THE CHEMICAL PROPERTIES OF RICE BRAN OILS AND ITS EFFICIENCY ON CHOLESTEROL, TRIGLYCERIDE, INFLAMMATION AND OXIDATIVE STRESS IN HYPERCHOLESTEROLEMIA RAT

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ABSTRACT

Rice bran oil (RBO) is the oil that is full with essential nutrients, especially antioxidant agents including γ-oryzanol, tocopherol and tocotrienol. At present, the RBO industry used white rice bran as the main material for production. There is another group of rice have health benefits, which is pigmented rice. The aim of this thesis was to study the chemical and antioxidant properties of RBO produced from the bran of three rice verities; Khao Dawk Mali 105 (KDML105; white rice), Red Jasmine rice (RJM; red rice) and Hom-nin rice (HN; black rice) using three extraction methods including cold-press extraction (CPE), solvent extraction (SE) and supercritical CO_2 extraction (SC-CO_2) and to compare the efficiency of the selected RBO from pigmented rice and non-pigmented rice and two RBO from a commercial produced by CEO Agrifood Co., Ltd. on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rats. It was found that the RBO yields obtained from SE, SC-CO_2 and CPE extractions were 17.35-20.19%, 14.76-18.16% and 3.22-6.22%, respectively. The RBO from the bran of pigmented rice samples exhibited high antioxidant activities. They also contained higher amount of γ-oryzanol, phytosterol and vitamin E (tocopherol and tocotrienol) than those of white rice sample. The RBO produced from HN using SC-CO_2 extraction method showed the best chemical and antioxidant properties.

Therefore, the HN-SC-CO_2 and two commercial RBO from CEO Agrifood Co., Ltd. were used to evaluate the efficiency on its ability to decrease cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rats. The thirty-six male Sprague-Dawley rats weighing 300±10 g were used in this experiment. They were divided into two groups; six rats were control group fed with normal diet (AIN93M) and thirty rats were induced to hypercholesterolemia and fed with the high fat diet (2% cholesterol mixed in normal diet) for 2 months. After the induction of hypercholesterolemia, the induced rats were randomly divided into five groups consisting of 1) the HC (hypercholesterolemia control) group fed with high fat diet (negative control), 2) the HMDG group, the hypercholesterolemia rats fed with high fat diet and 0.1 ml of molecularly distilled RBO (gavage), 3) the HRFF group, the hypercholesterolemia rats fed with high fat diet mixed with 4% of refined RBO, 4) the HHNG group, the hypercholesterolemia rats fed with high fat diet and 0.1 ml of HN-SC-CO_2 (gavage), and 5) the HHNF group, the hypercholesterolemia rats fed with high fat diet mixed with 4% of HN-SC-CO_2. The rats of groups HMRG and HHNG ate the supplement form (0.1 mL gavage), while those of groups HRFF and HSRF ate the cooking form (4% RBO replacing oil in normal diet). All groups were treated for 2 months. After treatment, all groups that received RBO, especially the HHNG group showed a decrease trend in total cholesterol, low density lipoprotein cholesterol and triglyceride compared to the untreated hypercholesterolemia group (HC). The liver weight of hypercholesterolemia rats was significantly higher ($p \leq 0.05$) than that of control group. Oxidative stress (MDA) in both plasma and liver, pro-inflammation cytokine (TNF-α) and antioxidant capacity (plasma ORAC) were improved in HMRG, HRFF and HHNG. Antioxidant enzyme (SOD) also showed an improved trend. From the results, it can be concluded that the selected HN-SC-CO_2 RBO and the company RBO could reduce free radicals, inflammation and oxidative stress in both plasma and liver of the hypercholesterolemia rats. In addition, the antioxidant enzyme could be simultaneously activated.

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ABBREVIATIONS

RBO	=	Rice bran oil
KDML 105	=	Khao Dawk Mali 105
RJM	=	Red jasmine rice
HN	=	Hom-nin
CPE	=	Cold-press extraction
SE	=	Solvent extraction
SC-CO_2	=	Supercritical carbon dioxide extraction
pH	=	Positive potential of the hydrogen ions
UV/VIS	=	Ultraviolet and Visible
M	=	Molar
FFA	=	Free fatty acid
AV	=	Acid value
PV	=	Peroxide value
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
IC_{50}	=	The half maximal inhibitory concentration
FRAP	=	Ferric reducing antioxidant power
ORAC	=	Oxygen radical absorbance capacity
SPSS	=	Statistical package for social science
ACF	=	Aberrant crypt foci
COX-2	=	Cyclooxygenase-2
PEG2	=	Prostaglandin E2
DNA	=	Deoxyribonucleic acid
mg	=	Milligram
mM	=	Millimolar
MDA	=	Malondialdehyde
STZ	=	Streptozotocin
^{o}C	=	Degree Celsius
Toc.	=	Tocopherol
T3	=	Tocotrienol

ABBREVIATIONS (CONT.)

TBARS	=	Thiobarbituric acid reactive substances
TE	=	Trolox equivalents
TNF-α	=	Tumor necrosis factor alpha
SOD	=	Superoxide dismutase
w/w	=	Weight by weight
µl	=	Microlitre
HPLC	=	High performance liquid chromatography
R^2	=	R-square value
S.D.	=	Standard deviation
TPTZ	=	2,4,6-tripyridyl-s-triazine
ELISA	=	Enzyme-linked immunosorbent assay
BHT	=	Butylated hydroxytoluene
KI	=	Potassium iodide
IV	=	Iodine value
TPC	=	Total phenolic content
CAFA	=	Cycloartenol ferulate
24MCAFA	=	24-methylene cycloartenol ferulate
CampFA	=	Campesteryl ferulate
SitoFA	=	β-sitosteryl ferulate
GC	=	Gas chromatography
SFA	=	Saturated fatty acid
MUFA	=	Monounsaturated fatty acid
PUFA	=	Polyunsaturated fatty acid
TC	=	Total cholesterol
TG	=	Triglyceride
HDL	=	High-density lipoprotein
LDL	=	Low-density lipoprotein
AOM	=	Azoxymethane

CHARPTER I

INTRODUCTION

Nowadays, most of rice bran oil (RBO) industries commonly use rice bran from whitish kernels or non-pigmented rice varieties for RBO production. These are rice varieties with white or slightly yellow tissue membrane such as Khao Dawk Mali 105, Pathum Thani 1, Phisanulok 2 and Chai Nat 1. There is another interesting group of rice duing to its health benefits is pigmented rice or colored rice. The pigments are produced and mainly stored in the part of rice bran causing the different colors of rice such as black, red or purple [1]. These are traditionally known to anthocyanin which have health benefits. The pigmented rice are more value in local markets and meet consumer need for health food. Anthocyanin presented has notable antioxidant properties and anti-inflammatory properties. Great interest has been shown that phenolic compounds have the ability to donate hydrogen and act as reducing agent. Besides, phenolic compounds also act as single oxygen and free radical hydrogen donors. Because of these properties, they provide protective effect on cell constituents against oxidative damage. Further studies have been shown that phenolic compounds can prevent cancer, cardiovascular and nerve diseases [2]. The well-known pigmented rice varieties found in Thailand are Hom-nin rice, Red jasmine rice and Rice berry. Those pigmented rice varieties are merrily produced in RBO industry both in domestic and abroad. Attention is currently being studied on the effectiveness and also the quality of pigmented rice in terms of nutritional quality with the emphasis on pigmented rice for a staple food and the main material for RBO industry.

Ministry of Health [3] reported that the degenerative diseases have risen up to triple times due to changing in lifestyle. With this lifestyle, daily foods normally consumed are easy foods and fast foods; for example, one dish meal, which is abundant with carbohydrate and oil. These food components can be absorbed to many organs in human body that cause degenerative diseases including hypercholesterolemia, hypertension, heart disease, diabetes and cancer. Thai people realize this fact therefore they have taken care of their health more and have chosen more healthy foods.

Moreover, vegetable oil is also one of alternative foods, which have been always chosen by many consumers. RBO is one of the vegetable oils that they have realized. The RBO is the oil that is full of essential nutrients, especially antioxidant agents including γ-oryzanol, tocopherol and tocotrienol [4]. These agents serve to eliminate free radicals, prevent free radicals to react with biomolecules causing damages to the body and also prevent certain diseases. Moreover, γ-oryzanol that features in reducing cholesterol levels in blood and the low density lipoprotein (LDL) can reduce the risk of coronary artery disease and can delay aging [5, 6, 7] and platelet aggregation [8]. In addition, RBO contains monounsaturated fatty acids, polyunsaturated fatty acids and phospholipids which are important in the building and repair of neurons and the brain. The ceramide is a critical component of the skin, making the skin elastic. Linoleic acid group and vitamin B complex allow for better functioning of the nervous system. Therefore, RBO industry is more interested in improving the quality of RBO. Results of many studies on product development of RBO have shown that RBO contains high vitamin E and γ-oryzanol content [9]. As a result, RBO industry is interested in the development of RBO from pigmented rice to enhance the nutritional value and to meet the needs of consumers who are interested in their health.

Despite research shows the health benefits of RBO as mentioned above, there is no report of an important ingredient in RBO that manufactured in Thailand. Consequently, the objective of this research is to study the chemical properties of RBO from 2 varieties of pigmented rice including Hom-nin rice and Red jasmine rice and 1 variety of non-pigmented rice, Khao Dawk Mali 105 rice using three extraction methods: cold-pressed method, organic solvent by n–hexane method and supercritical carbon dioxide extraction method. Obtained oil was purified and tested for the yield and quality for the comparison. The best treatment was selected. Commercial RBO was used in this study. Finally, the selected RBO and the commercial RBO were tested for its efficiency on cholesterol level, triglycerides, inflammation and oxidative stress in hypercholesterolemia rats. The data from this study can primarily be compared to humans due to the rat are mammals with a similar system to the human body.

Objectives of the study

1. To study the properties of RBO from 3 varieties of rice (Hom-nin rice, Red jasmine rice and Khao Dawk Mali 105) using 3 extraction methods (cold-press, solvent and supercritical fluid extraction).

2. To study and compare the efficiency of RBO from pigmented rice and non-pigmented rice and commercial RBO on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rats.

CHARPTER II

LITERATURE REVIEWS

Rice and by-products

Rice (*Oryza sativa* L.) is the main staple food in Thailand and many countries in Asia. It is also the main exported product of Thailand. About 20 million tons per year of rice are produced in Thailand, which produces about 1.6 million tons of rice bran [10]. Rice is considered a basic source of energy for more than half of the population worldwide [11]. In Thailand, rice exhibits many colors, such as white, black, red and purple depending on the pigments accumulated in the pericarp and bran layer of the rice kernels. Rice grain structure consist of hull, pericarp, seed coat, aleuronic layer, embryo and endosperm (Figure 1).

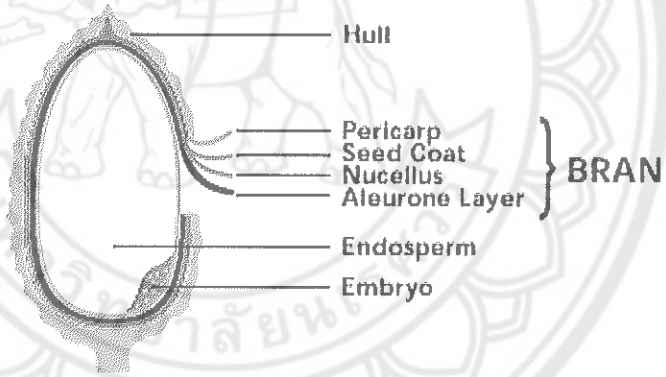


Figure 1 Component of rice grain [12]

1. Hull

Rice hulls, the hard outer covering brown rice which protect rice grain during growing season from insect and changing of external environment.

2. Pericarp

The most outer fibrous layer which thicks about 10 μm and locates the pigment in colored rice. Main function of pericarp is to serve as protective layer

molds and quality deterioration from oxidation and enzymes due to the movement of oxygen, carbon dioxide and vapor of water.

3. Seed coat

Seed coat is only a few cells in thickness 0.5 μm. This layer is rich in oil and protein contents but low in starch content.

4. Aleuronic layer

Aleuronic layer is part of endosperm which is the most outer. Therefore, bran portion of rice grain comprise of pericarp, seed coat, nucellus and aleuronic layer and typically includes embryo. Rice bran contains 18–22% oil. This part is removed by milling. Bran is the most nutritious part of rice and rich in fiber, protein, oil, vitamins and minerals although it has very low starch.

5. Embryo or germ

Embryo is very small because weight only 1–3% of total rice grain. It locates at central bottom portion of the garin. Embryo can grow to be a new plant because it is the living organism in the grain. Embryo is removed by milling result in indented shape at the end of the milled rice grain.

6. Endosperm

After removing the hull, the bran and the embryo, endosperm remains. Endosperm is the largest portion of the rice grain because weight 80% of total rice grain. In addition, endosperm is rich in carbohydrate but low in vitamin and mineral.

Thai non-pigmented rice varieties such as Khao Dawk Mali 105 or Thai jasmine rice, Pathum Thani 1, Phitsanulok 2 and Chai Nat 1, and pigmented rice (black, red and purple) such as Hom-nin rice, Red jasmine rice and Rice berry, have received the attention from consumers who are looking for healthier foods. The health benefits of pigmented rice are attributed to the presence of antioxidant compounds that possess anticarcinogenic, antiallergic, anti-inflammatory, anti-atherosclerosis, hypercholesterolemia and hypertension [13].

Rice bran is a by-product of the rice milling process, represents about 8–11% of the grain by weight and it contains various antioxidants that impart beneficial effects on human health. A major rice bran fraction contains 18–22% oil and highly unsaponifiable components (4.3%) including fibre, protein, minerals, vitamins [14], phytochemicals such as γ-oryzanol [15], tocols (tocopherols and tocotrienols) and

polyphenols [16]. These phytochemicals show antioxidant and free radical scavenging activities [17].

1. Nutritional values of rice by-products

Rice by-products generally contain higher amount of nutrients when compared to the polished rice. Table 1 shows the proximate composition and major minerals of several rice by-products. Rice bran, which is derived from the outer layer of the rice grain, is composed of an aleurone layer of the rice kernel, with some proportion of the endosperm and germ [18] accounting for approximately 10% of the weight of the rice grain [19]. This part is composed of both lipophilic antioxidants (tocopherols, tocotrienols and γ-oryzanol) and phenolics [20]. These substances protect against chronic diseases of the cardiovascular system and help to quench the free radicals and anticancer effects [21, 22].

In addition, the consumption of whole grains has been reported to protect against colorectal cancer in human interventions [23]. The chemopreventive properties of whole grain consumption have been attributed both to fibers and to other phytochemicals [24] that are mostly present in the bran layer. Table 2 summarizes several previous findings on the effect of rice by-products on cancer.

Table 1 Proximate composition and minerals content of rice by-products [25]

Nutrients	Content (% of dry matter)				
	Broken	Husk	Bran	Polishing	Straw
Dry matter	87.0-89.0	87.0-92.5	89-94	90	90.9
Protein	6.7-9.8	2.1-4.3	10.6-16.9	11.2-13.4	1.2-7.5
Crude fat	0.5-1.9	0.30-0.93	5.1-19.7	10.1-13.9	0.8-2.1
Crude fiber	0.6	30.0-53.4	7.0-18.9	2.3-3.6	33.5-68.9
Ash	5.0	13.2-24.4	8.8-28.8	5.2-8.3	12.2-21.4
Carbohydrate	-	22.4-35.3	90	51.1-55.0	39.1-47.3
Calcium	0.09-0.19	0.04-0.21	0.08-1.4	0.05	0.30-0.71
Phosphorus	0.03-0.04	0.07-0.08	1.3-2.9	1.48	0.06-0.16

Table 2 Summary of anti-cancer effect of rice by-products

Authors	Components	Findings
Kawabata et al. [26]	Rice germ	Inhibit ACF formation and reduce incidence of colonic adenocarcinoma
Huang et al. [27]	Isovitexin (rice husk)	Inhibit release of TNF-α, a pro-inflammatory cytokine and cyclooxygenase-2 (COX-2) expression reduced lipopolysaccharide-stimulated prostaglandin E2 (PEG_2)
Jeon et al. [28]	Methanol extracts (rice husk)	Possess significant reactive oxygen activity scavenging and metal chelating activities and protective against oxidative DNA damage using human lymphocytes
Kim et al. [29]	Methanol extracts (rice husk)	Highly cytotoxic, with IC_{50} values of 0.5 μg/mL *in vitro* reduced colonic pre-neoplastic ACF formation by 35%
Boateng et al. [30]	Rice bran	5% and 10% rice bran significantly ($p < 0.05$) reduce the incidence of azoxymethane (AOM) induced colon tumors in male Fisher 344 rats after 44 weeks feeding
Kannan et al. [31]	Peptides (rice bran)	Inhibit 84% of colon cancer cells (Caco-2 and HCT-116) growth, 80% for breast cancer cells (MCF-7, MDA-MB-231) growth and 84% for liver cancer cells (HepG-2) growth

2. Varieties of rice

In this research, 3 varieties of rice were used; Hom-nin rice, Red jasmine rice and Khao Dawk Mali 105 for extraction of RBO.

1. Hom-nin rice

Among the black rice, the most popular is probably Hom-nin rice or fragrant purple rice developed by Kasetsart University (Thailand's agricultural university). It looks black when raw but is actually deep purple when cooked. More importantly, it contains a substance called proanthocyanidin, which gives the rice its dark color, and is a more potent antioxidant than non-pigmented rice. Many studies have reported that black rice is rich of anthocyanin and other polyphenolic compounds much more abundantly than white rice [32, 33]. Table 3 shows the nutritional information of Hom-nin rice.

Table 3 Nutritional information of un-milled Hom-nin (Black) rice [34]

Phytonutrients	Content
Protein	10-12.5%
Complex carbohydrate	70-86%
Amylose	7-16%
Amylopectin	80-57%
Omega-3 (Fat)	1-2%
Digestible fiber	2-10%
Sodium	0.001%
Iron	2.25-3.26 mg/100 g
Zinc	2.9 mg/100 g
Copper	0.1 mg/100 g
Calcium	4.2 mg/100 g
Potassium	20 mg/100 g
Vitamin B1	0.34 mg/100 g
Vitamin B2	0.05 mg/100 g
Vitamin B3	4.7 mg/100 g
Vitamin B6	0.62 mg/100 g
Folic acid	339.4 µg/100 g
Antioxidants-Bioflavonoids: anthocyanin (cyanidin), proanthocyanidin (procyanidin), and vitamin E	293 µmol/100 g

2. Red jasmine rice

Red jasmine rice have been popular among health-conscious consumers. Harvested from mid-November through mid-December, red rice is primarily cultivated in Surin, a province in the North-East of Thailand near the border of Cambodia. This rice has an antioxidant pigments called anthocyanins. This antioxidant is beneficial to reduce symptoms of inflammation, allergy relief, cancer prevention and weight loss management. Red jasmine rice is a rich source of fiber, iron, thiamin, calcium and vitamin B1 (Table 4). Red jasmine rice is believed to reduce cholesterol and aid in performance of the Circulatory system [35].

