	145.16±3.08[c]	118.69±1.12[c]
γ-oryzanol extract*	116.53±5.50[c]	142.76±1.06[c]	117.27±2.20[c]
Encapsulated γ-oryzanol extract powder*	3.23±6.13[a]	8.347±0.91[a]	6.16±1.12[a]
α-tocopherol	148.44±13.62[d]	194.98±0.82[d]	167.44±0.58[d]
Commercial BHT	36.98±9.72[b]	80.86±0.60[b]	74.43±0.12[b]

[a] Each data point represents the mean±standard deviation of triplicate analysis

* Each sample calculated antioxidant activity base on γ-oryzanol content in dry sample

Part 3 Determination shelf-life of encapsulated γ-oryzanol extract powder

4.8 Accelerated Shelf-life evaluation

The purpose of this section was to establish shelf-life of encapsulated γ-oryzanol extract powder using accelerated condition as 30, 40 and 50 °C. Evaluation of stability was conducted at 10 days intervals for color, moisture content, a_w, lipid oxidation, antioxidant activity and γ-oryzanol content. The sample was selected the best condition from Part 2, which spray dried from oil-in-water rice bran oil emulsion.

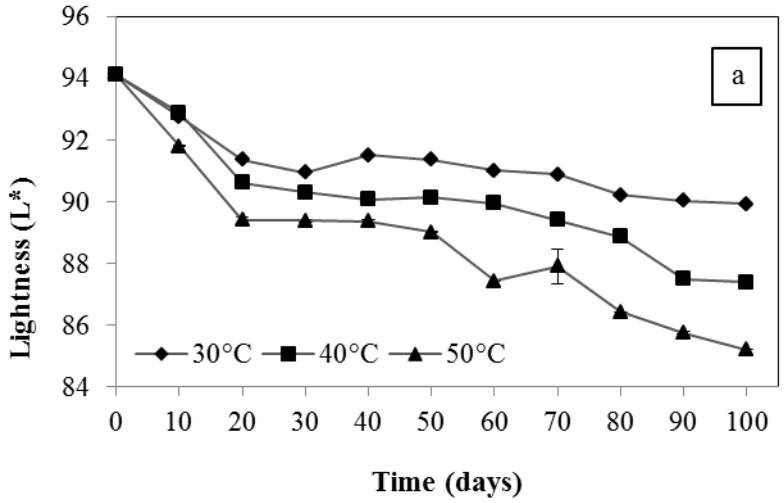
4.8.1 Color changes

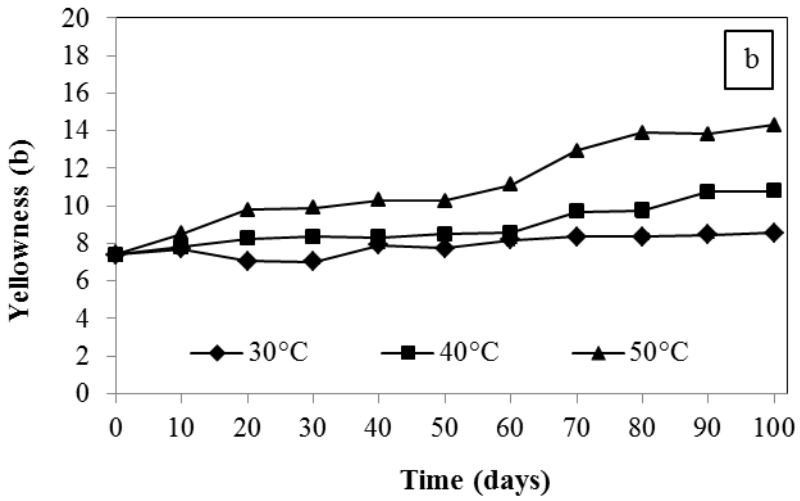
Color (L*, a*, b*) changes in encapsulated γ-oryzanol extract powder were investigated at different storage temperature (30, 40, and 50 °C) during storage time (100 days). The results found that lightness (L*) value decreased from 94.12 at 0 day to 89.93 (30 °C), 87.40 (40 °C), and 85.22 (50 °C) at 100 days. At higher storage temperature (50 °C), the lightness (L*) decreased significantly from 94.12 at 0 day to 89.41 at 20 days, and then slightly decreased from 89.41 at 20 days to 85.22 at 100 days. The encapsulated γ-oryzanol extract powder stored at low temperature (30, and 40 °C) showed higher L* value (Figure 4.4 (a)).

In the same way, the yellowness (b*), the powders stored at low temperature showed lower yellowness than the powders stored at high temperature and that increased significantly due to storage time (Figure 4.4(b)). No significant difference in b* value was observed the sample stored at 30 °C for all storage time (0 to 100 days). In the other hand, b* value slightly increased from 7.38 at 0 day to 10.77 at 100 days (store at 40 °C). Moreover, b* value (Figure 4.4 (b)) increased significantly from 7.38 at 0 day to 14.28 at 100 days (store at 50 °C). Therefore, storage temperature and storage time had an effect on the color change in encapsulated γ-oryzanol extract powder. Klinkesorn et al. (2005b) also emphasized that color change in spray-dried tuna oil encapsulated during storage time at different storage temperature and humidity. Those results found that at high storage temperature showed higher color change than lower storage temperature. Maillard reaction products can form during both drying and ambient storage of food powder (Thomas, Scher, & Desobry-Banon, 2004). As a result of the maillard reaction, the development of brown color can occur.

Furthermore, the effect of storage time and storage temperature on changing of product color was reported in term of color difference value (ΔE) and was compared in Figure 4.4(c). As indicated in the Figure, the highest storage temperature (50 °C) than others storage temperature (30 and 40 °C). An increase in ΔE value was observed with an increase in storage temperature and storage time, which had different of color from before keep. The ΔE value was increased significantly ($p \leq 0.05$) after the storage of 60 days and 80 days at storage temperature 40, and

50 °C, respectively. Thus, from this experiment, we can explain that the higher storage temperature can be developing of color.





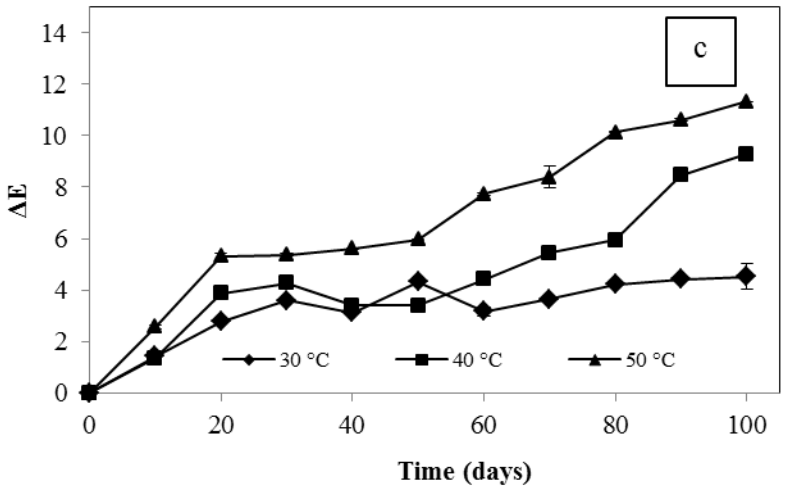


Figure 4.4 Change of color Lightness (a), Yellowness (b), and color difference (ΔE) (c) in encapsulated powder during storage for 100 days with different storage temperature (30, 40 and 50 °C).