Table 4 Nutritional information of red jasmine rice [35]

Phytonutrients	Content
Calories	216 kcal
Fat	2.70 g/100 g
Dietary fiber	2.65 g/100 g
Protein	6.66 g/100 g
Iron	8.99 mg/kg
Zinc	19.22 mg/kg
Calcium	180 mg/kg
Vitamin B1	0.14 mg/100 g
Vitamin B2	<0.25 mg/100 g
Vitamin B6	0.28 mg/100 g
Vitamin E	0.12 mgaTE/100g
Polyphenol	3.95 GAE mg/g

3. Khao Dawk Mali 105 (Jasmine rice)

Jasmine rice was first developed in Thailand between 1949 and 1950 [36] by breeding and selection of indigenous aromatic rice varieties and released to farmers in Thailand around 1959 [37]. The best variety of Jasmine rice was named "Khao Dawk Mali 105". Since then, the varieties of Thai aromatic rice has collectively been known

as "Khao Hom Mali" and has been translated into English as "Jasmine rice". The nutritional information of Khao Dawk Mali 105 was shown in Table 5.

Table 5 Nutritional information of Khao Dawk Mali 105 [37]

Phytonutrients	Content
Calories	356 kcal
Protein	6.20 g/100 g
Fat	1.10 g/100 g
Carbohydrate	80.40 g/100 g
Dietary fiber	0.60 g/100 g
Vitamin B1	0.11 mg/100 g
Vitamin B2	0.04 mg/100 g
Niacin	0.90 mg/100 g
Calcium	3 mg/100 g
Phosphorus	66 mg
Iron	Little

RBO extraction

RBO can be extracted by many various methods such as cold-press extraction, solvent extraction, supercritical fluid extraction, ultrasonic extraction, and microwave extraction but, this research used three methods to extract RBO, i.e. cold-press extraction, solvent extraction (n-hexane) and supercritical fluid extraction.

1. Cold-press

Cold-pressed oil is the oil which has been produced by application of a low heat technique and without using chemicals. The introduction of heat to the process will degrade the flavor, nutritional value, and color of the oil. Heat, however, increases the yield. For this reason, cold-pressed oil tends to be more expensive, but it has higher quality.

Commercial of mechanical pressing (cold-pressing) of RBO in Thailand has been used in small and medium factories. It is simple, ecologically friendly and does not require much energy; therefore it is suitable for small and medium scale industry.

industry. Mechanical pressing equipment manufactured in Thailand was shown in Figure 2. Its main components are: (a) driving motor (1 hp, 220 V); (b) maddock screw type; (c) barrel (longitudinal barrel with 16 bar cage inside upon which the screw rotates); (d) feed hopper with adjustable speed feed motor; (e) power transmission and gear reduction unit; and (f) frame. The screw press acts by the effect of its rotation within the stationary cylindrical barrel. As the screw rotates towards the discharge end of the barrel, the bran is conveyed under pressure along and to the end of the barrel. The extracted oil drains through perforations in the barrel's bottom while the pressed rice bran cake is discharged through the circular aperture between the tapered end of the screw and the barrel [38].

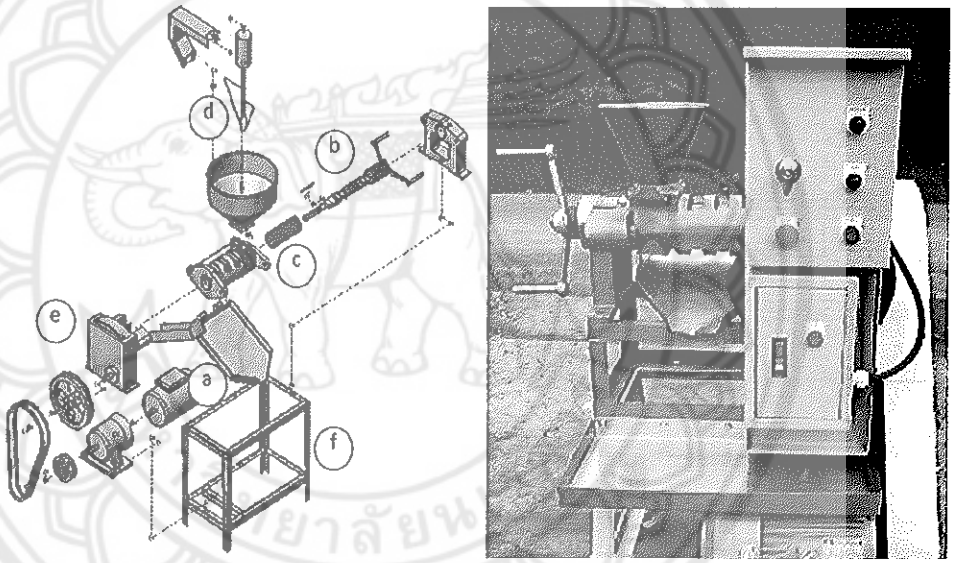


Figure 2 Schematic diagram of cold-pressing machine

Note: (a) driving motor (1 hp, 220 V), (b) maddock screw type, (c) barrel (longitudinal barrel with 16 bar cage inside upon which the screw rotates), (d) feed hopper with adjustable speed feed motor, (e) power transmission and gear reduction unit, (f) frame [38].

2. Solvent extraction

Commercial RBO is extracted using organic solvents [39-41]. Solvent extraction is an oil-extraction process using organic solvents such as hexane, ethanol, petroleum ether, or methanol. Amongst others, hexane has been used as the solvent for rice bran extraction by many researchers and industrialists due to the availability, high oil extractability (98%) and easy operation [41, 42].

3. Supercritical carbon dioxide extraction

Supercritical carbon dioxide Extraction ($SC\text{-}CO_2$) is based on the fact that, near the critical point of the solvent, its properties change rapidly with only slight variations of pressure. $SC\text{-}CO_2$ is the process of separating one component (the extractant) from another (the matrix) using supercritical fluids as the extracting solvent. Figure 3 displays a schematic flow diagram of $SC\text{-}CO_2$. Carbon dioxide is the most commonly used in SFE because of its low critical temperature (31°C), inertness, low toxicity and reactivity and high purity at low cost. Extraction conditions for supercritical carbon dioxide are above the critical temperature of 31°C and critical pressure of 74 bar [43].

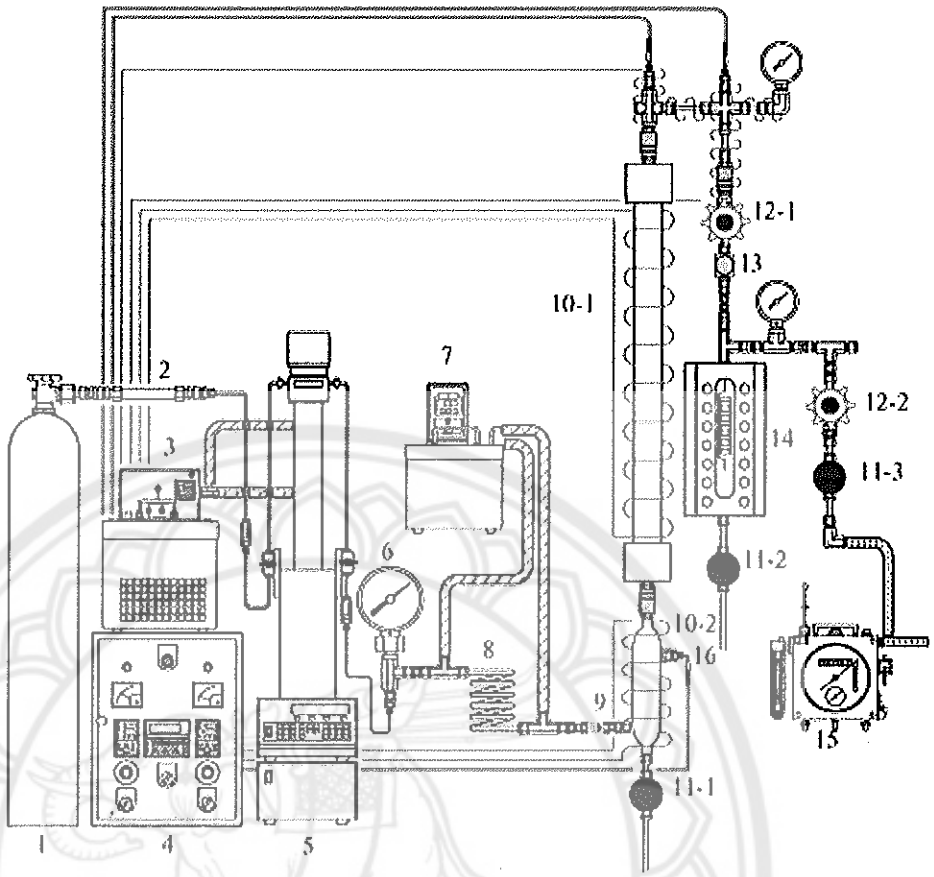


Figure 3 Schematic flow diagram of SC-CO_2 extraction of RBO [44]

Note: 1. CO_2 cylinder 2. CO_2 cleanup column
3. Cold liquid circulator 4. Temperature controller
5. High pressure pump 6. Pressure gauge
7. Hot liquid circulator 8. Preheater
9. Check valve 10-1. Extraction vessel
10-2. Reboiler 11-1~3. Metering valve
12-1~2. Backpressure regulator 13. Needle valve
14. Separator 15. Wet gas meter
16. Thermocouple 17. Line connector

From the above methods, it can be seen that each method of oil extraction has its own advantages and disadvantages. The advantages and disadvantages of three extraction methods are summarized in Table 6.

Table 6 Advantages and disadvantages of three extraction methods [45, 46, 47]

Methods	Advantages	Disadvantages
Cold-press	- Virgin oil	- Time and labor intensive
	- No potential for solvent contamination	- Relatively low oil yields
	- Low consumable costs	- Low and inconsistent oil production
	- No environmental problem regarding the use of screw press	- High oil loss
Solvent extraction	- High oil yields	- High potential for solvent contamination
	- Relatively simple and quick	- Safety issues and environmental concerns
	- Suitable for bulk oil extraction	
	- Low capital investment	
	- No especial equipment required	
	- Hexane can be recovered and reused, reducing cost significantly	
Supercritical CO_2 extraction	- Can be easily recycled	- Very high cost
	- Has good solvent characteristics for non-polar and slightly polar solutes	
	- The dissolving power and selectively can be controlled by selection of suitable pressure, temperature combination	
	- Decreasing the risk of damage of thermalabile compounds	
	- The pressure excess in the equipment prevents oxygen entry while extraction occurs, oxidation reactions do not happen	

Refining process

Refining produces edible oil with characteristics that consumers desire such as bland flavour and odour, clear appearance, light colour, stability to oxidation and suitability for frying. Two main refining routes are chemical refining and physical refining (steam stripping, distillative neutralisation) which are used for removing the free fatty acids. The refining process includes degumming, neutralization, bleaching, dewaxing and deodorization [48] (Figure 4). Degumming removes phospholipids and lipoproteins, through hydration, by adding water and either citric or phosphoric acid, followed by centrifugation [49, 50]. During neutralization, free fatty acids are removed by precipitation with a sodium hydroxide solution [51] and the sodium salts of the free fatty acids (soaps) are separated by centrifugation [49]. The pigments naturally present in the crude oil (including chlorophylls and carotenoids) are removed by adsorption on bleaching earth [52]. During dewaxing, the oil is maintained at low temperatures to provoke wax crystallisation; then solidified waxes are removed by filtration or centrifugation [50]. Finally, during deodorization, volatile substances that are responsible for undesirable odors are removed; for this purpose, the oil is heated to 200–250 °C at low pressures (3--5 mm Hg) [49, 53].

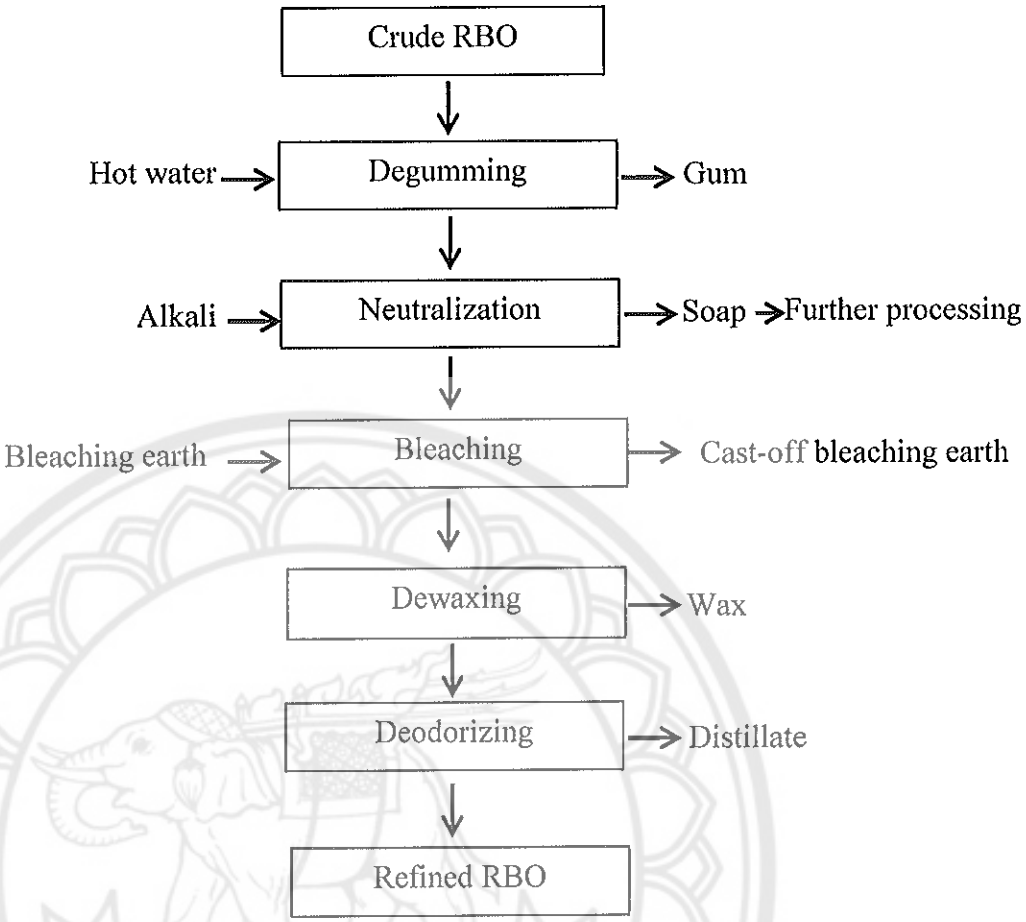


Figure 4 Flow chart of RBO chemical refining [48, 54]

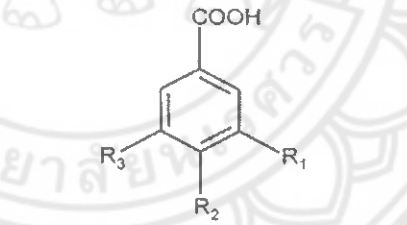
Antioxidants

Rice bran is a rich source of natural antioxidants which can be used as free radical scavengers. It is widely recognized that many of the today's diseases are due to the oxidative stress that resulted from an imbalance between formation and neutralization of free radicals. The antioxidants are very efficient in reducing low density lipoprotein and total serum cholesterol. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation

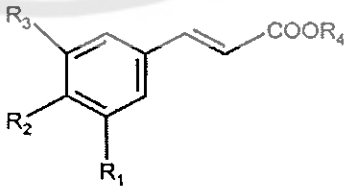
reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as γ-oryzanol, anthocyanins, phenolic acids and vitamin E [55].

1. Phenolic compounds

Phenolic compounds, a group of chemical compounds that are widely distributed in nature. Evidence indicates that phenolic compounds have potent antioxidant properties and free radical scavenging capabilities [56]. Phenolic compounds are known to exert various physiological effects in humans, such as preventing oxidative damage of lipid and low density lipoproteins [57], inhibiting platelet aggregation [58] and reducing the risk of coronary heart disease and cancer [59, 60]. Cereal grains contain unique free phenolic compounds and their glycosides, which exist in solution, and a significant amount of insoluble phenolic compounds, most of which are bound to polysaccharides in the cell wall [61]. These compounds are concentrated in the bran layers and are lost with the separation of seed coat during processing. Similarly, most phenolic compounds in rice, which is a major staple cereal all over the world, particularly in Asia, are also lost with rice bran. Figure 5 illustrates the chemical structures of phenolic compounds in rice.



Protocatechuic acid: R_1=H, R_2=OH, R_3=OH
Hydroxybenzoic acid: R_1=H, R_2=OH, R_3=H
Vanillic acid: R_1=H, R_2=OH, R_3=OCH_3
Syringic acid: R_1=OCH_3, R_2=OH, R_3=OCH_3



Caffeic acid: R_1=OH, R_2=OH, R_3=OH, R_4=H
p-Coumaric acid: R_1=H, R_2=OH, R_3=H, R_4=H
Ferulic acid: R_1=OCH_3, R_2=OH, R_3=H, R_4=H
Sinapinic acid: R_1=OCH_3, R_2=OH, R_3=OCH_3, R_4=H
Chlorogenic acid: R_1=OH, R_2=OH, R_3=H, R_4=quinate

Figure 5 Chemical structures of phenolic compounds in rice [62]

2. 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. The chemical structures of γ-oryzanol are illustrated in Figure 6. Norton [63] reported that the complete oryzanol group is unique to RBO and the exact composition of oryzanol depends on the rice cultivars. γ-Oryzanol, a mixture of phytosteryl ferulates comprises 3 major components cycloartenyl ferulates, 24-methylenecycloartanyl ferulate and campesteryl ferulate. The content of γ-oryzanol in the extracted RBO is approximately 1.8-30 g/kg dry rice bran [64]. These γ-oryzanol have several biological and physiological effects, such as serving as antioxidation, anti-blood cholesterol and anti-carcinogenic agents [65, 66, 67].

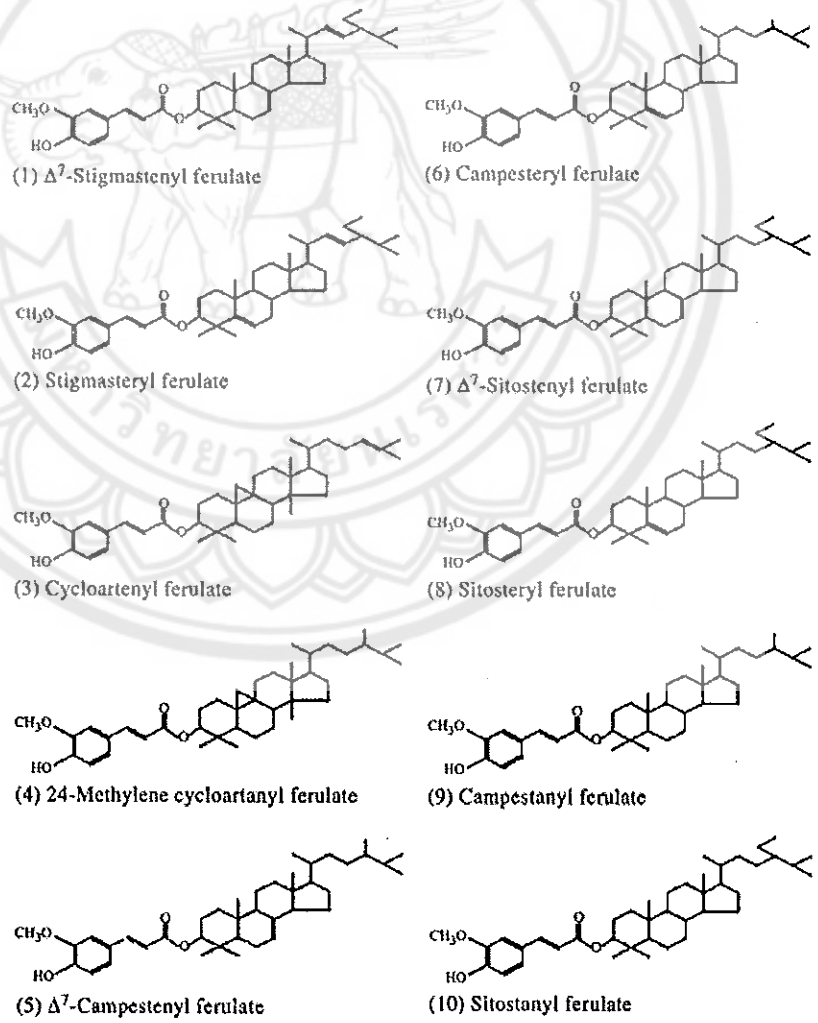


Figure 6 Chemical structures of γ-oryzanol [68]

3. Vitamin E

Vitamin E is a fat-soluble nutrient found in many foods. In the body, it acts as an antioxidant, helping to protect cells from the damage caused by free radicals. Vitamin E is a generic term for a group of four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ), of which α-tocopherol has the highest biological activity [69]. RBO contains about 0.1–0.14 mg/g vitamin E components [70]. Tocopherols constitute a series of related benzopyranols (or methyl tocols) that occur in plant tissues and vegetable oils and are powerful lipid-soluble antioxidants. In the tocopherols, the C16 side chain is saturated, but in the tocotrienols it contains three trans double bonds. Together, these two groups are termed the tocochromanols. In essence, the tocopherols have a 20-carbon phytyl tail (including the pyranol ring), with variable numbers of methyl groups attached to the benzene ring, and the tocotrienols a 20-carbon geranylgeranyl tail with double bonds at the 3', 7' and 11' positions, attached to the benzene ring. The side-chain methyl groups of natural tocopherols have R,R,R stereochemistry. The four main constituents of the two classes are termed : alpha (5,7,8-trimethyl), beta (5,8-dimethyl), gamma (7,8-dimethyl) and delta (8-methyl). The tocotrienols have a single chiral centre (Figure 7).

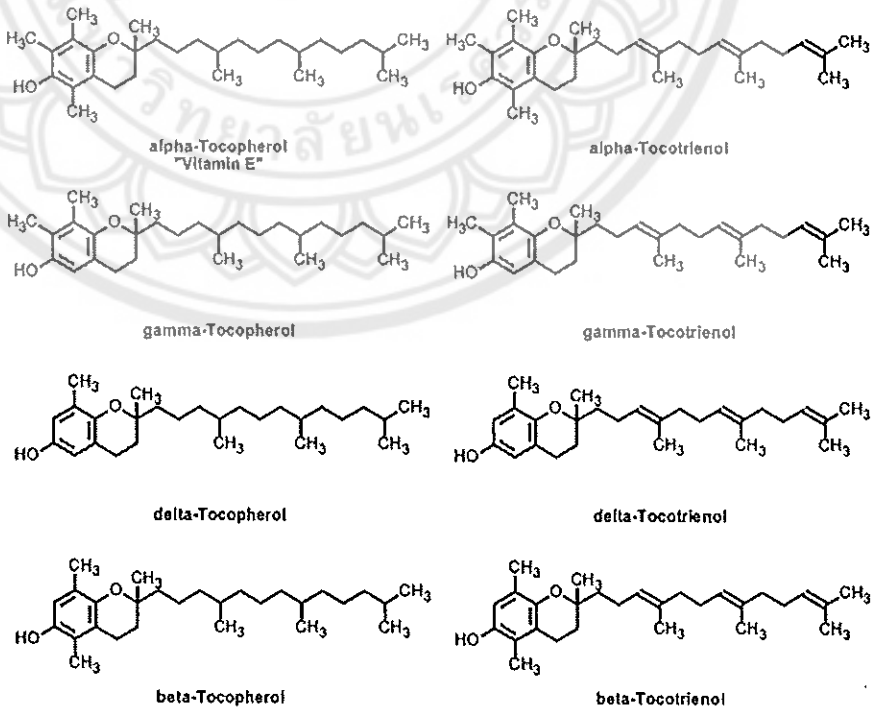


Figure 7 Chemical structures of tocopherols and tocotrienols [71]

4. Phytosterol

Phytosterols or plant sterols are a family of molecules similar to cholesterol. They are found in the membranes of plant. The structure of phytosetrols consists of a steroid skeleton with a hydroxyl group attached to the C-3 atom of the A-ring and an aliphatic side chain attached to the C-17 atom of the D-ring. Sterols have a double bond, typically between C-5 and C-6 while cholesterol has a side-chain composed of eight carbon atoms (Figure 8.) More than 100 types of phytosterols have been reported in plant species, but the more abundant are sitosterol, stigmasterol and campesterol [72, 73, 74]. Phytosterols have been described as bioactive molecules. The nutritional role of these compounds to reduce cholesterol levels in humans have been known since the 1950s [75]. Moreover, these substances are also used in other, such as cosmetics (creams, lipstick).

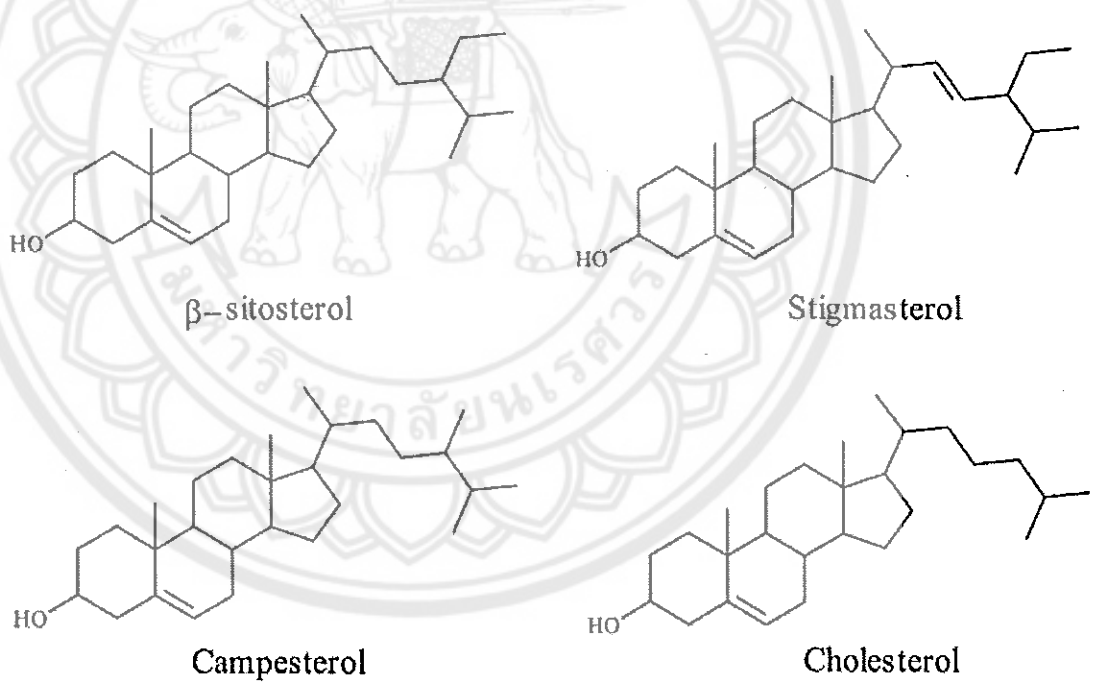


Figure 8 Chemical structures of β-sitosterol, stigmasterol, campesterol and cholesterol [76]

Health benefits of RBO

1. Efficiency of RBO on lipid-lowering

RBO is now considered to be a good substitute for vegetable oils. Moreover, RBO is a healthy oil. It is well established that RBO is one of the most nutritious and health-beneficial edible oils, as it contains high level of physiologically active compounds (Table 7). γ-Oryzanol and phytosterols have the capacity to lower blood cholesterol and decrease cholesterol absorption. Tocotrienols and γ-oryzanol are known as powerful antioxidants, which are associated with the prevention of cardiovascular diseases [77, 78, 79, 80, 81, 82]. Simultaneously, several studies have shown the beneficial of RBO in improving lipid profiles both in animal and humans as show in Table 8.

Table 7 Cholesterol lowering activity of RBO in comparison to other edible oils [25]

Edible oils	Cholesterol level (%)
Safflower	-16
Sunflower	-12
Soybean	+3
Sesame	+2
Corn	-15
Rice Bran	-17
Groundnut	+5

Many studies attempt to find out the health benefit of RBO in animal and human trials. There are detailed as below.

Chou et al. [83] studied in diabetic rats fed with the RBO diet (15% RBO) for 5 weeks. The results showed significant reduced in plasma total cholesterol/high density lipoprotein cholesterol (TC/HDL-C) concentration when compared with the diabetic control rats (diabetic control rats were fed 15% soybean oil).

Chen, & Cheng [84] studied similar model of Chou et al. [83], where the diabetic rats fed with the diets containing RBO for 4 weeks. It also showed significant decrease in plasma and hepatic total triglyceride levels in diabetic rats when compared with the rats fed with control diet.

Wilson et al. [66] observed hypercholesterolemia rats fed with 10% RBO for 10 weeks. The results showed significantly reduced plasma TC, LDL and total triglyceride concentration when compared to control rats fed with corn oil. In addition, RBO is also contained tocotrienols, which is reported to inhibit endogenous cholesterol biosynthesis by destroy HMG-CoA reductase activity in hypercholesterolemia rats.

Lai et al. [85] studied the efficiency of RBO on type 2 diabetes patients. Thirty patients were random to take 18 g soybean oil or 18 g RBO for 5 weeks. After 5 weeks, patients consumed RBO had significantly reduced serum TC levels when compared to soybean oil group. Moreover, it also showed a trend in decreasing serum LDL-C concentration.

Most et al. [78] studied in 14 adults that have hypercholesterolemia disease that consumed RBO and control oil diet for 5 weeks. The results showed that serum TC and LDL-C were reduced for those who consumed the diet containing RBO.

Berger et al. [86] studied the efficiency of RBO on 30 men with hypercholesterolemia disease for 4 weeks. The results showed a decrease in plasma TC and LDL-C concentration, as well as in LDL/HDL-C ratio.

Table 8 Efficiency of RBO on lipid-lowering in animal and human studies

Condition	Induction	Duration (week)	Dose (g or % dietary fats)	Pharmacological effect	Reference
Animal					
Diabetic rats	STZ/nicotinamide	5	15%	↓ Plasma TC/HDL-C ↑ Plasma HDL-C	Chou et al. [83]
Diabetic rats	STZ/nicotinamide	4	10–15 g	↓ Plasma TC/HDL-C	Chen, & Cheng [84]
Hypercholesterolemic rats	Hypercholesterolemic diet	10	10%	↓ Plasma TC, TG, LDL-C ↑ Plasma HDL-C	Wilson et al. [66]
Human					
Type 2 diabetes	-	5	18 g	↓ Serum TC	Lai et al. [85]
Hypercholesterolemia	-	5	12%	↓ Plasma TC, LDL-C	Most et al. [78]
Hypercholesterolemia	-	4	50 g	↓ Plasma TC, LDL-C	Berger et al. [86]

2. Efficiency of RBO on antioxidant activity

The effect of RBO on antioxidant activity in animal is shown in Table 9. Details are as followed.

Ha et al. [87] studied the efficiency of RBO on hypercholesterolemia rats that fed with high fat diet containing RBO and without RBO for 4 weeks. The results showed a significant decrease in hepatic lipid peroxide (TBARS) levels in hypercholesterolemia rats that fed with high fat diet containing RBO when compared to untreated RBO group.

Rana, Vadera, & Soni [88] studied in stress reduced rats. Healthy rats were divided into two groups that fed with diet containing 20% RBO and groundnut oil for 4 weeks, and then half the rats of each group were induced stress by N-nitrosodiethylamine injection. The results showed that RBO significantly reduced malondialdehyde (MDA) levels in erythrocytes, liver, heart and spleen in rats induced with stress when compared to control rats.

Minhajuddin, Beg, & Iqal [89] studied in hyperlipidemia rats. The rats were induced with atherogenic diet (5% hydrogenated fat, 0.5% cholic acid and 0.1% cholesterol) for 3 weeks and treated tocotrienol (T3) isolated from RBO at difference doses (0–50 mg/kg/d) for 1 week. The results showed significantly reduced hepatic TBARS and plasma LDL-C levels in hypercholesterolemia rats when compared to control group.

Ghatak, & Panchal [90] investigated the effect of γ-oryzanol fraction from RBO at dose 50 and 100 mg/kg body weight in hypercholesterolemia rats. The results showed that γ-oryzanol decreased hepatic MDA and increased hepatic reduce glutathione.

Table 9 Antioxidantive effect of RBO and its components

Condition	Induction	Treatment	Duration (week)	Pharmacological effect	Reference
Hypercholesterolemia rats	High fat diet	RBO	4	↓ Hepatic TBARS	Ha et al. [87]
Stress induced rats	N-nitrosodiethylamine	RBO	4	↓ MDA in erythrocyte, liver, heart, lung and spleen	Rana, Vadera, & Soni [88]
Hyperlipidemia rats	High fat diet	T3	1	↓ Hepatic TBARS, plasma LDL-C oxidation	Minhajuddin, Beg, & Iqal [89]
Hyperlipidemia rats	Triton WR-1339	γ-Oryzanol	3	↓ Hepatic MDA ↑ Hepatic reduce glutathione	Ghatak, & Panchal [90]

CHARPTER III

RESEARCH METHODOLOGY

Materials

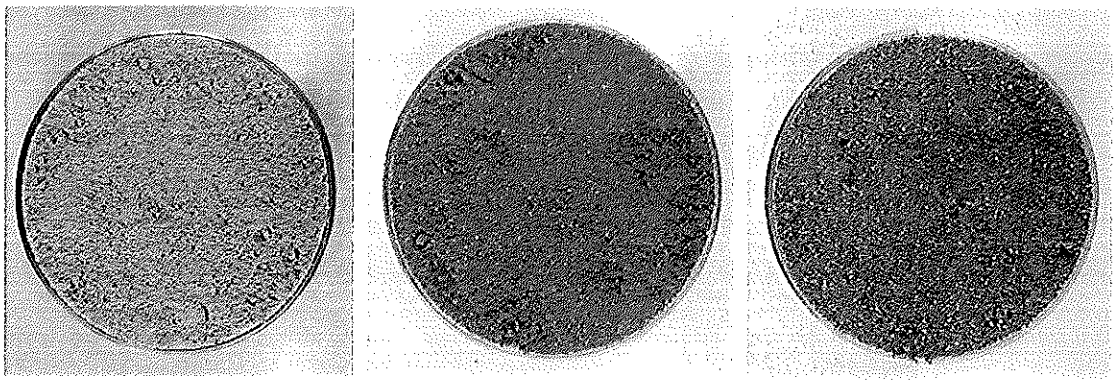
Materials used in this research were divided into 2 groups: 1) pigmented and non-pigmented rice and 2) RBO samples. Details of the materials are as follow.

1. Pigmented rice (Hom-nin rice, Red jasmine rice) and non-pigmented (Khao Dawk Mali 105) were brought from the community enterprise in Phichit Province. They were harvested between December 2014–February 2015. The paddy was dried to reach a moisture content of 13% by hot air oven. The dried paddy was milled and polished to obtain the rice bran at Tambon Nongsano, Samngam District, Phichit Province. The appearances of rice bran are shown in Figure 9. The rice bran was divided into 3 portions for using in the extraction (cold-press extraction, solvent extraction and supercritical CO_2 extraction). For cold-press extraction rice bran was extracted within 24 h. The other two samples were stored at –20°C by packing rice bran in a 3 kg vacuum aluminum foil bag until further use for extraction by solvent extraction and SC-CO_2 extraction methods.

2. Two RBO samples from CEO Agrifood Co., Ltd., including molecularly distilled RBO and processed RBO or refined RBO.

2.1 Molecularly distilled RBO was the oil extracted by solvent extraction and refined by a molecular distillation technique.

2.2 Processed RBO or refined RBO was the oil extracted by solvent extraction and refined by chemical refining process.



Rice bran of Khao Dawk Mali 105 | Rice bran of Red Jasmine | Rice bran of Hom-nin

Figure 9 The appearance of three rice bran varieties

Chemicals

1. Butylated hydroxytoluene (BHT) (Sigma-Aldrich, st. Louise, U.S.A)
2. Chloroform (RCI Labscan, Thailand)
3. Ethanol (Merck, Germany)
4. Ferric chloride (Sigma-Aldrich, st. Louise, U.S.A)
5. Folin-Ciocalteu reagent (Sigma-Aldrich, st. Louise, U.S.A)
6. Gallic acid (Sigma-Aldrich, st. Louise, U.S.A)
7. Glacial acetic acid (RCI Labscan, Thailand)
8. Hexane (RCI Labscan, Thailand)
9. Hydrochloric acid (RCI Labscan, Thailand)
10. Methanol (Merck, Germany)
11. Petroleum ether (RCI Labscan, Thailand)
12. Potassium phosphate (Fisher Scientific, U.K.)
13. Sodium carbonate (Ajax Finechem, Auckland, New Zealand)
14. Sodium hydroxide (RCI Labscan, Thailand)
15. Sodium thiosulphate (Ajax Finechem, Auckland, New Zealand)
16. 2,2-diphenyl-l-picrylhydrazyl (DPPH) (Sigma-Aldrich, st. Louise, U.S.A)
17. 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma-Aldrich, st. Louise, U.S.A)
18. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, st. Louise, U.S.A)

Apparatuses

1. Centrifuge (Eppendorf, model 4503, Germany)
2. Gas chromatography (GC) (GL Sciences, model GC4000, Japan)
3. Fluorescence spectrophotometer (PerkinElmer, Model LS 55, USA)
4. High performance liquid chromatography (HPLC) (Agilent Technologies, model 1100 series, USA)
5. High performance liquid chromatography mass spectrometry (HPLC ms/ms) (AB Sciences, model 4000 QTRAP, Japan)
6. Lovibond spectrocolorimeter (Lovibond colour, Model PFX-880L, Germany)
7. Magnetic stirrer (IKA, model C-MAG HS 7, USA)
8. Rotary evaporator (Heidolph, model Hei-VAP value, Germany)
9. Soxhlet apparatus (Electrohermai, model EME6, England)
10. Supercritical CO_2 extractor (Chengdong, model TH221-24, China)
11. UV-Vis spectrophotometer (Hitachi, model U2900/2910, Japan)
12. Vacuum pump (Rocker, model 300, Taiwan)
13. Vortex mixer (Finevortex, model Fine PCK, Korea)

Research methodology

This study, the research methodology was divided into 4 sections as follows.

1. Extraction and refining of RBO from pigmented and non-pigmented rice

1.1 Method of extraction

1.1.1 Cold-press extraction (CPE): The extraction process was conducted at Nongsano Community, Phichit Province where the screw press machine was available. Rice bran was fed into the hopper of the screw-press. The crude oil was forced through the slits along the barrel length. The compressed rice bran was simultaneously discharged through a choke at the end of the barrel (Figure 10). The oil was filtered through filter paper (Whatman No.4, 20 μm) by vacuum pump. The yield of oil was recorded. The crude oil was further used in the refinery process.