4.8.2 Moisture content and water activity

Changes of moisture content and water activity have been shown in Figure 4.5 (a and b). Moisture content and water activity increased with increasing of storage time. The results found that moisture content increased from 1.89% at 0 day to 2.51% (30 °C), 2.78% (40 °C), and 3.26% (50 °C) at 100 days. At the higher storage temperature, 50 °C moisture content increased significantly after storage 50 days.

Changes of a_w that found slightly increased with increasing during storage time. The results found that a_w increased from 0.412 at 0 day to 0.494 (30 °C), 0.518 (40 °C), and 0.537 (50 °C) at 100 days. The water activity is the most important parameter of water in terms of food safety and food stability. The free or available water in food supports microbial growth and participates in and supports chemical and enzymatic reactions and spoilage processes. The a_w about 0.4 to 0.5 can be increased the rate of oxidation of fats.

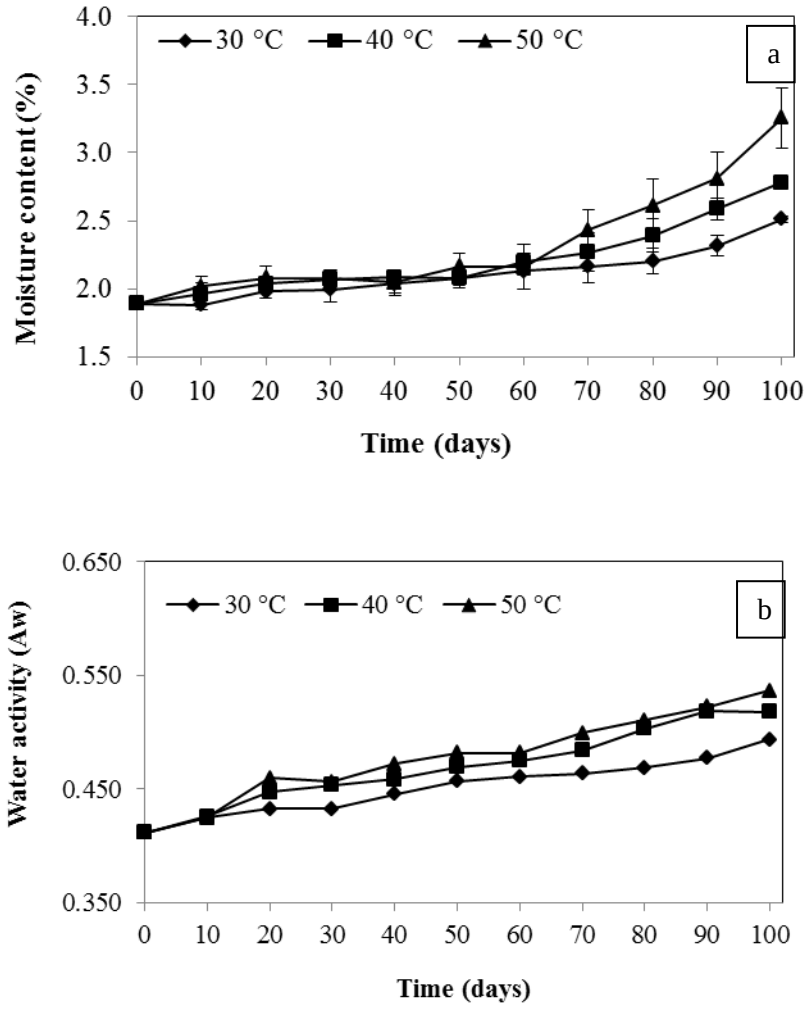


Figure 4.5 Change of moisture (a) and water activity (b) in encapsulated powder during storage for 100 days with different storage temperature (30, 40 and 50 °C)

4.8.3 Lipid oxidation

To monitor lipid oxidation during storage at various temperatures, the oxidative stability of encapsulated γ-oryzanol extract powder was evaluated by measuring the primary and secondary oxidative products using lipid hydroperoxides and headspace analysis (hexanal).

The oxidative stability of dried powder in this work was determined on the total lipid because that lipid oxidation in encapsulated γ-oryzanol extract powder does not correlate well with free or extractable fat (Klinkesorn et al., 2005b). The

information of lipid oxidation products (lipid hydroperoxide and hexanal) was then monitored during storage. There was an appreciable increase in the production of primary and secondary lipid oxidation products in all of the reconstituted emulsion during storage (Figure 4.6 and 4.7 (a,b)).

Figure 4.6 showed that the formation of lipid hydroperoxides and hexanal in encapsulated powder without γ-oryzanol (control) during storage at 30°C was observed after the storage of 20 days and 30 days, respectively. Both lipid hydroperoxides and hexanal increased significantly over 30 days and 40 days, respectively.

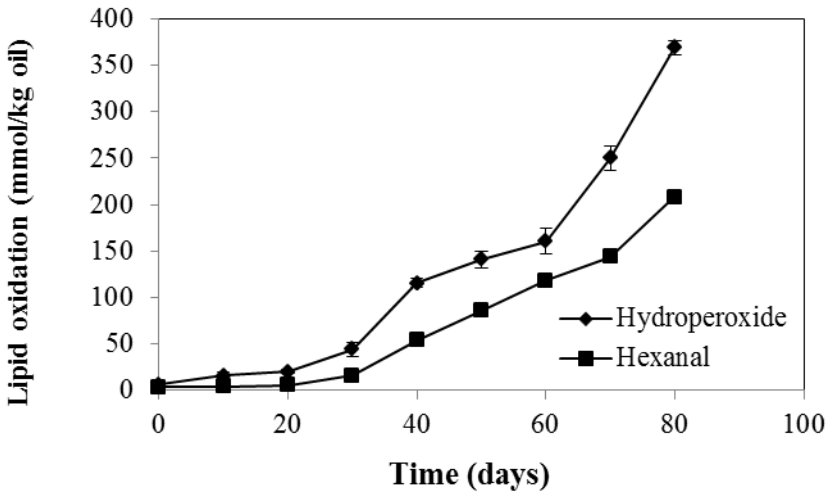


Figure 4.6 Change of hydroperoxide and head space of hexanal in encapsulated powder without γ-oryzanol (control) during storage at 30°C

Figure 4.7 (a,b) showed that the formation of lipid hydroperoxides increased with increasing storage temperature (Figure 4.7a). A similar trend was observed in the development of hexanal during storage (Figure 4.7b).

The decomposition of hydroperoxide was observed after the storage of 60 days (20.94 mmol/kg oil), 40 days (21.24 mmol/kg oil), and 30 days (16.71 mmol/kg oil) at storage temperature 30, 40, and 50 °C, respectively. These results are similar to those reported by Klinkesorn et al., 2005b who reported that encapsulation of spray dried tuna oil with multilayer (lecithin, chitosan, and corn syrup solid) was significant