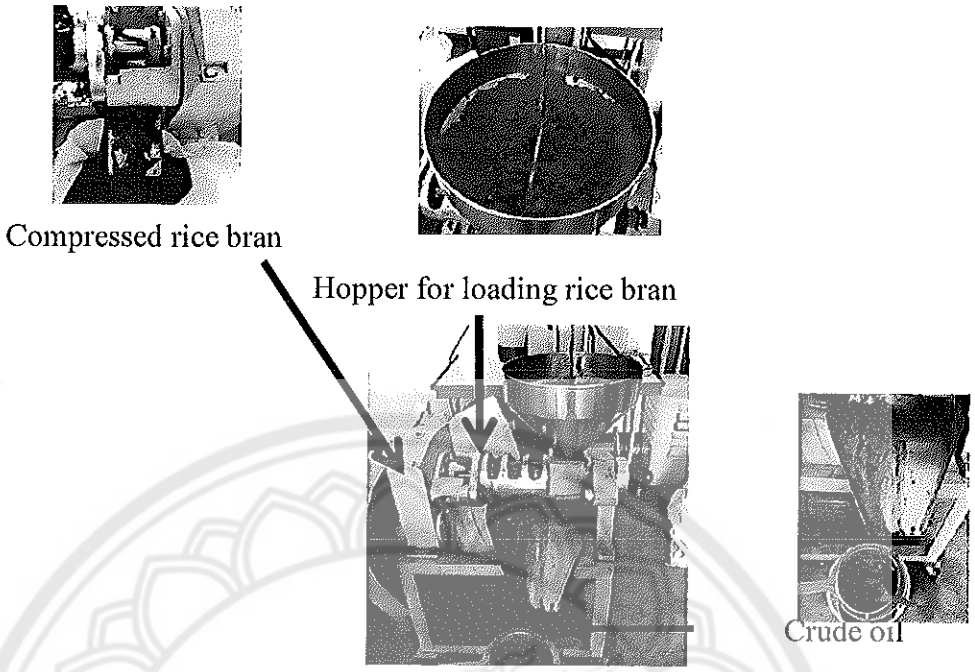
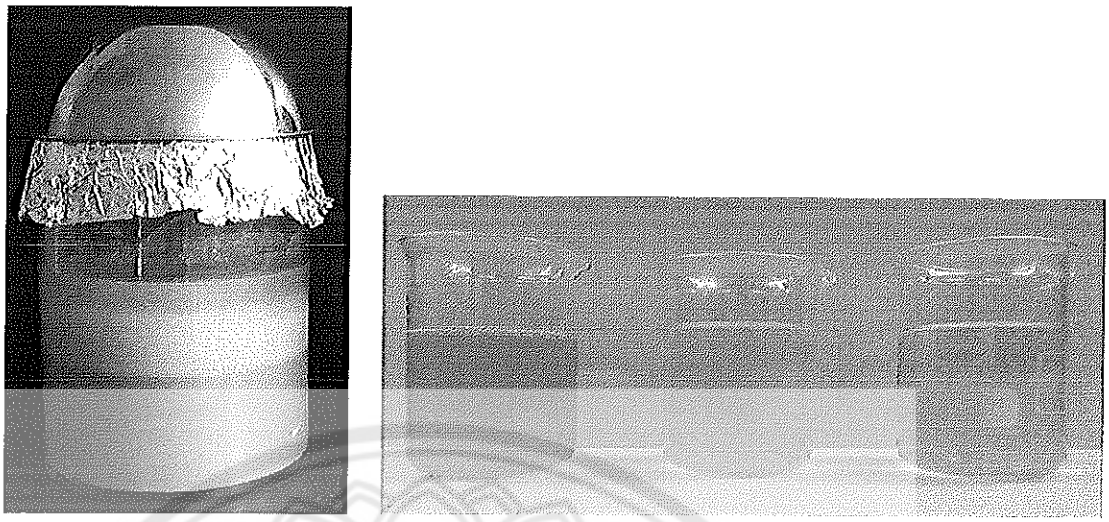


Figure 10 Cold-press extraction

1.1.2 Solvent extraction (SE): The extraction process was conducted in the laboratory at Naresuan University. The ratio of rice bran : hexane of 1 : 3 (w/v) was used for extraction at room temperature for 3 h and the extraction was repeated 2 times. Figure 11 shows pictures during a solvent extraction. The solid was removed by filtration using a filter paper (Whatman No. 4, 20 μm) under vacuum. The solvent received after filtration is called "miscella". The miscella was evaporated to remove hexane out of the oil. The yield of crude oil was calculated. The crude oil was then further used in the refinery process.



Rice bran soak in hexane Miscella from extraction 1, 2 and 3 times, respectively

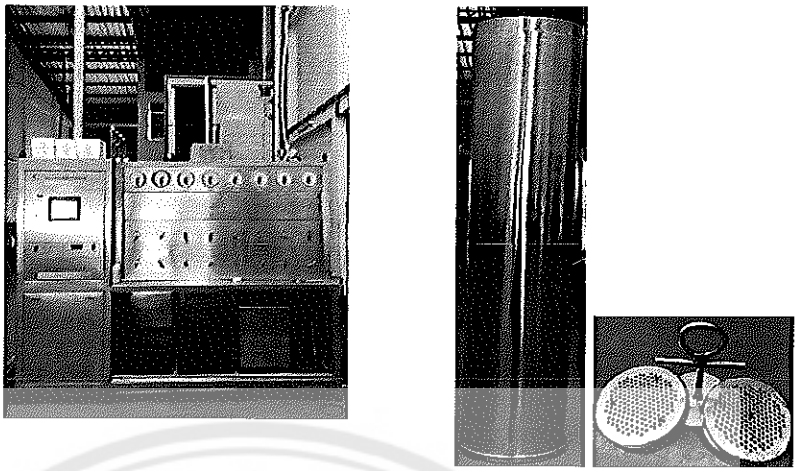
Figure 11 Solvent extraction

1.1.3 Supercritical carbon dioxide extraction (SC-CO_2): The extraction process was conducted at Origin Plant Co., Ltd., Samutprakan Province. The extractor is shown in Figure 12. The ratio of rice bran and glass bead used was 1:1 which was packed inside a tubular extractor. Rice bran was extracted at 60°C and high pressures of 300 bar for 4 h and the CO_2 flow rate was 35 L/h. The yield of crude oil from SC-CO_2 was recorded and refinery process was further conducted.

The yield of rice bran after polishing and oil extraction from 3 rice varieties using 3 extraction methods was calculated by the following equation (1) and (2), respectively.

$$\text{Yield of rice bran (\%)} = \frac{\text{Weight of rice bran (kg)}}{\text{Weight of paddy rice (kg)}} \times 100 \text{ -------- (1)}$$

$$\text{Yield of oil (\%)} = \frac{\text{Weight of oil extracted (g)}}{\text{Weight of rice bran sample (g)}} \times 100 \text{ -------- (2)}$$



Tubular extractor

Figure 12 Supercritical CO_2 extractor

1.2 Determination of acid value, free fatty acids content and peroxide value of crude RBO after extraction

The obtained RBO from each extraction method was analyzed for acid value (AV), Free fatty acids content (FFA) (Method Ca 5a-40) and Peroxide value (PV) (Method Cd 8-53) according to AOCS [91] official methods.

For analysis of AV, 1 g of oil samples was dissolved with 25 mL of diethyl ether and 25 mL of 95% ethanol. Phenolphthalein (2 drops) was used as an indicator and then it was titrated with 0.1 N potassium hydroxide solution until to pink end point appeared. AV was calculated follow the equation (3).

$$AV = \frac{56.1 \times V \times C}{m} \qquad \text{-------- (3)}$$

Where 56.1 is the molecular weight of KOH, V is the volume of KOH solution used, C is the concentration of KOH solution used (0.1 N) and m is weight of samples (1 g).

FFA was conducted the same method with AV but different in calculation. FFA was calculated as oleic acid and expressed as percentage of total lipids, follow of the equation (4).

$$FFA = 28.2 \times V \times C \quad \text{--------} (4)$$

Where 28.2 is the molecular weight of oleic acid divided by ten.

Analysis of PV was conducted by using 5 g of oil samples into 250 mL flask, 30 mL of solvent mixture (glacial acetic acid and chloroform (3:2)) was added. One mL of saturated potassium iodide (KI) solution was added, then stored in a dark for 5 min. Then, 75 mL of distilled water was added before titrated with 0.01 N sodium thiosulfate solution using starch indicator (1 mL) until the yellow color was discharged. A blank was prepared alongside the oil samples. PV was calculated by equation (5).

$$PV = \frac{1000 \times (V1-V2)}{m} \quad \text{--------} (5)$$

Where V1 is volume of $Na_2S_2O_3$ for determination of test samples in mL, V2 is volume of $Na_2S_2O_3$ for determination of blank in mL and m is weight of samples (5 g).

1.3 Refining process

The refining process of crude RBO carried out in this experiment consisted of 4 steps namely dewaxing, degumming, bleaching and deodorization as described in Figure 13.

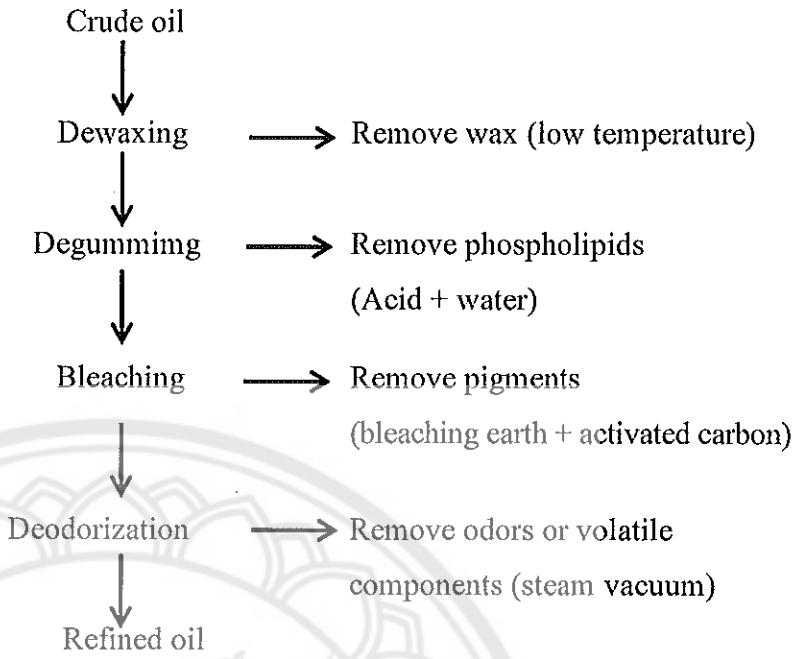


Figure 13 Refining process of crude RBO

1.3.1 .Dewaxing

The oil was cooled at 4°C for 4 h and then centrifuged at 6,000 rpm for 15 min in order to separate the wax. Figure 14 illustrates the wax obtained after dewaxing of crude RBO.

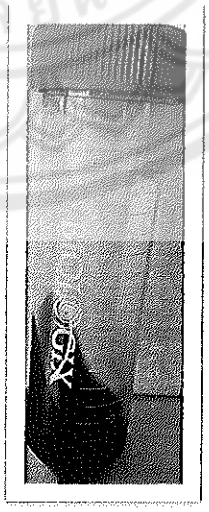


Figure 14 Wax in RBO

1.3.2 Degumming

Degumming is the process for removing gum and phospholipid out of the dewaxed RBO. The process was adding 3% of 85% of phosphoric acid by weight of sample with hot water at 80°C, mixed by magnetic stirrer for 30 min and centrifuged at 6,000 rpm for 15 min. After that, the dewaxed RBO was removed to another beaker for separating gum and phospholipid. The intermediate layer of gum after centrifugation is shown in Figure 15. The oil was washed by hot water (80°C) until isolated water has a pH 7 (neutralize).

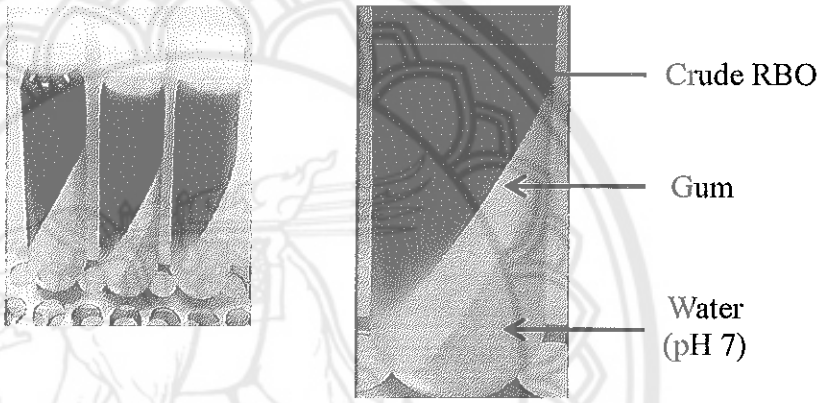


Figure 15 The intermediate layer of gum after centrifugation

1.3.3 Bleaching

Bleaching is the process for improvement the color of crude RBO. Bleaching earth 7% and activated carbon 0.7% of crude RBO were added to the round flask containing degummed RBO. The degummed RBO was bleached at 120°C, under vacuum with constant stirring by magnetic stirrer for 30 min. The bleaching equipment is shown in Figure 16. The obtained oil was then filtered through a filter paper Whatman No. 42 (2.5 μm).

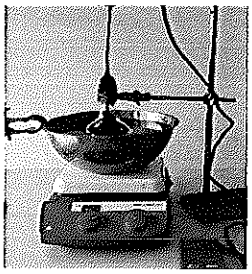


Figure 16 Bleaching of degummed RBO

1.3.4 Deodorization

The bleached RBO was added in the three-necked round flask. A thermometer was inserted through the first neck and the second neck of the flask was fitted with a three bent adaptor, which was connected to a water condenser and vacuum pump. The 2% steam was added to the round flask through the third neck. The bleached RBO was heated on the oil bath (thermal oil, boiling point 300°C). The series of deodorization process are shown in Figure 17. The bleached RBO in the flask was heated to 220°C and distilled for 60 min under low pressure (5 mmHg). At the end of the deodorization process, the bleached RBO became the refined RBO. The appearance of the refined RBO is shown in Figure 18.

Figure 17 The equipment set up for deodorization of crude RBO

Figure 18 Refined RBO

After finishing of the refinery process, the refined RBO was analyzed for the chemical properties including AV, FFA, PV (following the methods described in section 1.2), Iodine value (IV) and color.

IV was performed according to Wijs method. 0.2 g of oil sample was mixed with 20 mL mixture solution (n-hexane: acetic (1:1)), 25 mL of Wijs solution was added and then allowed to stand in a dark place for 60 min. Then 20 mL of 15% KI, 100 mL of distilled water, 2 mL of 1% starch solution was added before titrated with 0.1 M sodium thiosulfate. IV was calculated according to equation (6).

$$IV = \frac{12.69 \times C \times (V1-V2)}{m} \quad \text{--------} \quad (6)$$

Where 12.69 is the equivalent weight of iodine, C is the concentration of $Na_2S_2O_3$ (1 M), V1 is volume of $Na_2S_2O_3$ for determination of blank in mL, V2 is volume of $Na_2S_2O_3$ for determination of test samples in mL and m is weight of samples (0.2 g).

The color of RBO samples was measured by Lovibond spectrocolorimeter (Model; PFX-880L) in 1 in. cell in the transmittance mode and expressed as red (R), yellow (Y), blue (B) and neutral (N) values.

2. The physicochemical properties of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company

The 2 groups of RBO samples were used for this study.

1. RBO from 3 varieties of pigmented and non-pigmented rice using 3 extraction methods (9 samples from section 1).

2. RBO from CEO Agrifood Co., Ltd., (2 samples) including molecularly distilled RBO and refined RBO.

All RBO samples were analyzed for the followings.

2.1 Determination of total phenolic content (TPC)

The total phenolic content (TPC) was measured using the Folin-Ciocalteu colorimetric method [6]. The reaction mixture contained 200 μL of refined oils, 800 μL of Folin-Ciocaltue reagent (diluted with water 1:10 v/v and freshly prepared) and 2 mL of 7.5% sodium carbonate. The final mixture was diluted to 7 mL with deionized water. The mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. Then the absorbance at 765 nm was measured using a UV-VIS spectrophotometer (Hitachi, U2900/2910, Japan). Gallic acid was used as a standard and the results were calculated as gallic acid equivalents (g/100 g) of oils.

2.2 Determination of γ-oryzanol

The γ-oryzanol was determined by RP-HPLC method [6, 92, 93]. Briefly, the refined oils (50 mg) were dissolved in methanol (3 mL) and mixed vigorously for 3 min at room temperature before filtering through a syringe filter with PTFE (0.2 μm). The RP-HPLC consisted of an Agilent 1100 series (USA), column oven equipped with Hypersil ODS (4.0 x 250 mm, 5 μm, Agilent Technologies, USA), and a variable wavelength UV-VIS detector (model G1379A) at 330 nm. The mixture of methanol: acetonitrile: dichloromethane: acetic acid at 50:44:3:3 v/v/v/v was used as a mobile phase with a flow rate of 1.0 mL/min. The content of total γ-oryzanol was calculated from the peak area of γ-oryzanol compared with standard γ-oryzanol.

2.3 Determination of the components of γ-oryzanol by HPLC-MS/MS

The concentration of γ-oryzanol which consists of cycloartenol ferulate (CAFA), 24-methylene cycloartenol ferulate (24MCAFA), campesteryl ferulate (CampFA) and β-sitosteryl ferulate (SitoFA) was determined by a HPLC-MS/MS

technique following the method described earlier [94]. Approximately 100 mg of refined RBO was dissolved in 2 mL of isopropyl alcohol (IPA). Then 10 µL of sample stock solution was diluted with IPA to make a final volume of 1,000 µL. The sample solution was filtered with 0.2 µm syringe filter and then 10 µL of the prepared solution was injected to HPLC-MS/MS (4000 QTRAP, AB SCIEX, Tokyo, Japan). HPLC-MS/MS was performed at 40°C using ODS column. The methanol was used as a mobile phase with a flow rate of 1.0 mL/min. All components of γ-oryzanol were detected in atmospheric pressure chemical ionization mode. The concentrations of individual component of γ-oryzanol in the refined RBO samples were determined using calibration curves of standard. The analysis time was about 25 min. The peaks were sorted as CAFA at 14.5 min, 24MCAFA at 15.6 min, CampFA at 17.3 min and SitoFA at 19.0 min.

2.4 Determination of α-tocopherol

The α-tocopherol of refined RBO was measured following the method of Speek, Schrijver and Scherurs [95] and AOAC [96]. The oil sample (0.5 g) was diluted with n-heptane (10 mL) in the volumetric flask and filtered through syringe filters (0.45 µm). The α-tocopherol was separated on a RP-HPLC (Agilent 1100 series equipped with a Mightysil RP-18 GP column (4.6 x 250 mm, 3 µm, Kanto Chemical Co., Inc., Tokyo, Japan) and a FLD G1321A fluorescence detector operating with excitation and emission wavelengths of 290 and 330 nm, respectively. The mobile phase was n-heptane and 2% isopropyl alcohol with a flow rate of 1.0 mL/min. The peak areas of standard α-tocopherol were used for calculating the α-tocopherol contents.