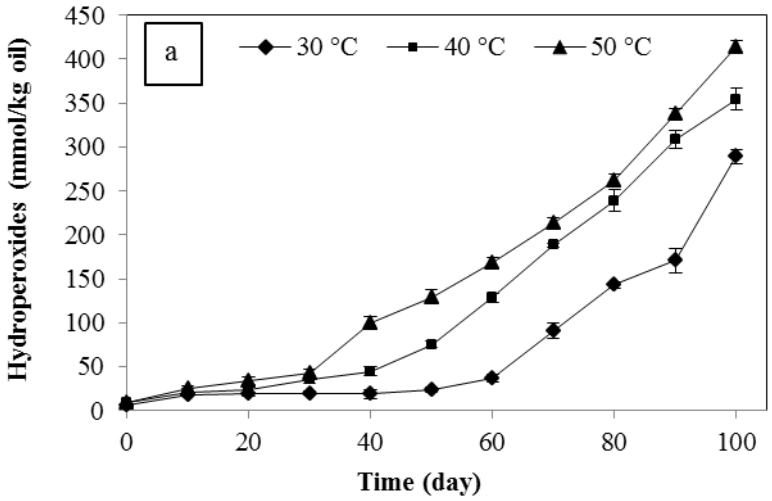
increase in the hydorperoxide and TBARS concentration after 18 day at 37 °C (Carneiro et al., 2013) encapsulated conjugated flaxseed oil using gum Arabic and maltodextrin as wall material presented peroxide concentration after one week, reaching value of 24.8 meq peroxide/kg oil. Moreover, Jiménez et al. (2011) also conjugated linoleic acid (CLA) using polymericmatrices as wall material presented peroxide concentration after 18 days, reaching a value of 15 meq peroxide/kg oil at 35 °C. Powders oxidative stability was strongly influenced by the wall material combination. Although the maltodextrin and whey protein concentrate mixture have shown the lowest encapsulation efficiency, this combination presented the highest oil protection against oxidation compared with maltodextrin and gum Arabic (Carneiro et al., 2013). Martinez et al. (2015) claimed that rosemary extract (1600 μg/g oil) significantly increased oxidative stability of walnut and chia oil encapsulated by hydroxypropyl methylcellulose and maltodrextrin, especially on the encapsulated chai oil, which also increases antioxidative ability. In addition, Tamm et al. (2016b) encapsulated rapeseed oil using the combination of Pea protein isolate (PPI) and glucose syrup by spray drying. They found the oil encapsulated within microcapsule with enzymatic hydrolyzed PPI show lower hydroperoxide content after 1 mount storage than the oil within microcapsule with native PPI.

Also, the formation of hexanal in encapsulated γ-oryzanol extract powder (Figure 4.6b) increased with storage temperature and time. The sample stored at low temperature storage showed lower hexanal values than the sample stored at high temperature during 100 days of storage. The powder stored at the different temperature (30, 40, and 50 °C) that showed the formation of hexanal increased significantly over 60 days, 50 days, and 30 days, respectively. No difference in the amount of hexanal was observed the sample stored at 30 °C during storage time for 60 days, after that hexanal value increase from 5.50 mmol/kg oil at 60 days to 66.71 mmol/kg oil at 100 days. Jiménez et al. (2011) studied the production of hexanal during storage of microencapsulated conjugated linoleic acid (CLA) and concluded that gum Arabic was not effective in protecting these compounds against oxidation, while WPC showed the best performance. Drusch et al. (2007) evaluated the oxidative stability of fish oil microcapsules obtained by spray drying and observed a propanal production lower than 100 mmol/kg oil in samples encapsulated with proteins, while

for samples encapsulated with gum Arabic, the propanal production exceeded 200 mmol/kg oil after 30 days of storage. Degradation products of oil such as aldehydes and short-chain hydrocarbons are volatile and impart off flavors, including warmer-over flavor. Pentanal and hexanal are the major volatile products of linoleic acid oxidation (Jiménez et al., 2011).

Moreover, the results of formation lipid oxidation in term of hydroperoxide and hexanal compared with the encapsulated γ-oryzanol powder and the encapsulated powder without γ-oryzanol (control), which stored at 30 °C. The result showed the amount of hydroperoxide was observed for control sample (20 days) and encapsulated γ-oryzanol powder (60 days). Therefore, fortification of γ-oryzanol in encapsulated powder can be inhibited lipid oxidation.

The result indicated that surface oil is defined as non-encapsulated oil on the surface of the dried microcapsule. It is important as it can easily react with oxygen to produce off-flavors and result in poor stability. In this case, we observed the free oil (2.92%), which can be explain the cause of lipid oxidation.



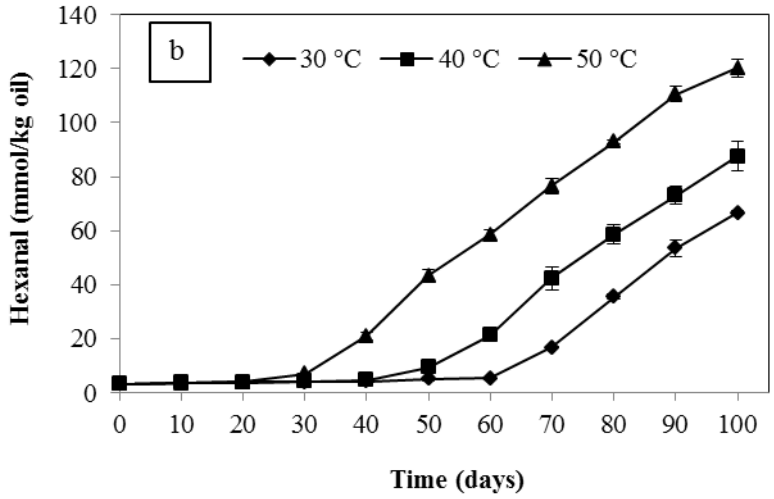


Figure 4.7 The change of hydroperoxide (a) and head space of hexanal (b) in encapsulated γ-oryzanol powder during storage at different temperatures (30, 40 and 50 °C).

4.8.4 γ-oryzanol content

Figure 4.8 show the effect of storage temperatures on the γ-oryzanol content in encapsulated γ-oryzanol powder for 100 days. The γ-oryzanol content of all powder samples decreased as the storage time increased. The decreasing rate of the γ-oryzanol powder during storage at 50 °C was higher than those at 30 and 40 °C. The γ-oryzanol contents slightly declined during the stages of initial storage (0-60 days), and then sharp declined after 60 days of storage. This result indicated that the decreasing of γ-oryzanol content in the powder samples was affected both storage temperature and storage time. A decrease in γ-oryzanol content of the samples with increasing storage time may also relate with lipid oxidation (peroxide) produced.

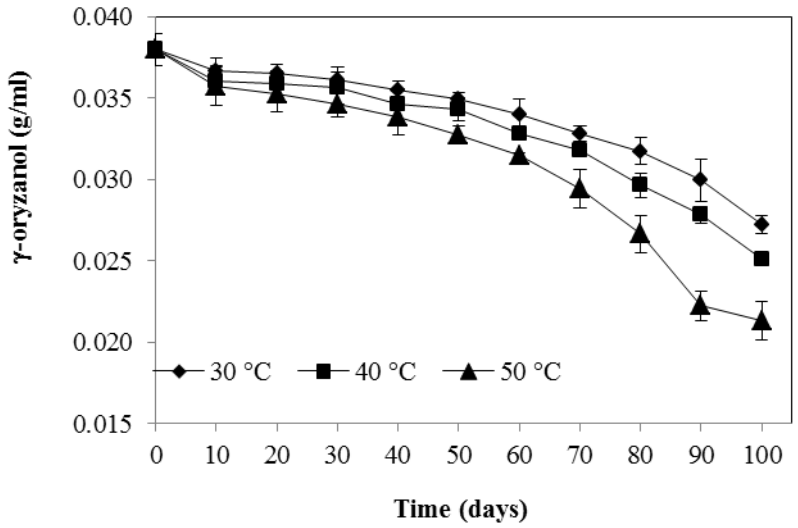


Figure 4.8 Change of γ-oryzanol content in powder during storage at different temperatures (30, 40 and 50 °C).

4.8.5. Determination of the deactivation kinetics

Most food quality deterioration has been found to fit either a zero or first order mathematical expression.

The characterization of dried powder was changed during storage, increased or decreased with time and temperature. Food quality was loss may be described by zero or first order (Pua et al., 2008). The data was best fit by zero or first order below;

$$\text{Zero order} \qquad C = C_0 - kt \tag{4.3}$$

$$\text{First order} \qquad \ln(C) = \ln C_0 - kt \tag{4.4}$$

Where : C is the initial quality at day 0 after spray drying

C_0 is the quality after t (time) of stability

k is degradation rate constant

t is storage time (days)

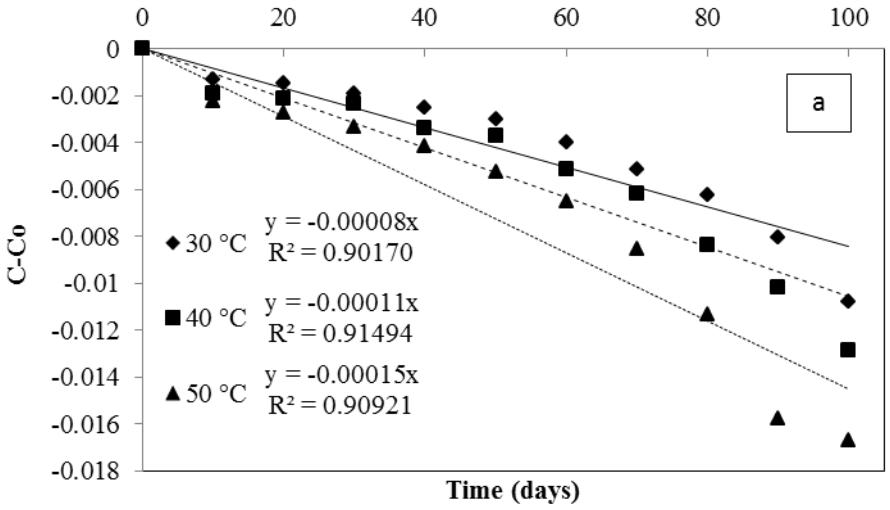
Figure 4.9 shows the change in γ-oryzanol content as a function of storage temperature during storage time 100 days. The concentration of γ-oryzanol in encapsulated γ-oryzanol extract powder decreased as the storage time increased for all temperature studied. Therefore, storage time and temperature had effect on γ-oryzanol content. For storage temperature at 30 °C, the γ-oryzanol content slightly decrease from 38.00 mg/ ml reconstituted at 0 day to 27.2 mg/ml reconstituted at 100 days. The lowest γ-oryzanol content in powder was 21.3 mg/ml reconstituted which storage at 50 °C for 100 days.

In order to determine the reaction order of a quality attribute, the experimental data obtained was fitted to linear equation which mentioned as equation 4.1 and 4.2. The changes in γ-oryzanol content in powders measured at the different storage temperature (30, 40, and 50 °C) versus storage time (100 days) were plotted and data obtained was fit by linear equation. If the data showed a good fit to a linear equation, then the data indicated a zero order reaction. On the other hand, if the ln(C-Co) versus time fits a linear equation, then the reaction was considered to be a first order reaction (Singh, 2000). The result shows (Figure 4.9b) that the γ-oryzanol content changes in powders followed zero order reaction kinetics (Table 4.20). Additionally, the results could be applied to approximately describe the degradation reaction of the γ-oryzanol in encapsulated γ-oryzanol extract powder and the temperature effects on the degradation rate of γ-oryzanol. The main reaction that caused the degradation of γ-oryzanol could be the oxidation of the compound by oxidation products of oil which formed during the storage time and temperature. A similar trend was observed by Khuwijitjaru et al. (2011) for the content of γ-oryzanol in rice bran oil during heating at 120 °C, which slightly augments during the first 5-7 h of treatment. Additionally, they found that degradation of γ-oryzanol in rice bran oil obeyed to first-order kinetics. Therefore, the rate constant and activation energy of the degradation reaction at different temperatures may be used to predict the retained amount of the compound during high temperature processing such as frying (Khuwijitjaru et al, 2004; Khuwijitjaru et al, 2011).

Table 4.20 R-square of zero order and first order kinetic model of γ-oryzanol degradation in encapsulated γ-oryzanol extract powder in various temperatures (30, 40, and 50 °C).

Quality	Temperature °C	R^2	
		Kinetic order reaction	
		n=0	n=1
γ-oryzanol content	30	0.924	0.899
	40	0.930	0.903
	50	0.925	0.887

The rate constant (k) obtained from the plots of loss quality versus time are 0.00009, 0.00011, and 0.00016 at 30, 40, and 50 °C storage temperature, respectively. The result showed that the effect of storage temperature on the degradation kinetics for loss quality in encapsulated γ-oryzanol extract powder. An increase in storage temperature led to an increase in the rate constant (k) of encapsulated γ-oryzanol extract powder.



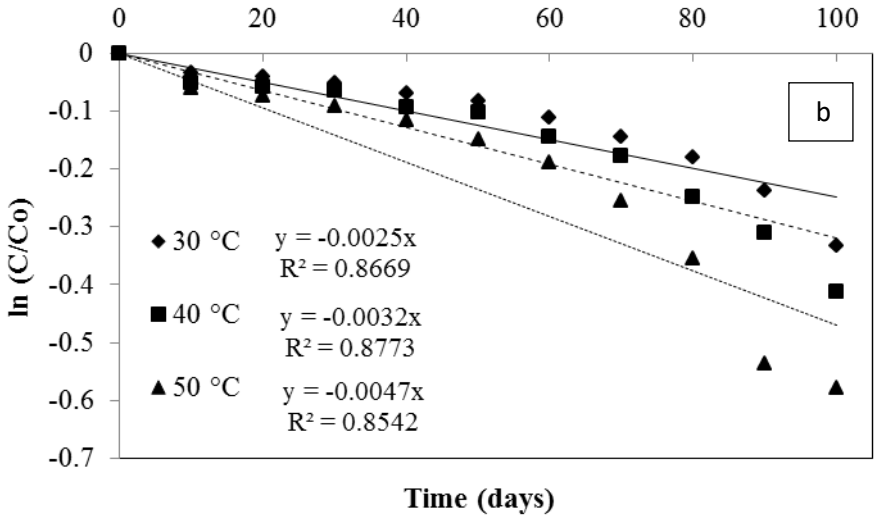


Figure 4.9 Kinetic reaction order for degradation of γ-oryzanol content in dried powder with different carrier (a) zero order reaction (b) first order reaction

Furthermore, activated energy (Ea) of degradation of γ-oryzanol in encapsulated γ-oryzanol extract powder was 23324.9 J/mole. Activated energy (Ea) is the minimum amount of energy that is required to activate chemical reaction. Therefore, degradation of γ-oryzanol required a greater activated energy (Ea). Wu et al., 2014 reported the reaction rate constant (k) that result the greatest loss were observe for free sulforaphane, indicating that the microencapsulated system offers greater protection to the sulforaphane. The reason may be effective microencapsulation of γ-oryzanol within the wall material.