2.5 Determination of α-, β-, γ-, δ- tocopherols and –tocotrienols

The concentrations of α-, β-, γ-, δ-tocopherols (Toc) and -tocotrienols (T3) in RBO samples were determined by a HPLC-MS/MS technique following the method described earlier [97, 98]. Approximately 100 mg of refined RBO was dissolved in 2 mL of IPA. Then 50 µL of the solution was further diluted with IPA to make a final volume of 1,000 µL. The prepared solution was filtered with 0.2 µm syringe filter and then 20 µL of the prepared solution was injected to HPLC-MS/MS (4000 QTRAP, AB SCIEX, Tokyo, Japan). The separation was performed at 40°C using a silica column (ZORBAX Rx-SIL, 4.6 × 250 mm; Agilent, Palo Alto, CA). The mixture of hexane: 1,4-dioxane:2-propanol at 100:4:0.5 v/v/v was used as a mobile phase with a flow rate of 1.0 mL/min. The Toc and T3 were detected in atmospheric pressure chemical

ionization mode. All vitamin E derivatives were successfully separated without peak overlapping, and the analytical time was about 18 min. The peaks were sorted as α-Toc at 6.8 min, α-T3 at 7.8 min, β-Toc at 9.1 min, γ-Toc at 9.8 min, β-T3 at 10.9 min, γ-T3 at 11.6 min, δ-Toc at 13.5 min and δ-T3 at 16.1 min. The concentrations of Toc and T3 were calculated from the peak area of the multiple standards.

2.6 Determination of phytosterols

The concentration of phytosterol including campesterol, stigmasterol and sitosterol was determined by GC technique as described elsewhere [99]. Each 0.1 g of RBO samples was weighed into a test tube with screw cup. The internal standard, 5-α-cholestane, was added to the sample in the test tube before saponification. The sample was saponified with 2.5 mL of 2 M KOH in ethanol solution and then heated at 60°C for 1 h. Then 2 mL of saturated NaCl and 3 mL of hexane were added to the reaction mixture, mixed and centrifuged at 3,000 rpm for 10 min to separate the layers. The hexane phase was transferred into another test tube and it was extracted with 3 mL of hexane again. The combined hexane phases were evaporated to dryness. Then 0.3 mL of bis(trimethylsilyl)-trifluoracetamid containing 1% trimethylchlorsilane (BSTFA-TMCS) was added to the sample in the test tube and heated at 70°C for 15 min. The BSTFA-TMCS was evaporated to dryness under the nitrogen stream and 1 mL of chloroform was added. The sample was injected to the GC (GL Sciences Inc., Japan, Tokyo) with ZB-5MS column (Phenomenex, USA, CA 30m×0.25 mm, thickness 0.25 μm) and FID detector. The mobile phase was helium with a flow rate of 1.0 mL/min. The components of phytosterols were separated isothermally at 300°C. Campesterol, stigmasterol and sitosterol were calculated from the peak area of the multiple standards.

2.7 Determination of fatty acid composition

The fatty acid compositions were determined by GC following the methods described by AOAC [96] and Jham, Teles and Compos [100]. 0.2 g of oil samples were mixed with 1 mL of 0.5 M KOH in methanol, closed the cap of test tube and incubated in water bath at 100°C for 5 min to saponify the lipid. Thereafter, 400 μL of 7% boron tri fluoride-methanol reagent was added, heated in water bath at 100°C for 15 min, then, 2 mL distilled water was added. After that, the upper layer was collected and extracted with petroleum ether 2 times. After extracted, the solvent was dried in hot air oven at 60°C. The residue was dissolved with 1 mL of chloroform and a portion of

the extract was injected to GC system. Capillary GC (Agilent 6850 Series) equipped with a capillary column (DB-23 Agilent; 50% cyanopropyl-methylpolysiloxane; 60 m×0.25 mm–0.25 μm film) was used. The GC conditions operated at the initial temperature of 60°C for 2 min, then increased to 60°C–190°C at 10°C/min, 190°C–200°C at 0.5°C/min and at the final temperature of 200°C–240°C at 50°C/min with a total run time of 75 min. The flow rate of gas (Nitrogen) was 2 mL/min. A split ratio of 1:10 and an injection volume of 1 μL were used. The fatty acid composition was obtained by comparison of the peak retention times with the respective fatty acid standards.

2.8 Determination of antioxidant capacity

2.8.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging effects were determined according to the method of Brand-Williams, Cuvelier and Berset [101]. The reaction mixture contained 2 mL DPPH solution (0.0394 g DPPH in 1 L methanol) and 300 μL oil samples. The mixture was shaken and incubated for 30 min in the dark at room temperature. The absorbance was determined at 517 nm by UV-VIS spectrophotometer (Hitachi, U2900/2910, Japan). The data were obtained and 50% inhibition (IC_{50}) was calculated using Microsoft® EXCEL®.

2.8.2 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay which is based on the reduction of the Fe(III)-TPTZ complex was measured by a spectrophotometer [102]. 150 μL of oil sample was mixed with 3 mL of FRAP solution (25 mL; 300 mM acetate buffer (pH 3.6), 2.5 mL; 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl and 2.5 mL; 20 mM ferric chloride). The mixture was shaken and incubated for 30 min in the dark at room temperature. The absorbance was determined at 593 nm by UV-VIS spectrophotometer (Hitachi, U2900/2910, Japan). The Trolox was used as a standard.

2.8.3 Oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) assay was determined according to the method described by Ou, Hampsch-Woodill and Prior [103]. Analyses were conducted in phosphate buffer pH 7.4 at 37°C. Peroxyl radical was generated using 2,2'-azobis (2-amidino-propane) dihydrochloride which was

2.8.3 Oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) assay was determined according to the method described by Ou, Hampsch-Woodill and Prior [103]. Analyses were conducted in phosphate buffer pH 7.4 at 37°C. Peroxyl radical was generated using 2,2'-azobis (2-amidino-propane) dihydrochloride which was prepared fresh for each run. Fluorescein was used as the substrate. The fluorescence conditions were set at excitation of 493 nm and emission of 515 nm. The standard curve was the linear of Trolox. The results were expressed as mM TE/g fresh mass.

The selection index

The sample that exhibited the highest antioxidant content (vitamin E, γ-oryzanol, total and phenolic) and antioxidant activity from 9 refined RBO samples from pigmented and non-pigmented rice was selected. It was then used for the study of its efficiency on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rats compared with 2 commercial RBO samples from CEO Agrilfood Co., Ltd.

3. Shelf life of refined RBO

Sixteen plastic bottles of 50 mL refined RBO were kept at room temperature for different range of times for study the shelf life at day 0, 1, 2, 3, 4, 5, 6, 7, 21, 35, 49, 63, 93, 123, 153 and 183. The refined RBO samples were analyzed for AV, FFA and PV as described in the section 1.2.

4. The efficiency of refined RBO on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rats

One refined RBO sample selected from section 2 and two commercial RBO samples from CEO Agrifood Co., Ltd. were used in this study.

4.1 Diet development with a mixture of RBO and its chemical analysis

The three RBO samples were formulated in the diet standard (AIN93M) for replacement of soybean oil as shown in Table 10. All diets were analyzed for the chemical properties including:

4.1.1 Proximate analysis following the AOAC [96] methods.

1) Moisture contents of the diets

Approximately 5 g diet was weighed into a moisture can (W1) and dried at 105°C to a constant weight. The moisture can containing samples was

2) Crude protein content of the diets

Crude protein content was determined by Kjeldahl method. Approximately 1 g sample was put into digestion flask and 7 g of catalyst (mixture of potassium sulfate, K_2SO_4 and copper sulfate, $CuSO_4$; (100:7)) and 20 mL of conc. H_2SO_4 were added. The digestion flask was placed in the block of digest position and heated at 420°C until solution was clear. After digestion, the digestion flask was cooled and diluted with 60 mL distilled water cautiously. After that, the condenser for distillation of NH_3 with 2% boric acid was connected. It was titrated with 0.1 N HCl and the amount of HCl used was recorded. The results were calculated follow equation (8).

$$\text{Protein (\%)} = \frac{(A-B)\times N\times 14\times 6.25}{\text{Weight of sample}} \quad \text{---------- (8)}$$

A is volume (mL) of 0.1 N HCl used in sample titration
B is volume (mL) of 0.1 N HCl used in blank titration
N is Normality of HCl
14 is atomic weight of nitrogen
6.25 is the protein-nitrogen conversation factor

3) Ash content of the diet

The diet (5 g) was weighed into the crucible. Then it was heated on the hot plate until fume is no longer produced. It was then placed in the furnace where it was heated at 550°C until the sample turn to gray. After complete heating, it was cooled down in the desiccator. After that, it was weighed and calculated for the ash content using the following equation (9).

$$\text{Ash (\%)} = \frac{\text{Weight of samples after burning}}{\text{Weight of sample}} \times 100 \quad \text{---------- (9)}$$

4) Fat content of the diet

Approximately 5 g sample was weighed in the extraction thimble before placing in the Soxhlet tube. 250 mL of petroleum ether was added into

the extraction flask and placed on the heating mantle. The extraction was performed for 4 h. Then the petroleum ether was evaporated. After evaporation, it was transferred into the hot air oven at 105°C for 30 min. It was cooled in the desiccator and weighed (weight of fat). The crude fat content of the diet was calculated follow equation (10).

$$\text{Fat (\%)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100 \quad \text{---------- (10)}$$

5) Fiber content of the diet

Approximately 3 g sample was weighed into the fritted glass crucibles and transferred to the hot extractor. The samples were hydrolyzed with 0.128 M S_2HO_4 and followed by 0.223 M KOH in the hot extractor. The residue was washed with hot water 3 times and washed with acetone. The residue and crucibles were dried at 105°C for 2 h and weighed (W1) before being ignited in a muffle furnace at 550°C until the residue turns to gray. The residual ash was cooled in the desiccator and weighed (W2). The percentage of crude fiber was calculated follow equation (11).

$$\text{Crude fiber (\%)} = \frac{\text{W1}-\text{W2}}{\text{W of sample}} \times 100 \text{ ---------- (11)}$$

6) Carbohydrate content of the diet

The total carbohydrate was calculated follow equation (12).

Total carbohydrate (%) = (100 – (moisture + protein + ash + fat + fiber)) -------- (12)

4.1.2 Determination of TPC, γ-oryzanol, α-tocopherol, fatty acid composition and antioxidant capacity (DPPH, FRAP and ORAC)

The analysis of TPC, γ-oryzanol, α-tocopherol, fatty acid composition and antioxidant capacity (DPPH, FRAP and ORAC) was conducted in the same condition as described in sections 2.1, 2.2, 2.4, 2.7 and 2.8, respectively.

4.2 The efficiency of RBO on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat

This research used five-weeks-old male Sprague-Dawley rats (300±10 g) that obtained from the National Laboratory Animal Centre, Salaya campus, Mahidol University, Thailand. All rats were maintained in accordance with the guidelines of the Animal Care Ethical Committee of National Laboratory Animal Center, Mahidol University. Rats were individually housed in stainless cages (Figure 19) at ambient humidity (60±5%), temperature (22±2°C) and light-dark (12:12) standard environmentally controlled room. Rats were accustomed to the new environment for 1 week with free access rat diet and water. After 1 week of acclimation, they were divided into two groups; six rats were control group fed with normal diet (AIN93M) and thirty rats were induced to hypercholesterolemia and fed with the high fat diet (2% cholesterol mix in normal diet) for two months. After the inductions of hypercholesterolemia, induced rats were randomLy divided into five groups (Figure 20). Thus, the rats had 6 groups consisted of;

1. The control group where the rats were fed with normal diet (Normal, C).
2. The group that rats were induced to have high cholesterol and fed with high fat diet (HC, negative control group).
3. The group that rats were induced to have high cholesterol and fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage) (HMDG).
4. The group that rats were induced to have high cholesterol and fed with high fat diet mixed with 4% of refined RBO (HRFF).
5. The group that rats were induced to have high cholesterol and fed with high fat diet and 0.1 mL of selected RBO (gavage) (HHNG).
6. The group that rats were induced to have high cholesterol and fed with high fat diet mixed with 4% of selected RBO (HHNF).

All 6 groups of rats were fed with experimental diets (Table 10) for 60 days.

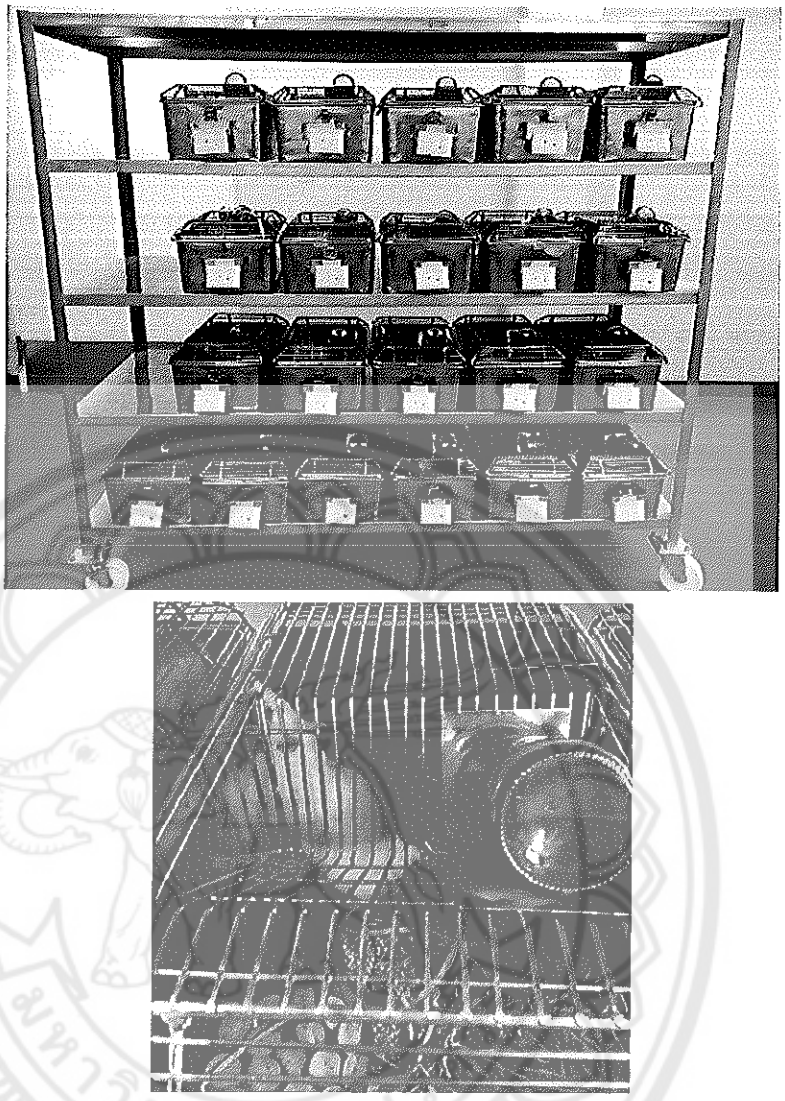


Figure 19 The stainless cages of rats

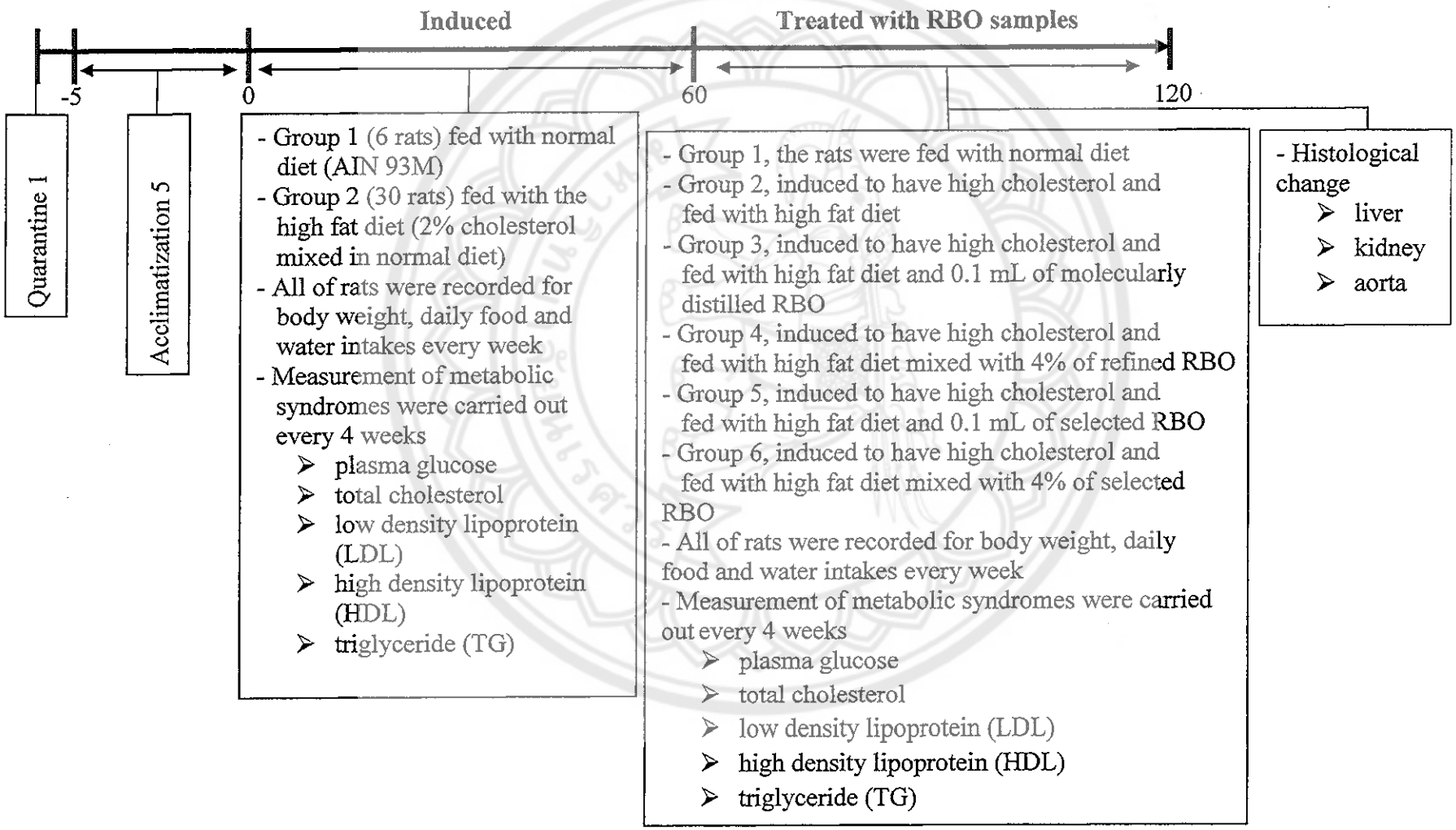


Figure 20 The *in vivo* protocol

Table 10 AIN93M diet mixture with RBO for feeding the rats

Components (g/100 g)	Normal	HC	HMDG	HRFF	HHNG	HHNF
Casein	14.00	14.00	14.00	14.00	14.00	14.00
L-Cystine	0.18	0.18	0.18	0.18	0.18	0.18
Corn starch	46.56	46.56	46.56	46.56	46.56	46.56
Maltodextrin	15.50	15.50	15.50	15.50	15.50	15.50
Sucrose	10.00	9.00	9.00	9.00	9.00	9.00
Soybean oil	4.00	4.00	4.00	-	4.00	-
Cellulose	5.00	5.00	5.00	5.00	5.00	5.00
Mineral mix	3.50	3.50	3.50	3.50	3.50	3.50
Vitamin mix	1.00	1.00	1.00	1.00	1.00	1.00
Choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25
TBHQ, antioxidant	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008
Cholesterol (%)	-	2	2	2	2	2
Molecularly distilled RBO (gavage)	-	-	0.1	-	-	-
Refined RBO (Mixed with diet)	-	-	-	4.00	-	-
Selected RBO (gavage)	-	-	-	-	0.1	-
Selected RBO (Mixed with diet)	-	-	-	-	-	4.00

All rats were examined for biological markers in below.