Table 4.21 Estimate kinetic reaction constant value for spray-dried powder

Temperature °C	**Kinetic reaction constant (γ-oryzanol content)**
	n=0
30	0.00009
40	0.00011
50	0.00016
Ea/R	2805.5
Ea (J/mole)	23324.93

4.8.5.1 Arrhenius equation

As the reaction rate, constants were determined at three different temperatures, the theory behind the accelerated shelf life testing method (ASLT) can be applied in order to predict the shelf-life of spray-dried powder. The concept of the ALST is based on collecting experimental data that describe the phenomenal at high temperatures and using such results in order to extrapolate the system behavior at some lower temperature. In most case, this temperature effect can be described by the Arrhenius law:

$$k=k_0 e^{-Ea/RT} \quad (4.3)$$

Where k = rate constant for deteriorative reaction at temperature T,

k_0 = constant, independent of temperature

Ea = activation energy (J/ mole)

R = ideal gas constant ($8.314 JK^{-1} mol^{-1}$), and

T = absolute temperature (K).

In such case, a simple graphical representation of ln(k) versus (1/T) determined the activation energy. In our case, the Arrhenius behavior was found applicable for temperatures ranging fron 30 to 50 C as a fairly acceptable linear trend is detected when ln(k) is plotted as a function (1/T).

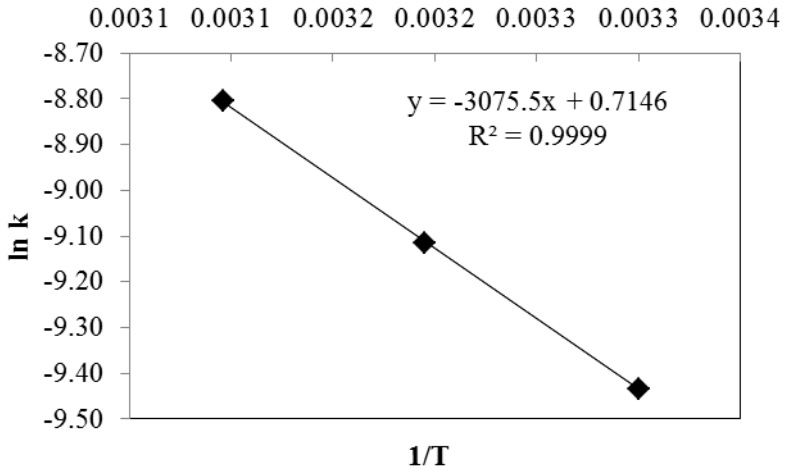


Figure 4.10 Effect of storage temperature on the apparent kinetics of the degradation in encapsulated γ-oryzanol extract powder.

4.8.5.2 Determination shelf-life

The linear shelf-life plot, also known as the shelf-life plot was made by plotting ln (t_s) versus 1/T. Shelf-life was calculated with a half of decreasing in γ-oryzanol content (Table 4.22).

Table 4.22 Parameter of shelf-life evaluation in encapsulated γ-oryzanol extract powder

Temp °C	Temp K	1/T	k	ln k	t_s (days)	ln t
30	303	0.0033	0.00008	-9.4335	237.500	5.470
40	313	0.0032	0.00011	-9.1150	172.727	5.152
50	323	0.0031	0.00015	-8.8049	126.667	4.842

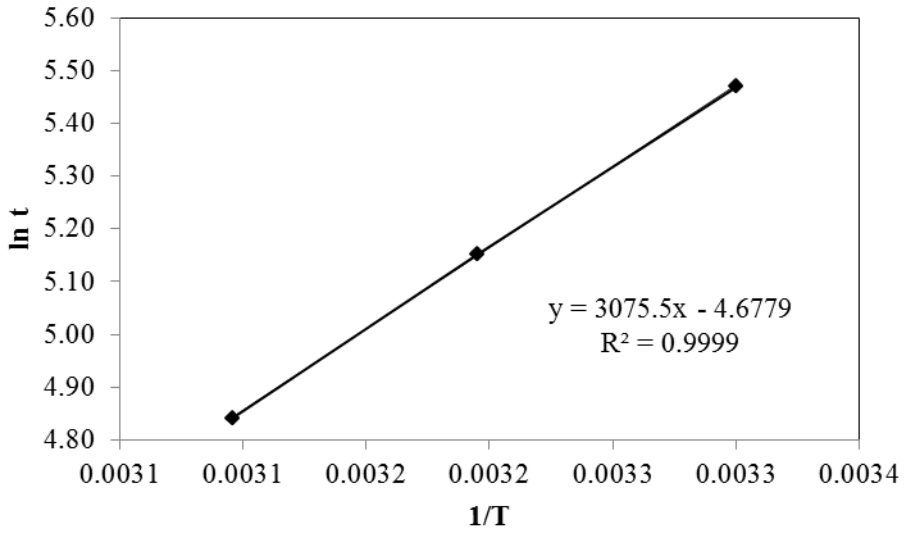


Figure 4.11 The linear shelf-life plot relationship between ln t_s and 1/T for different storage temperature of encapsulated γ-oryzanol extract powder.

Estimate shelf-life of encapsulated γ-oryzanol extract powder at difference storage temperature were predicted and found in Table 4.22. The estimated shelf-life equation was y = 3075.5x – 4.6779 (y= ln (time (days) of shelf-life, x= Storage temperature^{-1}, K). The encapsulated γ-oryzanol extract powder can be kept in 25 °C for 277 days and 4 °C for 613 days.

Table 4.23 Estimate shelf-life of encapsulated γ-oryzanol extract powder

Shelf-life Equation	Storage temperature (°C)	Estimate shelf-life (days)
y = 3075.5x – 4.6779 (y= ln (time of shelf-life, days)) (x= Storage temperature^{-1}, K)	25	277
	4	613

4.8.6 DPPH radical scavenging activity

After spray drying process, the γ-oryzanol content of the encapsulated γ-oryzanol extract powder was 38.00 mg/ml reconstituted. The effect of encapsulated γ-oryzanol extract powder as an antioxidant was evaluated during storage at different temperatures (Figure 4.12). It was found that the methanol extract of γ-oryzanol from the encapsulated γ-oryzanol extract powder showed a decreasing DPPH radical scavenging activity when both storage times and storage temperatures were increased. DPPH radical scavenging activity slightly decreased during 50 days of storage time, and then strongly decreased for over 50 days. The methanol extract of γ-oryzanol during storage at 50 °C showed lower DPPH radical scavenging activity than those of 30 °C and 40 °C of storages. This result might be due to the different hydrogen donation ability of the samples. It is generally accepted that the electron-donating ability of the samples results in their antioxidant activity toward lipid oxidation. DPPH radical scavenging test is the methods for investigation of the hydrogen donating potency of antioxidants.

The results are similar to those reported by Monika et al. (2013) reported DPPH radical scavenging activity of γ-oryzanol in rice bran oil. Refined rice bran oil was observed 2,803 mg/kg of γ-oryzanol and that found 53.9% inhibition of DPPH radical activities. Bopitiya et al. (2014) also reported the antioxidant activity as measured using DPPH radical scavenging assay of γ-oryzanol in rice bran oil extract, which extract from white rice at 50 °C. It possessed strong antioxidant activity (0.027 mg/ml IC_{50} value). Kumar et al. (2014), who showed the DPPH radical scavenging activity (%) of γ-oryzanol fortified fat in biscuits, which baked at 200 °C for 9 min and kept at 38 °C. The DPPH radical scavenging activity did not alter during baking and storage time (120 days).