1. Body weight, daily food and water intake were measured every week.

2. Measurement of metabolic syndromes such as plasma glucose, total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglyceride (TG) were carried out every 4 weeks.

3. Measurement of oxidative stress and antioxidant capacity at the end of the 60 days experimental period.

3.1 Lipid peroxidation by measurement of plasma and liver malondialdehyde (MDA)

Plasma and liver malondialdehyde were performed using a commercial kit (TBARS assay kit; KA 1381; Abnova company) according to the manufacturer's instructor. Approximately 25 mg of liver was weighed into a 1.5 mL centrifuge tube and 250 μL of RIPA buffer (mixture solution of 100 mM, Triton X-100, 1 mM EDTA and 10% glycerol) was added. After that, the centrifuge tubes containing samples were homogenized on the ice and centrifuged at 1,600 × g at 4°C for 10 min. Supernatant of each sample (100 μL) was added into the test tube. For the plasma analysis, dilution of sample was not conducted. After adding both plasma and liver sample into the test tube, 100 μL SDS solution was added and swirled. After mixing, 4 mL of the color reagent (cap tube) was added and boiled in a boiling water for 1 h. Then the test tube was removed and placed into ice bath for 10 min to stop the reaction. After 10 min, it was centrifuged at 1,600 × g at 4°C for 10 min. The tube was placed at room temperature for 30 min and 150 μL of each samples was placed into 96-well microplate. The absorbance was monitored at excitation wavelength of 530 nm and an emission wavelength of 550 nm using fluorescence spectrophotometer. The MDA value was calculated from absorbance of the samples compared with TBA malondialdehyde standard curve.

3.2 Antioxidant enzyme of superoxide dismutase (SOD)

The percent inhibition of superoxide dismutase (SOD) was performed using a commercial kit (superoxide dismutase assay kit; KA0783; Abnova company) according to the manufacture's instructor. 20 μL of plasma was added to blank 2 and 20 μL of water was added to each blank 1 and blank 3 wells (Table 11). After that, 200 μL of WST working solution was added to each well and 20 μL of dilution buffer to was added each blank 2 and blank 3 wells. Next, 20 μL of enzyme working solution was added to each sample and blank 1 well and swirled. The mixture was incubated at 37°C for 29 min. The absorbance was measured at 450 nm using a microplate reader.

Table 11 Amount of each solution for sample, blanks 1, 2 and 3

	Sample	Blank 1	Blank 2	Blank 3
Sample solution	20 µL	-	20 µL	-
ddH_2O	-	20 µL	-	20 µL
WST working solution	200 µL	200 µL	200 µL	200 µL
Enzyme working solution	20 µL	20 µL	-	-
Dilution buffer	-	-	20 µL	20 µL

3.3 Inflammation cytokine of tumor necrosis factor alpha (TNF-α)

The TNF-α was measured using a commercial kit (Rat TNF-α ELISA MAX™ Deluxe set; 438204; BioLegend company) according to the manufacture's instructor. The ELISA plate was coated by using 100 µL of diluted capture antibody solution. After that, it was sealed and incubated at 4°C overnight. After overnight, it was washed 4 times with 200 µL 1X assay diluent A to each well, sealed and incubated at room temperature for 1 h while shaking. After incubating, it was washed 4 times with buffer (wash buffer was phosphate buffer saline, PBS; 8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl dissolved in deionized water to 1 L, mixed with 0.05% tween-20) and 50 µL of the standard dilutions (Rat TNF-α standard). Sample was added to the appropriate well. After adding standard and samples, it was sealed and incubated at room temperature for 2 h while shaking. After 2 h, it was washed 4 times with wash buffer. Then 100 µL diluted detection antibody solution was added to each well. It was sealed and incubated at room temperature for 1 h while shaking. After 1 h, it was washed 4 times and 100 µL diluted avidin-HRP solution was added to each well. The plate was sealed and incubated at room temperature for 30 min while shaking. After that, it was washed 5 times with every times soaking for 1 min per wash and 100 µL mixed TMB substrate solution was added to each well. The plate was incubated in the dark for 25 min. The absorbance was measured at 450 nm and 570 nm within 15 min. The TNF-α value was calculated from absorbance of the samples compared with standard curve.

3.4 Histological change (liver, kidney and arota) by light microscopy

The liver, kidney and arota were quickly excised and flushed with ice-cold phosphate-buffer saline pH 7.4 (PBS) and fixed in the 10% formalin for subsequent histological examination.

All of the procedures in this animal study were performed in accordance with the Mahidol University policy for care and use of animals for scientific proposes and approved by the Animal Care Ethical Committee of the Central Animal Facility Research Division, Faculty of Science, Mahidol University, Thailand (Approval No. RA 2016-16, Validity dates: September 2016-September 2017).

Statistical analysis of data

The experimental data were subjected to a one-way analysis of variance for a completely random design to determine the least significant difference at the level of 0.05. The data values were expressed as mean ± SD.

CHARPTER IV

RESULTS AND DISCUSSION

This chapter provides the results and discussions of the study which were divided into 4 sections as follows.

Section 1, extraction and refining of RBO from pigmented and non-pigmented rice.

Section 2, the physicochemical properties of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from CEO Agrifood Co., Ltd.

Section 3, shelf life of refined RBO.

Section 4, the efficiency of RBO on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat.

Extraction and refining of RBO from pigmented and non-pigmented rice

1. The yield of rice bran and RBO samples

The paddy of KDML 105, RJM and HN were milled and polished in order to obtain rice bran for extraction of oil by 3 extraction methods namely cold-pressed, solvent and supercritical CO_2 extraction. The yields of rice bran were calculated and shown in Table 12. The results shown that, the yields of rice bran from KDML105, RJM and HN rice were 8.40%, 7.83% and 11.10%, respectively. HN provided the highest yield while RJM provided the lowest yield. Then, rice bran was extracted by 3 extraction methods. The yields of RBO from 3 rice varieties extracted by 3 extraction methods are shown in Figure 21. The SE method provided higher yield than CPE and SC-CO_2 methods approximate 75% and 10%, respectively, which is in agreement with the report of Noppawat et al. [104]. While considering the varieties of rice, it was found that excepted for SE, rice bran from RJM provided significantly higher yield of oil than CPE and SC-CO_2 methods ($p \leq 0.05$). For SE, rice bran from RJM and KDML 105 had significantly higher yield of oil than HN ($p \leq 0.05$). It was shown that the content of oil

from RJM was significantly highest followed by KDML 105 and HN, respectively ($p \leq 0.05$).

Table 12 The yields of rice bran after polishing

Paddy	Yield of rice bran (%)
KDML 105	8.40
RJM	7.83
HN	11.10

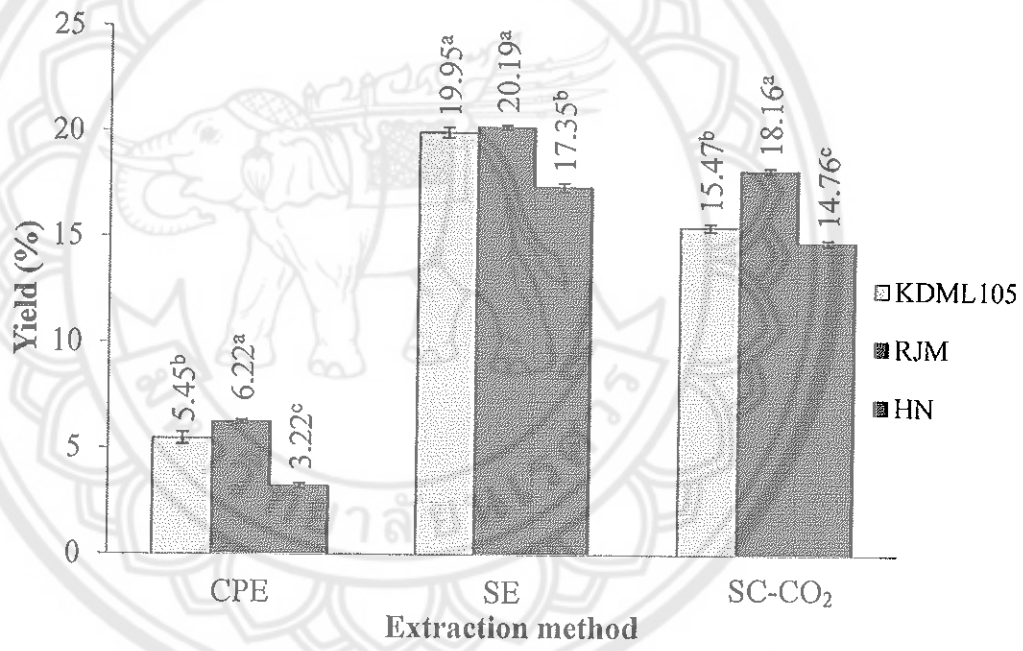


Figure 21 Yield of RBO from three rice varieties extracted by different extraction methods

Bars with different letters on top are significantly different in retained percentage ($p \leq 0.05$). CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

2. The properties of 3 rice varieties of crude and laboratory refined RBO

The crude RBO were determined for acid value (AV), free fatty acid (FFA) content and peroxide value (PV) straight away after extraction in order to know the initial values of crude oil. The crude RBO samples were then refined by a number of refining steps. The refined RBO samples were analyzed for its properties (AV, FFA, PV, IV and color) and compared with the results obtained from the crude RBO samples. The results are shown in Figures 22–25 and color of the refined RBO samples is presented in Table 13.

2.1 Acid value (AV)

The AV of crude and refined RBO samples from 3 rice varieties and 3 extraction methods are shown in Figure 22. The AV is detected as a result of hydrolysis reaction of triglyceride to glycerol and FFA by lipase activity, which is increased rapidly after the milling process [105]. It was found that the AV of crude RBO samples ranged between 5.03–12.01 mg KOH/g oil while the AV of the refined RBO samples were 4.43–9.44 mg KOH/g oil and were significantly lower ($p \leq 0.05$) than the crude oil samples. The result of AV after refining process showed a decrease in values. This was occurred at deodorization step where FFA was removed and the oxidation reaction was prevented by heating [106]. However, the AV of both crude and refined RBO samples were higher than that of the CODEX standard of fats and oils. According to the CODEX standard, the maximum level of AV is 0.6 mg KOH/g oil [107]. The RBO samples obtained in this study had the AV higher than the recommendations, due to the raw material (rice bran) in this study was not stabilized before use for destroy lipase activity [105, 108] and the refining process in the laboratory was not a closed system and continuous, thus resulting of the refining process have lower quality and can not reduce the AV to conform to the standard. The HN rice showed the highest AV followed by KDML 105 and RJM, respectively for all extraction methods. The AV of crude RBO samples were similar to the study of Thanonkaew et al. [109] who reported that the AV of crude RBO ranged between 6.30–11.11 mg KOH/g oil.

2.2 Free fatty acid (FFA)

The FFA content was analyzed in the same manner as AV, therefore, showed similar trends to the results that explained in AV. It was found that the FFA content of all crude RBO samples ranged between 2.52–6.03% while the FFA of the

refined RBO samples were 2.22–4.74% (Figure 23). In addition, the prevention of food adulteration act of India and Rules, 1954 [110] suggested the FFA content for the refined RBO should be less than 0.25%. The RBO samples obtained in this study had the FFA contents higher than the recommendations, the same trends of AV. However, the FFA content of crude RBO in this study was lower than the reported of Maria et al. [111], who studied the physicochemical properties of RBO extracted by solvent extraction. It was found the FFA content of 9.1%. In addition, it was close to the report of Thanonkaew et al. [109], who reported the FFA range between 3.17–5.58% in crude RBO. For refined RBO samples, there was a conflict with Bijay and Patel' [112] study, which reported that the FFA content was in the range of 0.4–0.8%. Their FFA content was low due to the use of NaOH for neutralization process, while our study used hot water (80°C). The reason for not using NaOH in this study due to the previous study had reported that there was a lot of RBO losses (18–22%; w/w of total oil) [112].

2.3 Peroxide value (PV)

The PV is the foremost initial reaction product of lipid oxidation. It was found that the PV of all crude RBO samples ranged between 3.08-8.41 mg eq/kg oil, while those for refined RBO samples were 0.77–2.24 mg eq/kg oil (Figure 24). The PV of crude RBO samples obtained from SE method had significantly different ($p \leq 0.05$) with another two extraction methods. This was probably due to the process of SE method had to incubate before extraction and filter many times, so the RBO was contacted with oxygen, resulting in oxidation reaction. However, the PV of both crude and refined oils were lower than the CODEX standard of fats and oils [107] which states that the maximum level of PV of RBO is 10 mg eq/kg oil. Moreover, the RBO samples obtained in this study had the PV lower than the report of Thanonkaew et al. [109], who reported the PV of crude RBO range between 12.13–18.85 mg eq/kg oil.

2.4 Iodine value (IV)

Analysis of IV is to determine the amount of unsaturation contained in the oil. The high IV indicates more unsaturated fatty acids present in the oil. The CODEX standard recommended between 90–105 g Iodine/100 g oil. The results of this study had the IV of 92.10–100.63 g Iodine/100 g oil which was conformed to the CODEX standard [107] and showed significantly different ($p \leq 0.05$) among samples. The results are shown in Figure 25. The RBO sample from HN rice provided the highest

IV, followed by KDML 105 and RJM rice, respectively. The IV of RBO samples obtained in this study were consistent with the report of Rani et al. [113], who reported the IV of crude RBO 95 g Iodine/100 g oil.

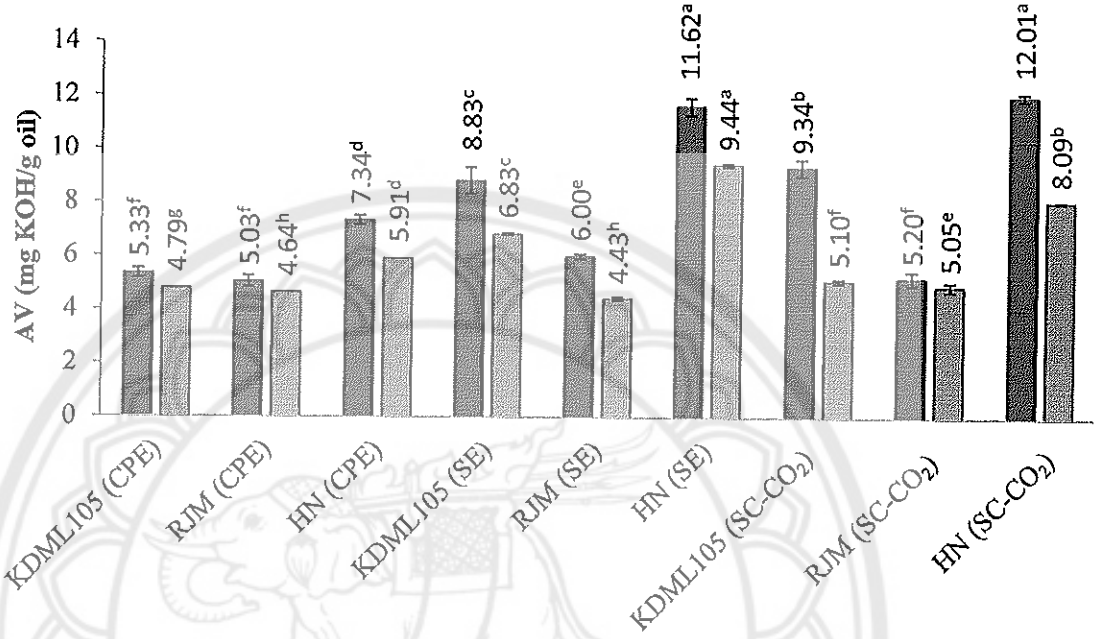


Figure 22 Acid value (AV) of RBO from three rice varieties extracted using different extraction methods

Note: ▩ : crude RBO, ▩ : refined RBO. Bars with different letters on top are significantly different in retained percentage ($p \leq 0.05$)

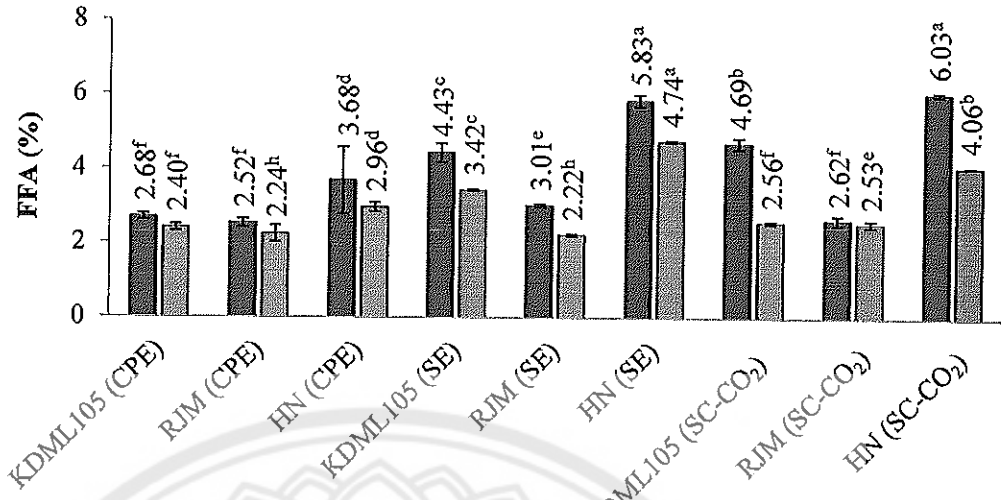


Figure 23 Free fatty acid (FFA) content of RBO from three rice varieties extracted using different extraction methods

Note: ■ : crude RBO, ■ : refined RBO. Bars with different letters on top are significantly different in retained percentage ($p \leq 0.05$)