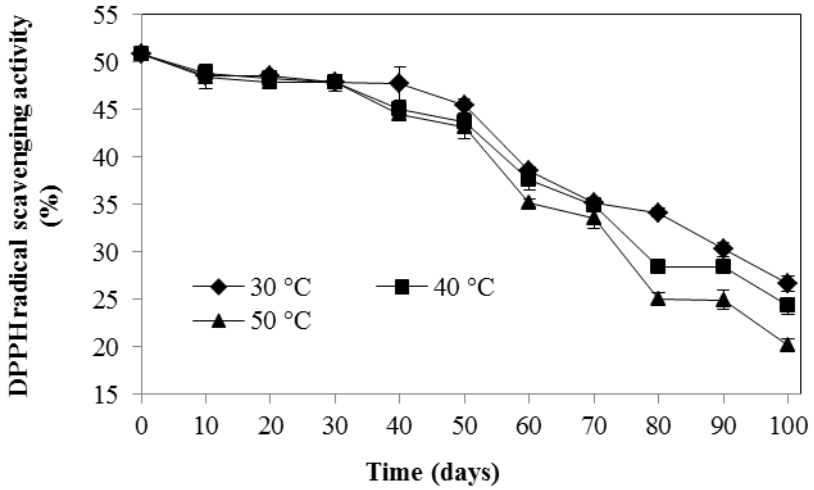


Figure 4.12 Change of antioxidant activity with DPPH scavenging in powder during storage at different temperatures (30, 40 and 50 °C).

Part 4 Application of encapsulated γ-oryzanol extract powder as food ingredient

In this part, there were two different formulas as control sample without encapsulated γ-oryzanol extract powder and with 5% of encapsulated γ-oryzanol extract powders. Formulations of salad dressing are given in Table 4.24. Chemical composition, characteristics and sensory evaluation of salad dressing are presented in Table 4.25-4.26 and Figure 4.13-4.16.

Table 4.24 Formulation for two salad dressings with and without encapsulated γ-oryzanol powders

Ingredient (%)	Control	Add encapsulated γ-oryzanol powders
Rice bran oil	65	60
Egg York	10	10
Sugar	5	5
Vinegar	5	5
Salt	2	2
Water	10	10
Citric acid	3	3
Encapsulated γ-oryzanol extract powders	-	5

The chemical compositions of the two salad dressings are presented in Table 4.25. It found that the moisture content, water activity, protein and ash of control and salad dressing with encapsulated γ-oryzanol powders were not significantly different ($p>0.05$). The control sample contained a higher content of fat (63.96%) than those of salad dressing with encapsulated γ-oryzanol powders (58.43%), while the salad dressing with encapsulated γ-oryzanol powders (0.14 g/100 g of sample) had a higher content of γ-oryzanol than control sample (0.02 g/100 g of sample). Similar results were reported by Naknaen et al., (2010). They prepare the salad dressing fortified with different amount of Gac fruit and monitored lipid oxidation during storage at 30 °C for 4 week. The chemical composition including fat (42.39%), protein (1.80%), ash (1.58%), moisture (17.18%) and carbohydrate (37.05%) in the control sample (without Gac fruit).

Table 4.25 Chemical compositions of control and salad dressing with 5% **of encapsulated γ-oryzanol extract powders**

Chemical Properties (%)	**Control**	**salad dressing with 5% of encapsulated γ-oryzanol extract powders**
Moisture content ^{ns}	17.62±0.75	18.68±0.92
Water activity ^{ns}	0.77±0.01	0.744±0.02
Fat content	63.96±1.93^{b}	58.43±2.81^{a}
Protein content ^{ns}	8.98±0.41^{a}	8.85±0.19^{a}
Ash ^{ns}	1.24±0.05^{a}	1.23±0.02^{a}
γ-oryzanol content (g/100 g of sample)	2.03±0.00^{b}	7.06±0.01^{a}

* Each data point represents the mean±standard deviation of triplicate analysis; the multiple comparisons were determined by Duncan at $p\leq0.05$.

* Bars with the different letter are significantly different ($p\leq0.05$) for each method of extraction.

* Factor 6.25 for estimate protein content

4.9.1 Change of color

The color of all samples was stable regardless of storage time, as shown in Table 4.2.6. The color of control and salad dressing with γ-oryzanol powder were not different ($p<0.05$) during storage. All samples-packages (tightly capped glass bottles) at 25°C for 120 days showed a lower L value and decreased from 64.30 to 56.13 and 63.20 to 52.18 in the control and 5% added γ-oryzanol sample, respectively. This result indicated that the color of all sample changed from light to dark color, when the storage time was increased. This may be due to occur oxidation of carotenoids in the egg yolk used. Similar results were reported by Waite et al. 2009. They found L value decreasing with storage temperature and storage time increased.

Adding encapsulated γ-oryzanol extract powders had not significant ($p>0.05$) effect on pH of the different sample. The pH of salad dressing slightly decreased with increasing storage time (Table 4.26). Therefore, fortification of γ-oryzanol extract powders no affected on pH. The pH of salad dressing ranges from 3.6 to 4.1 (Gorji et al., 2016). The result indicated that the pH less than 4.1 to reduce the risk of microbial growth during storage ambient temperature (Radford and Board, 1993). According to this theory, all the samples were stable during storage.

Table 4.26 Color change of different formula salad dressing of different formula salad dressing during storage at 25 °C

Treatment and Storage (days)	pH	Color		
		L*	a*	b*
Salad dressing (control)				
0	4.21±0.03	64.30±0.85	6.20±0.03	30.20±1.06
30	4.09±0.08	59.13±0.45	6.44±0.18	28.83±0.67
60	4.03±0.08	59.07±0.65	6.47±0.05	29.20±0.90
90	3.94±0.09	56.77±0.91	6.40±0.22	30.07±1.11
120	3.97±0.11	56.13±0.95	6.36±0.05	29.40±1.05
Salad dressing with 5% of encapsulated γ-oryzanol extract powders				
0	4.13±0.01	63.20±0.01	6.17±0.01	29.13±0.16
30	3.99±0.03	58.39±0.04	7.02±0.02	31.32±0.73
60	4.02±0.07	53.81±0.02	6.80±0.05	31.29±0.03
90	4.13±0.09	53.47±0.05	6.79±0.06	30.24±0.20
120	4.03±0.09	52.18±0.03	6.51±0.11	31.03±0.15

The viscosity of control and added encapsulated γ-oryzanol extract powders were similar during storage. The viscosity of all salad dressing increased with increasing storage time (Figure 4.13). The results were found while increasing storage time from 60 to 120 days, resulting in significant increase in the viscosity in 5% of encapsulated γ-oryzanol extract powders and control sample salad dressing from 5706 and 5497 cP to 7721 and 7604 cP, respectively. Waite et al., (2009) who reported the viscosity of salad dressing was slightly increased thickness during storage time for 4 weeks. These result indicated that all salad dressing (emulsion) were stable during storage.

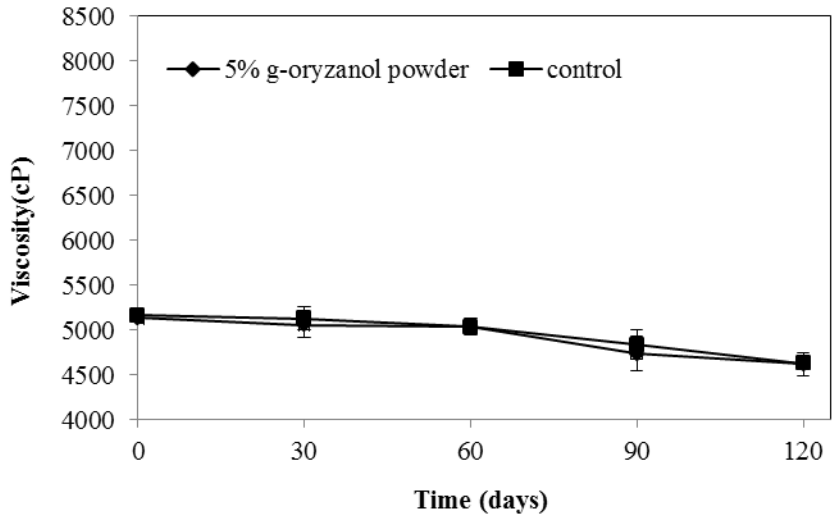


Figure 4.13 Viscosity of different formula salad dressing of different formula salad dressing during storage at 25 °C

The effect of encapsulated γ-oryzanol extract powders on γ-oryzanol content was showed in are shown in Figure 4.14. Salad dressing with 5% encapsulated γ-oryzanol extract powders had γ-oryzanol content (7.06 mg/100 g sample) higher than salad dressing without encapsulated γ-oryzanol extract powders (2.03 mg/100 g of sample). For the control salad dressing was observed γ-oryzanol content because it prepared by rice bran oil. However, all salad dressing were stability of the γ-oryzanol during storage. In salad dressing, oil droplets are surrounded by a membrane of emulsifier molecules that provides physical stability of the emulsion and oxidative stability by acting as a barrier against pro-oxidants such as metals and free radicals. Thus, the effect of emulsifier on the oxidative stability of salad dressing is important. Salad dressing is traditionally made from egg yolk, which plays the role of emulsifying agent. Salad dressing made with whole egg was the most sensitive to oxidation while soluble lecithin fractions were the best emulsifiers during storage period (Magda et al., 2003). One of the most effective means of retarding lipid oxidation in a complex food system such as salad dressing is to incorporate antioxidant. In this case, the salad dressing made with 5% encapsulated γ-oryzanol extract powders as a food ingredient.

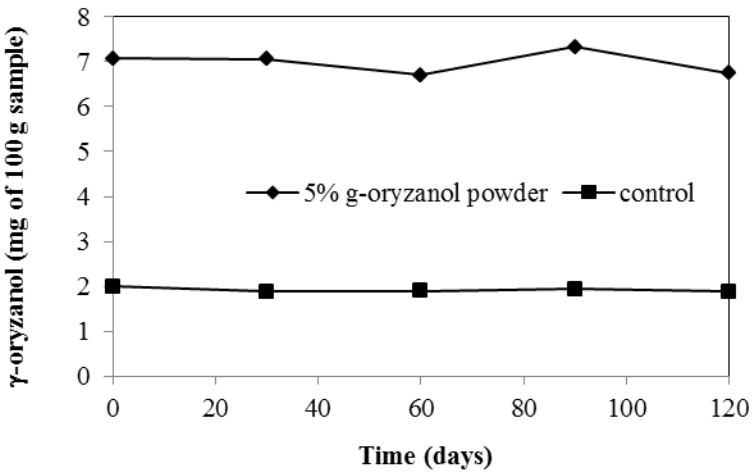


Figure 4.14 The γ-oryzanol content of different formula salad dressing of different formula salad dressing during storage at 25 °C

Antioxidant activity using DPPH assay was shown in Figure 4.15. Salad dressing with 5% encapsulated γ-oryzanol extract powders had DPPH radical scavenging activity (% inhibition) higher than salad dressing without encapsulated γ-oryzanol extract powders. The γ-oryzanol is natural antioxidant, which is antioxidant of ferulic acid. The γ-oryzanol content was observed 7.06 mg of 100 g sample and slightly decreased for storage period. Also, the DPPH scavenging activity was observed for the storage period. This results indicated that they can use encapsulated γ-oryzanol extract powders as a food ingredient, which act as a natural antioxidant.

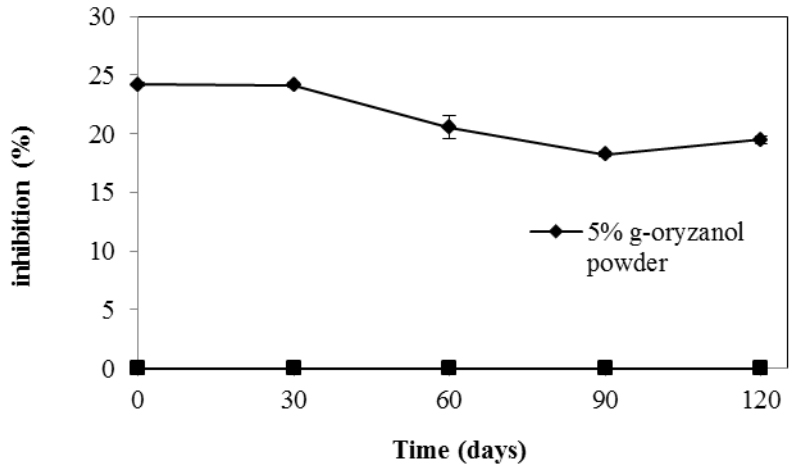


Figure 4.15 Inhibition activities (DPPH scavenging activity) of different formula salad dressing during storage at 25 °C

4.9.2 Sensory Test

The sensory analysis of salad dressing was carried out by a consumers (30 persons) by assigning score (9-point hedonic scale) for each quality attributes such as color, viscosity, odor, viscosity, taste, and overall quality, as shown in Table 4.27. All samples did not show any significant effect ($p>0.05$) on sensory attributes. The result indicated that the average score of color, taste and overall were good (a score of 7 on the scale) for the control sample. Similarly, the sensory quality of salad dressing fortified encapsulated γ-oryzanol extract powders, which were good in term of color, taste, and viscosity. Moreover, the overall acceptance score for the fortified encapsulated γ-oryzanol extract powders salad dressing was higher than the control sample. However, fortification of γ-oryzanol extract powders had no effect on consumer acceptable.

Table 4.27 Sensory evaluation with preference test (9-point hedonic scale)

Attribute	Control	Including 5% encapsulated γ-oryzanol extract powders
Color[ns]	7.35±0.67	7.20±0.83
Ordor[ns]	6.50±0.89	6.90±0.85
Viscosity[ns]	6.95±0.69	7.00±0.79
Taste[ns]	7.40±0.68	7.70±0.80
Overall[ns]	7.30±0.57	7.40±0.50

*Each data point represent the mean±standard deviation of triplicate analysis (n=30)

*Rows with the different letter are significantly different ($p \leq 0.05$).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

(1) Three different extraction techniques (soxhlet extraction, ultrasonic assisted extraction and combination between both techniques) were successfully used for the extraction of γ-oryzanol from rice bran oil soapstock (RBOS). The best conditions for soxhlet extraction were found at solid to solvent ratio of 1:12, extraction temperature of 70.1 °C, and extraction time of 7.26 h. that gave 87.52% γ-oryzanol recovery. The suitable condition for ultrasonic assisted extraction was found at a frequency of 12 kHz, extraction temperature of 74.1 °C, and extraction time of 60 min that obtained 54% γ-oryzanol recovery. In comparison of three methods, the combined technique (ultrasonic assisted and soxhlet extraction) gave the highest γ-oryzanol recovery (90.85%). γ-oryzanol was purified in high purity by two-step crystallization process, which gave the high purity (~87%).