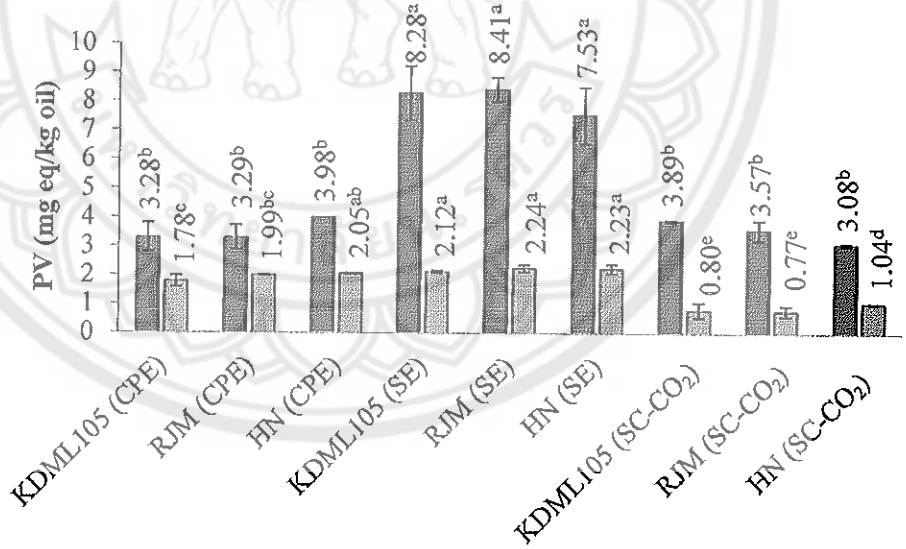


Figure 24 Peroxide value (PV) of RBO from three rice varieties extracted using different extraction methods

Note: ■ : crude RBO, ■ : refined RBO. Bars with different letters on top are significantly different in retained percentage ($p \leq 0.05$)

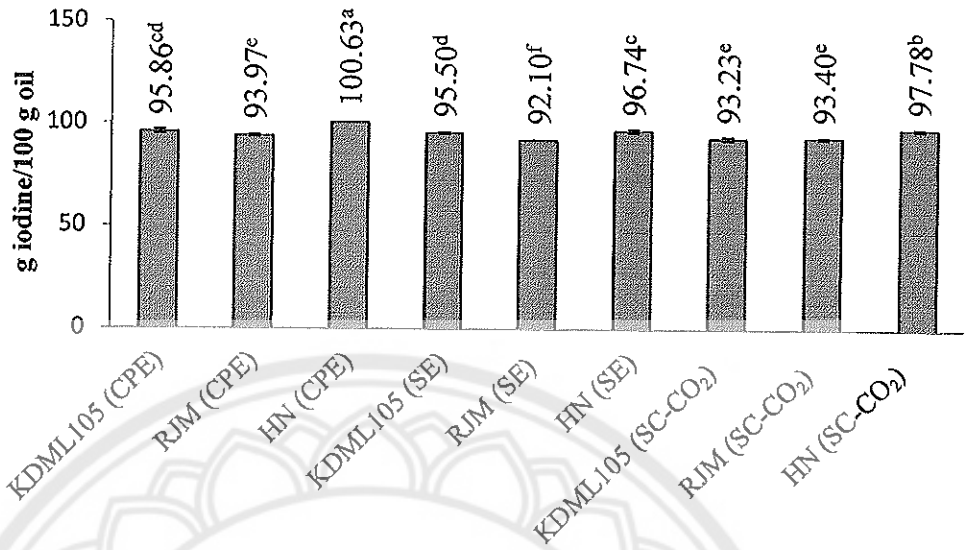


Figure 25 Iodine value (IV) of refined RBO from three rice varieties extracted using different extraction methods

Note: Bars with different letters on top are significantly different in retained percentage ($p \leq 0.05$).

2.5 Color

The color of RBO samples obtained in this study is shown in Table 13. Color is the important characteristic for visual inspection of RBO. The color standard for refined oil as recommended by the CODEX standard [107] is Y+5R < 20. In this study, it was found that all the refined RBO samples conformed to the CODEX standard [107]. The color (Y+5R) of the commercial refined RBO was 11.50 which was similar to the values found in the RBO samples obtained from KDML 105 (11.20) and RJM (10.10) rice extracted by CPE, and KDML 105 (10.40) extracted by SE. The RBO samples extracted by SC-CO_2 showed very low color values (Y+5R) indicating the excellent color quality.

Table 13 The color of refined RBO samples from three rice varieties extracted using different extraction methods and commercial refined RBO

Sample	Red (R)	Yellow (Y)	Blue (B)	Neutral (N)	Y+5R
KDML 105 (CPE)	1.00	6.20	0.00	0.10	11.20
RJM (CPE)	1.00	5.10	0.00	0.10	10.10
HN (CPE)	1.50	9.60	0.00	0.10	17.10
KDML 105 (SE)	1.00	5.40	0.00	0.10	10.40
RJM (SE)	1.00	4.70	0.00	0.10	9.70
HN (SE)	1.40	11.00	0.00	0.10	18.00
KDML105(SC-CO_2)	0.40	2.60	0.00	0.10	4.60
RJM (SC-CO_2)	0.40	2.60	0.00	0.10	4.60
HN (SC-CO_2)	0.60	3.80	0.00	0.10	6.80
Commercial oil	1.00	6.50	0.00	0.10	11.50

Note: CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

The characteristics of RBO samples in this study revealed that the AV and FFA content failed to conform to the CODEX standard [107] and the prevention of food adulteration act of India and Rules 1954 [110]. However, the PV and color (Y+5R) were conformed to CODEX standard [107].

The physicochemical properties of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company

The properties of refined RBO samples from three rice varieties extracted by three extraction methods and two samples from CEO Agrifood Co., Ltd. (molecularly distilled RBO and refined RBO) were analyzed for total phenolic content (TPC), total

γ-oryzanol, α-tocopherol and fatty acid composition. The results of each analysis are as followed.

1. The total phenolic contents (TPC)

The TPC contents of the refined RBO samples were determined by Folin-Ciocalteu method and reported as gallic acid equivalents [6]. As shown in Table 14, the TPC contents of refined RBO samples from three rice varieties extracted by three extraction methods ranged between 6.63–10.22 mg gallic acid/g oil and showed significantly different in both varieties and extraction methods ($p \leq 0.05$). The RBO samples of HN rice obtained by three extraction methods showed a significantly higher TPC content ($p \leq 0.05$) than other varieties. The highest concentration (10.22 mg gallic acid/g oil) of TPC was found in HN oil extracted by SC–CO_2 method ($p \leq 0.05$). The TPC contents found in this study were higher than those reported by Bopitiya and Madhujith [114] who studied the TPC content of RBO from two rice verities (BG 400; non-pigmented rice and LD 365; red rice) and found ranged between 2.54–2.63 mg gallic acid/g oil. In addition, the TPC contents in this study were also higher than those reported by Sompong et al. [115], who studied the TPC content of red and black rice from Thailand, Sri Lanka and China. The results were ranged between 3.40–6.91, 0.79–2.08 and 2.53 mg gallic acid/ g oil, respectively. They also reported that the TPC content of Thailand's rice had TPC content higher than Sri Lanka's rice and China's rice. Two RBO samples of CEO Agrifood Co., Ltd. had the TPC ranged between 37.57–42.62 mg gallic acid/ g oil which were higher than nine RBO samples from three rice varieties extracted by three extraction methods about five times. This was probably due to the factory has a continuing and well control extraction and refining process. As a result, the quality of oil in term of TPC from the factory was better than the oil produced in the laboratory. The samples obtained from the company did not include in the statistical analysis because in the section 2 of this study it was conducted to compare the properties of nine RBO samples for selection the best RBO sample to be used in the animal studies (Section 4).

Table 14 The TPC of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company

Extraction methods	Samples	TPC (mg gallic acid/g oil)
CPE	KDML 105	8.75±0.10^{c}
	RJM	6.84±0.09^{f}
	HN	9.26±0.15^{b}
SE	KDML 105	8.05±0.12^{d}
	RJM	7.33±0.03^{e}
	HN	9.28±0.16^{b}
SC-CO_2	KDML 105	7.19±0.06^{e}
	RJM	6.63±0.24^{f}
	HN	10.22±0.06^{a}
	Molecularly distilled RBO	42.62±1.11
	Refined RBO	37.57±0.12

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

2. The total γ-oryzanol contents by RP-HPLC method

The total γ-oryzanol contents of all RBO samples were determined using HPLC and calculated by the calibration curve of γ-oryzanol standard. There were four peaks of standard presented between 15.5–20.0 min. The chromatograms of γ-oryzanol standard and RBO sample are presented in Figures 26 and 27 (HN-SC-CO_2), respectively. The γ-oryzanol concentration of refined RBO samples from three rice varieties extracted by three extraction methods and RBO samples from CEO Agrifood Co., Ltd. were in the range of 106.90–281.95 and 213.55–275.08 mg/g oil, respectively (Table 15). The total γ-oryzanol contents obtained in the RBO samples were higher than

89.3 mg/g oil those reported by Yoshie et al. [116]. These results may have many contributing factors such as production process of RBO, rice variety and analysis methods, etc. The RBO of HN rice had the significantly highest concentration ($p \leq 0.05$) of total γ-oryzanol, followed by the RBO from RJM and KDML 105, respectively. This result showed the pigmented rice contained higher γ-oryzanol concentration than the non-pigmented rice which is in accordance with previously reported literatures [65, 117]. With regard to extraction methods, the oil obtained from SE method showed the highest content of γ-oryzanol, followed by those extracted by the SC-CO_2 and CPE methods, respectively. This was mainly due to γ-oryzanol is soluble in organic solvents [118]. The result was also consistent with Ramsay et al. [119] and Noppawat et al. [104], who reported that SE (hexane) provided higher γ-oryzanol than SC-CO_2.

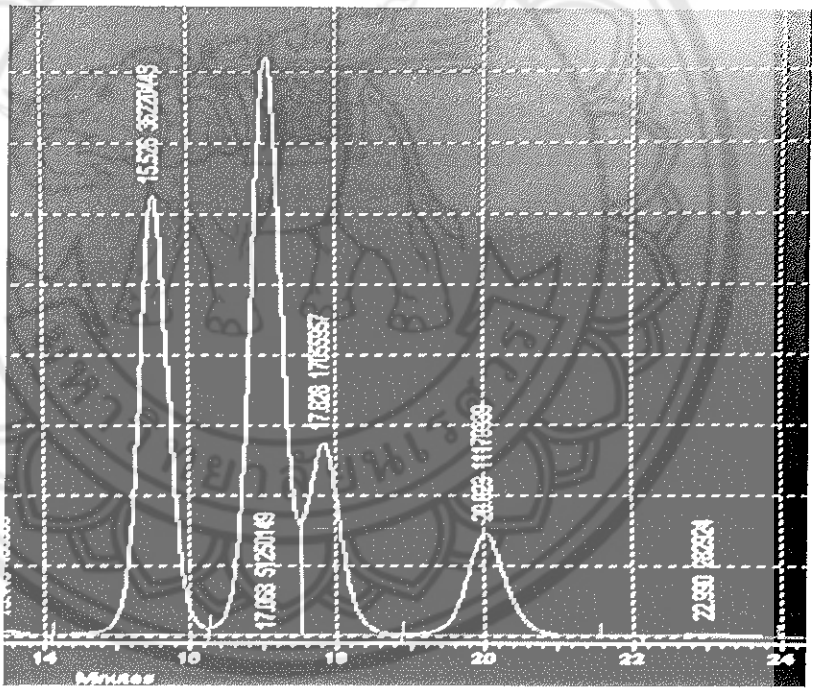


Figure 26 The chromatogram of total γ-oryzanol standard

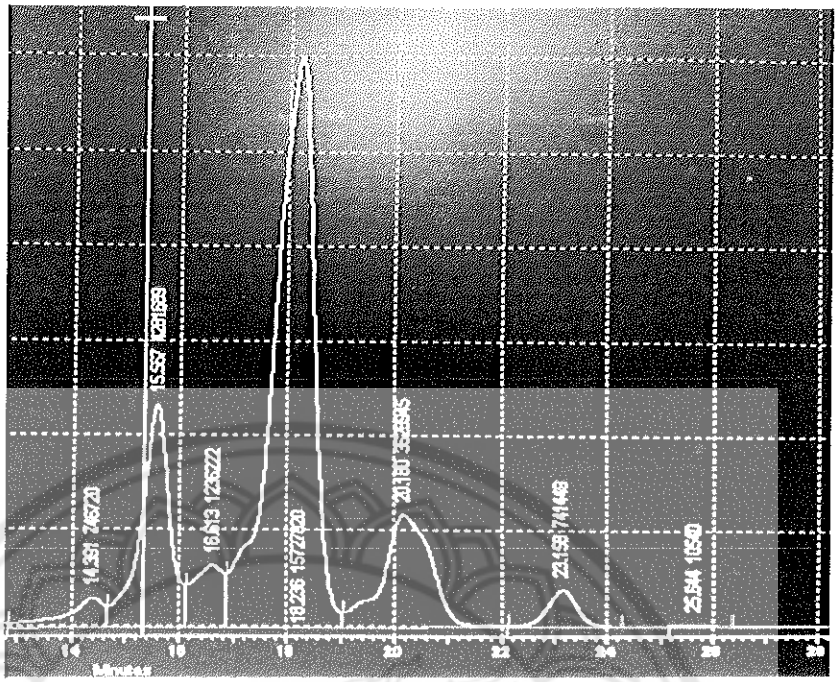


Figure 27 The chromatogram of total γ-oryzanol detected in RBO sample obtained from Hom-nin rice extracted by the SC-CO_2 extraction method

Table 15 The total γ-oryzanol of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company

Extraction methods	Samples	Total γ-oryzanol (mg/g oil)
CPE	KDML 105	106.90±0.74^{g}
	RJM	124.89±1.27^{f}
	HN	165.89±0.07^{e}
SE	KDML 105	120.20±2.16^{f}
	RJM	204.81±0.26^{c}
	HN	281.95±2.13^{a}

Table 15 (cont.)

Extraction methods	Samples	Total γ-oryzanol (mg/g oil)
SC-CO_2	KDML 105	119.75±2.97[f]
	RJM	177.50±1.37[d]
	HN	226.56±2.10[b]
	Molecularly distilled	275.08±1.56
	Refined RBO	213.55±4.35

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

3. The components of γ-oryzanol by HPLC-MS/MS

Chemically, γ-oryzanol is a mixture of ferulic acid esters of triterpene alcohols (phytosterols). There are ten components of γ-oryzanol which consists of Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, campesteryl ferulate, Δ^7-campestenyl ferulate, 24-methylenecycloartanyl ferulate, Δ^7-sitotenyl ferulate, sitosteryl ferulate, campestanyl ferulate, and sitostanyl ferulate. In this study, the only four main components were found in the RBO samples including cycloartenol ferulate (CAFA), 24-methylene cycloartenol ferulate (24MCAFA), campesteryl ferulate (CampFA) and β-sitosteryl ferulate (SitoFA) [120]. The peaks were sorted as CAFA at 14.5 min, 24MCAFA at 15.6 min, CampFA at 17.3 min and SitoFA at 19.0 min as shown in Figures 28 and 29 (HN-SC-CO_2). The concentration of individual component of γ-oryzanol in the refined RBO samples were determined by using the calibration curves of standard.

The concentrations of γ-oryzanol components in RBO samples are shown in Table 16. Total γ-oryzanol content was found to be approximately 163-544 mg/100 g oil. CampFA had the highest amount of γ-oryzanol, followed by CAFA, 24MCAFA and SitoFA, respectively. With regards to the varieties, the RBO from HN rice contained

significantly higher ($p \leq 0.05$) total γ-oryzanol content than those of KDML 105 and RJM when extracted by the CPE and SE methods. However, its total γ-oryzanol content was not significantly different ($p > 0.05$) to RJM when extracted by SC-CO_2 method. The RBO samples from KDML 105 and RJM rice had no significant different ($p > 0.05$) in total γ-oryzanol content for all extraction methods except SE method. The results suggested that the RBO from HN rice was the potential source of total γ-oryzanol. With regard to extraction methods, no significant difference ($p > 0.05$) was found in total γ-oryzanol content for all extraction methods within each rice variety except HN's RBO extracted by SC-CO_2 method, indicating a little effect of extraction methods on total γ-oryzanol content.

In terms of the components, CAFA contents in HN's RBO samples were significantly higher ($p \leq 0.05$) than other rice varieties for all extraction methods. Within the same rice variety, there was no significant difference ($p > 0.05$) of CAFA content in RBO samples extracted from all extraction methods, except HN from SE that had significantly higher ($p \leq 0.05$) CAFA content than those obtained from SC-CO_2 method. The results suggested that rice varieties contributed to the CAFA content in RBO.

For 24MCAFA, the values ranged from 49.62 to 99.23 mg/100 g oil. HN's RBO generally showed high contents of 24MCAFA, although no significant differences were found ($p > 0.05$) in all other samples.

For SitoFA, there was no significant difference ($p > 0.05$) in SitoFA content of all RBO samples from all rice varieties and extraction methods, except that HN's RBO sample obtained by SE method had significantly higher ($p \leq 0.05$) SitoFA content than that of RJM.

The CampFA content of HN's RBO sample was significantly higher ($p \leq 0.05$) than other rice varieties for CPE and SE methods. There was no significant difference ($p > 0.05$) in CampFA content of RJM and KDML 105 in all extraction methods. Within the same rice variety, there was no significant difference ($p > 0.05$) in CampFA content of RBO samples extracted by all extraction methods.

In this study, rice varieties were found to have the pronounced effects on phytochemicals than extraction methods. The contents of γ-oryzanol components were different depending on rice varieties. Generally, 24MCAFA and CampFA were found

to be the major ferulates which is in agreement with previously reported literatures [121, 122].

This study found that extraction methods had less effect than rice varieties on the contents of γ-oryzanol. However, previous work [123] has reported that SC-CO_2 extraction provided tremendously higher yield of γ-oryzanol than solvent extractions. This is understandable as different conditions were used between this paper and the mentioned one. Oryzanol contents were reported to be severely affected by alkali conditions. It has been reported that 83–95% of γ-oryzanol from its original content was lost during alkali refining [121]. Recently, SC-CO_2 extraction of lipids has received much attention as an alternative to organic solvent extraction and has been shown to be an ideal method for extracting certain lipids [124]. Most studies that used SC-CO_2 in lipid extraction have focused on the yield of extractable material. However, each compound possesses a unique extractability under different conditions of supercritical fluid extraction [125]. In addition, current publications indicated high phytochemicals including oryzanol in colored rice especially black rice, similar to HN rice in this study [115, 126-127]. However, more research is needed in this area.