(2) Encapsulated γ-oryzanol extract powder (EOEP) was successfully produced with spray-drying of an rice bran oil-in-water emulsion. The EOEP showed a spheroid powder, low moisture content and water activity. The encapsulated efficiency and yields were 91.55-93.22% and ~70%, respectively. The γ-oryzanol content in the EOEP were 10.76-11.27 mg/g of powder. The reconstituted of the EOEP in term of droplets size and ζ-potential were increase. The antioxidant activities (DPPH, $ABTS^{+}$ and FRAP) were similar effective as an antioxidant activity of the commercial γ-oryzanol.

(3) Shelf-life of EOEP was calculated with a half of decreasing in γ-oryzanol content. Storage time and temperature had an effect on γ-oryzanol content. The estimated shelf-life equation was $y = 3075.5x - 4.6779$ (y= ln (time (days) of shelf-life, x= Storage $temperature^{-1}$, K). The encapsulated γ-oryzanol extract powder can be kept in 25 °C for 277 days and 4 °C for 613 days. The estimation of Shelf-life for encapsulated extract γ-oryzanol powder indicated that good physicochemical of

powder such as low moisture content and water activity, high oil encapsulation efficiency.

(4) The EOEP can be used as food ingredients in salad dressing (W/O emulsion). There are two formulas of salad dressing with and without encapsulated γ-oryzanol extract powders, which did not show any significant effect on proximate analysis, sensory attribute, change of color, pH, and viscosity. However, adding encapsulated γ-oryzanol extract powders in salad dressing had good effect on γ-oryzanol content and antioxidant activity.

Recommendations

(1) As the composition of the starting soapstock itself was one of the important factors for the separation process of γ-oryzanol, more detailed information about the composition of the soapstock sample should be determined. Further studies are warranted for recovery of γ-oryzanol using novel extraction technologies.

(2) In this study, there observed significant loss of γ-oryzanol during the first crystallization to separated impurity composition, which should therefore be minimized. This could be achieved by improving rapid freezing process. Because of the impurity contains wax, triglyceride and sterol, which crystal produced at very low temperature, whereas, γ-oryzanol became the crystal at around 5 °C. Therefore, if the temperature rapidly reduced in first crystallization, that may be decrease γ-oryzanol loss in impurity crystal.

(3) The hydrophobic antioxidant activity testing is necessary as Oxygen radical absorbance capacity (ORAC), which can confirm another antioxidant ability of γ-oryzanol extract. The γ-oryzanol were found in rice bran oil soapstock, which including or 9.97% γ-oryzanol on the wet basis. The characterization analyses confirmed the potential of the RBOS as an interesting γ-oryzanol source. The process of separation starting saponification of the RBOS to convert the remaining glycerides into soap, adjust pH 9.5, and followed by extraction of γ-oryzanol from the dried matter with organic solvent (ethyl acetate). Three different techniques namely soxhlet extraction, ultrasonic assisted extraction and combination between both techniques were the effective methods for the isolation γ-oryzanol.

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APPENDICES

APPENDIX A
PREPARATION OF DEHYDRED SOAPSTOCK

APPENDIX A

PREPARATION OF DEHYDRED SOAPSTOCK

Rice bran oil soapstock mixing with NaOH

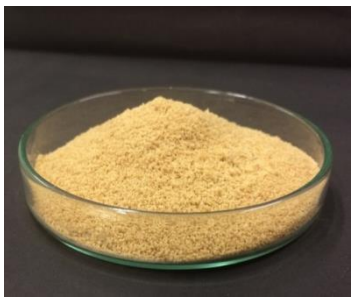
↓

Heat and stirrer at 80 °C for 30 minute

↓

Dehydrated at 90 °C

↓

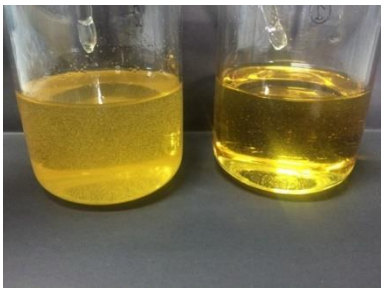


Ground and sieving (60 mesh)

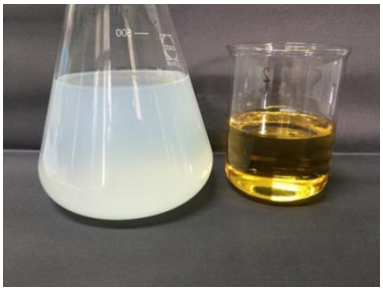
APPENDIX B
PRODUCTION OF γ-ORYZANOL EMULSION

APPENDIX B

PRODUCTION OF γ-ORYZANOL EMULSION



Rice bran oil and γ-oryzaol

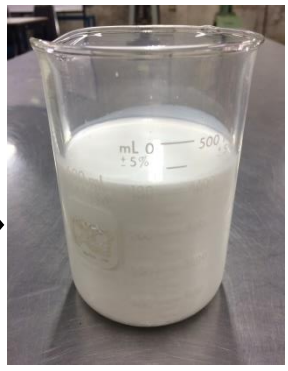


Oil phase: Rice bran oil and γ-oryzaol extract

Aqueous phase: Whey protein concentrate and Maltodextrin

Mixing

Homogenize

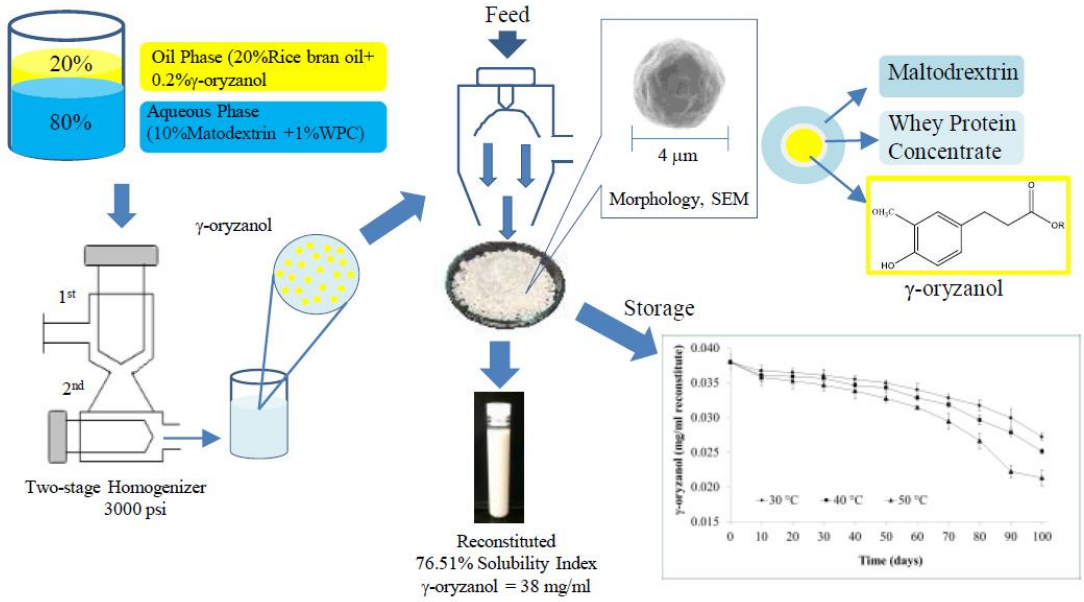


γ-oryzaol extract rice bran oil emulsion

APPENDIX C
PRODUCTION OF γ-ORYZANOL ENCAPSULATED POWDER

APPENDIX C

PRODUCTION OF γ-ORYZANOL ENCAPSULATED POWDER



Appendix Figure C1 Production of encapsulation and spray dry

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