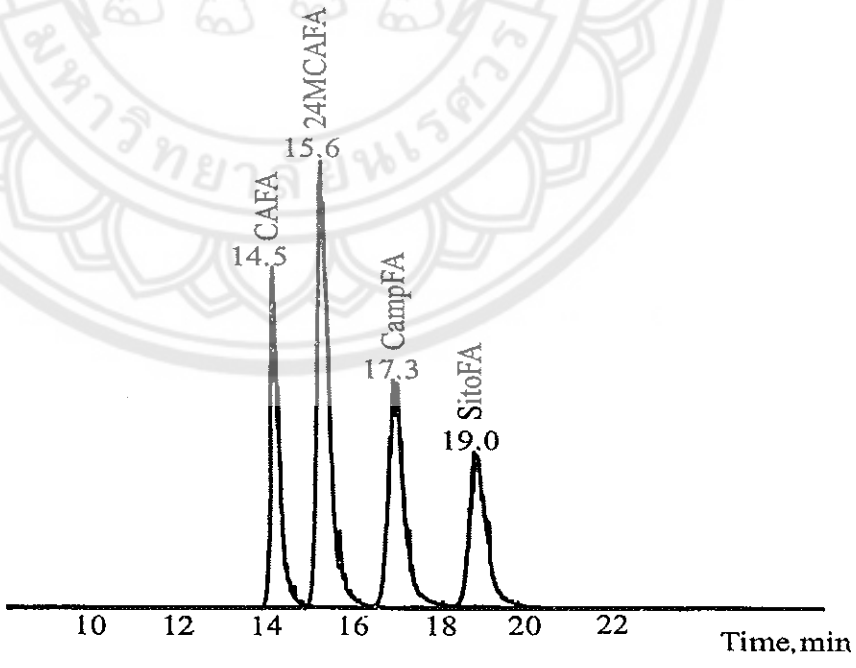


Figure 28 The chromatogram component of γ-oryzanol standard

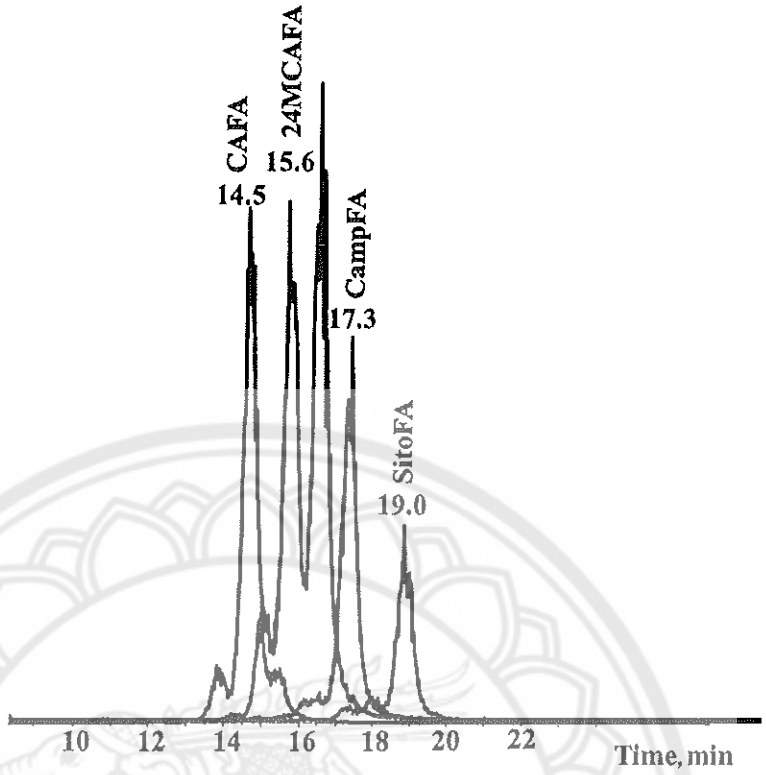


Figure 29 The chromatogram component of γ-oryzanol detected in RBO sample obtained from Hom-nin rice extracted by the SC-CO_2 extraction method

Table 16 γ-Oryzanols component of RBO samples from three rice varieties extracted using different extraction methods

RBO samples	CAFA (mg/100g oil)	24MCAFA (mg/100g oil)	Camp FA (mg/100g oil)	Sito FA (mg/100g oil)	Total (mg/100g oil)
KDML 105 (CPE)	89.99±5.9^{c}	75.16±5.36^{abc}	149.88±9.74^{bc}	11.59±1.88^{ab}	326.62±7.58^{bc}
RJM (CPE)	75.04±8.28^{d}	76.09±6.82^{abc}	114.32±7.33^{bc}	9.63±1.69^{ab}	275.09±6.05^{bcd}
HN (CPE)	167.03±2.53^{a}	99.23±6.23a	264.51±5.69^{a}	13.09±2.37^{a}	543.78±7.41^{a}
KDML 105 (SE)	88.78±7.98^{c}	85.21±9.48^{abc}	146.53±5.10^{bc}	12.15±2.07^{a}	332.67±7.15^{bc}
RJM (SE)	42.25±6.51^{f}	49.62±3.79^{c}	64.84±9.30^{c}	6.23±0.44^{b}	162.94±5.11^{d}
HN (SE)	171.26±1.75^{a}	90.13±6.35^{ab}	268.22±6.20^{a}	13.18±3.33^{a}	542.79±6.60^{a}
KDML 105 (SC-CO_2)	57.61±3.50^{e}	54.77±5.88^{bc}	94.25±5.71^{c}	8.56±2.47^{ab}	215.19±5.56^{cd}
RJM (SC-CO_2)	83.27±4.65^{cd}	64.31±6.05^{abc}	118.34±6.28^{bc}	9.50±0.83^{ab}	275.42±5.44^{bcd}
HN (SC-CO_2)	139.48±6.12^{b}	67.84±4.46^{abc}	190.92±3.66^{ab}	9.89±1.92^{ab}	408.13±6.16^{ab}
Molecularly distilled RBO	229.73±9.45	880.00±6.92	256.56±8.28	14.59±0.65	1380.88±16.03

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin.

4. The α-tocopherol content by RP-HPLC method

The α-tocopherol content of all RBO samples was determined using HPLC and calculated by calibration curve of standard. The peak was shown at 4.5 min and the chromatogram is illustrated in Figure 30. The α-tocopherol content of refined RBO samples from three rice varieties extracted by three extraction methods and RBO samples from CEO Agrifood Co., Ltd. were in the range of 0.37–1.84 and 0.23–0.37 mg/g oil, respectively (Table 17). The α-tocopherol content in this study was found to be slightly higher than those of Chotimakorn et al. [128], who reported that the α-tocopherol content of five long-grained rice bran extracts from commercially available cultivars in Thailand were 0.12–0.38 mg/g. However, it was found that the α-tocopherol from their study was close to the RBO from CEO Agrifood Co., Ltd. The result of α-tocopherol content was shown the same trend of total γ-oryzanol. The HN oil had the highest level, followed by RJM and KDML 105, respectively. However, it had a different trend in the extraction methods. The CPE method showed the highest content, followed by those extracted by the SE and SC-CO_2 methods, respectively. The results illustrated that each extraction method was more suitable use for extraction of a particular compound, not all the compounds.

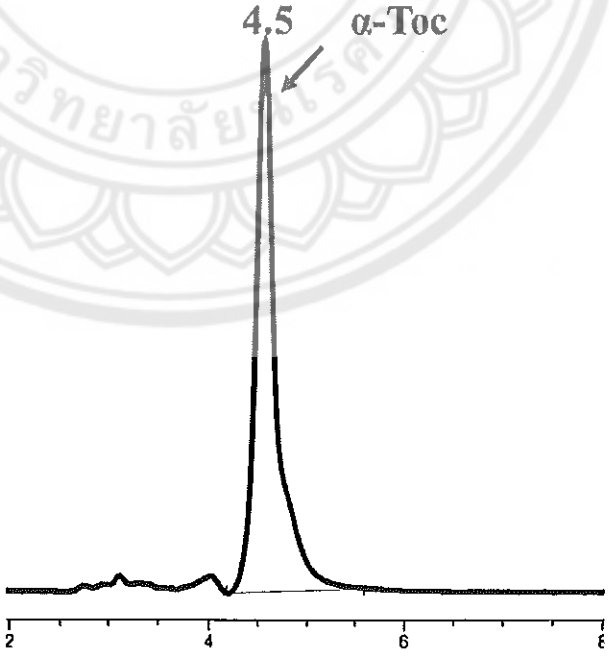


Figure 30 The chromatogram of α-tocopherol standard

Table 17 The α-tocopherol of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company

Extraction methods	Samples	α-Tocopherol (mg/g oil)
CPE	KDML 105	0.96±0.01^{e}
	RJM	1.01±0.01^{e}
	HN	1.84±0.07^{a}
SE	KDML 105	0.54±0.01^{f}
	RJM	0.97±0.01^{e}
	HN	1.10±0.05^{b}
SC-CO_2	KDML 105	0.37±0.01^{g}
	RJM	0.69±0.01^{e}
	HN	0.81±0.03^{d}
	Molecularly distilled RBO	0.37±0.07
	Refined RBO	0.23±0.06

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

5. Identification of isomers of vitamin E (by HPLC-MS/MS)

The chemical structure of vitamin E comprised eight different forms: α-, β-, γ-, δ- tocopherol (Toc) and α-, β-, γ-, δ-tocotrienols (T3). The concentration of Toc and T3 are shown in Table 18. The peaks were sorted as α-Toc at 6.8 min, α-T3 at 7.8 min, β-Toc at 9.1 min, γ-Toc at 9.8 min, β-T3 at 10.9 min, γ-T3 at 11.6 min, δ-Toc at 13.5 min and δ-T3 at 16.1 min (Figures 31 and 32 (HN-SC-CO_2)). The concentrations of individual component of vitamin E in the refined RBO samples were calculated from the peak area of the multiple standards. Among vitamin E homologues, γ-T3 was the predominant while β-T3 was not detected in any RBO sample. The total Toc and T3

contents of HN's RBO samples extracted by all methods were significantly higher ($p \leq 0.05$) than those of KDML 105 and RJM, indicating the effect of varieties on total Toc and T3 contents. The HN's RBO sample extracted by CPE had significantly higher ($p \leq 0.05$) total Toc and T3 content than SC-CO_2 and SE methods, respectively, indicating that CPE is more effectively preserved total Toc and T3 contents in the samples more than other methods.

In terms of vitamin E derivatives, this paper successfully separated all derivatives without peak overlapping. In general, HN's RBO samples had significantly higher ($p \leq 0.05$) α-Toc, β-Toc and δ-Toc contents than other rice varieties for all extraction methods. Comparing within the HN variety, the sample extracted by CPE had significantly higher ($p \leq 0.05$) α-Toc, β-Toc and δ-Toc contents than SC-CO_2 and SE methods. For γ-Toc, HN's RBO samples also exhibited higher γ-Toc content than other rice varieties. The RBO samples extracted from HN and RJM rice using CPE method showed the highest γ-Toc content, followed by SC-CO_2 and SE methods, respectively. It can be concluded that HN rice and CPE method provided RBO samples with the highest ($p \leq 0.05$) concentration of all four derivatives of Toc.

For T3 derivatives, α-T3 could be detected in HN samples using all extraction methods. While in RJM, it could be detected only by using CPE and SC-CO_2 methods. For KDML 105, it could only be found by the SE method. In HN's RBO sample, the CPE method provided the highest ($p \leq 0.05$) α-T3 content, followed by SC-CO_2 and SE methods, respectively. In contrast with β-T3, it was not detected in any samples in this study. For γ-T3, significant differences ($p \leq 0.05$) of contents were found in samples extracted from three rice varieties using all extraction methods. The RBO sample obtained from HN rice extracted by SC-CO_2 method showed the highest γ-T3 content. Similar to δ-T3, HN's RBO samples had significantly higher ($p \leq 0.05$) δ-T3 content than other rice varieties for all extraction methods. In addition, when considering the extraction methods, SC-CO_2 method provided the highest δ-T3 content. Therefore, it can be summarized that RBO from HN rice had the highest T3 content than those from RJM and KDML 105. For α-T3, CPE seemed to be the most appropriate extraction method while for γ-T3 and δ-T3, SC-CO_2 seemed to be the best extraction method.

It is now well established that vitamin E refers to eight different isoforms that belong to two categories, four saturated analogues (α, β, γ, and δ) called Toc and four unsaturated analogues referred to as T3. While the Toc has been investigated extensively, little is known about the T3. There is an evidence, however, that T3 may be superior in its biological properties, and that its anti-inflammatory and antioxidant activities could prevent cancer, diabetes, and cardiovascular and neurodegenerative diseases. RBO was described as one of the richest sources of Toc and T3 [129]. Quantities of Toc and T3 were found to be varied according to the origin of the rice bran [97] which is supported the findings from this study. In this study, all forms of Toc and T3 except β-T3 were detected in RBO samples extracted using different methods. Generally, HN's RBO extracted using CPE were found to be the best in preserving both Toc and T3. Mild condition of CPE could limit the loss of vitamin E. Currently, it has been reported that black rice provided higher bioactive compounds than other pigmented rice [115] which is consistent to the result found in this study.

Table 18 Contents of eight vitamin E homologues in RBO samples from three rice varieties extracted using different extraction methods

RBO samples	Content (mg/100 g)								
	α-Toc	β-Toc	γ-Toc	δ-Toc	α-T3	β-T3	γ-T3	δ-T3	Total Toc and T3
KDML105 (CPE)	0.83 ± 0.06^{gh}	0.25 ± 0.04^{g}	1.77 ± 0.11^{f}	0.16 ± 0.02^{e}	ND	ND	12.28 ± 1.05^{e}	0.92 ± 0.17^{d}	16.21 ± 1.40^{e}
RJM (CPE)	7.82 ± 0.19^{b}	0.42 ± 0.01^{d}	8.09 ± 0.24^{b}	0.32 ± 0.01^{d}	2.31 ± 0.06^{b}	ND	25.00 ± 0.58^{c}	0.61 ± 0.05^{e}	44.57 ± 0.28^{b}
HN (CPE)	11.41 ± 0.63^{a}	1.30 ± 0.07^{a}	10.38 ± 0.38^{a}	0.73 ± 0.04^{a}	2.84 ± 0.20^{a}	ND	29.58 ± 0.95^{b}	1.42 ± 0.08^{b}	57.66 ± 2.10^{a}
KDML105 (SE)	1.79 ± 0.02^{e}	0.34 ± 0.01^{ef}	2.97 ± 0.03^{e}	0.19 ± 0.04^{e}	1.28 ± 0.06^{d}	ND	23.20 ± 1.16^{c}	1.08 ± 0.18^{cd}	30.85 ± 1.03^{c}
RJM (SE)	1.28 ± 0.03^{fg}	0.19 ± 0.00^{h}	1.05 ± 0.03^{g}	0.13 ± 0.02^{e}	ND	ND	2.98 ± 0.26^{g}	0.28 ± 0.05^{f}	5.92 ± 0.28^{f}
HN (SE)	5.49 ± 0.25^{d}	0.52 ± 0.05^{c}	2.76 ± 0.18^{e}	0.50 ± 0.03^{c}	1.92 ± 0.23^{c}	ND	9.27 ± 0.88^{f}	1.38 ± 0.14^{b}	21.84 ± 1.71^{d}
KDML105 (SC-CO_2)	0.80 ± 0.03^{h}	0.28 ± 0.02^{fg}	1.93 ± 0.48^{f}	0.16 ± 0.05^{e}	ND	ND	17.83 ± 1.92^{d}	1.29 ± 0.19^{bc}	22.29 ± 2.39^{d}
RJM (SC-CO_2)	1.53 ± 0.09^{ef}	0.38 ± 0.03^{de}	4.75 ± 0.48^{d}	0.31 ± 0.03^{d}	0.83 ± 0.06^{e}	ND	25.58 ± 1.95^{c}	0.83 ± 0.15^{de}	34.21 ± 2.58^{c}
HM (SC-CO_2)	6.08 ± 0.30^{c}	1.03 ± 0.02^{b}	6.87 ± 0.35^{c}	0.65 ± 0.03^{b}	2.35 ± 0.13^{b}	ND	35.69 ± 2.76^{a}	1.90 ± 0.28^{a}	54.57 ± 2.67^{a}
Molecularly distilled RBO	3.17±0.18	0.43±0.02	3.60±0.22	0.41±0.04	1.59±0.06	ND	35.69±2.76	2.68±0.34	47.57±2.74

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

ND = Not detected (detection limit is 5 μg/g).

CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction;

KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin.

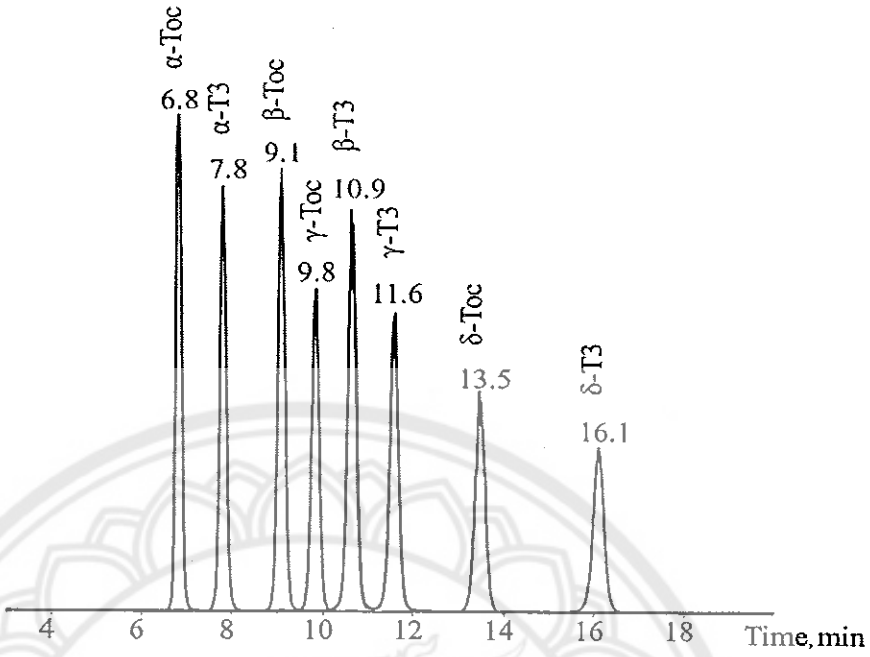


Figure 31 The chromatograms of tocopherol and tocotrienol standards

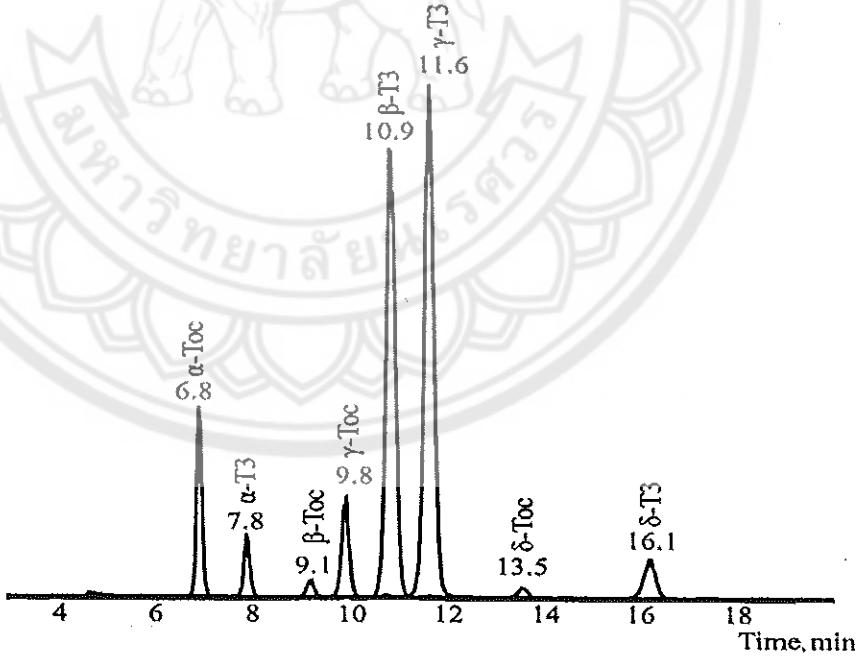


Figure 32 The chromatograms of tocopherol and tocotrienol detected in RBO sample obtained from Hom-nin rice extracted by the SC-CO_2 extraction method

6. Identification of isomers of phytosterols

Phytosterols were identified by GC technique. The contents of three major phytosterols were found in RBO from three rice verities extracted by three extraction methods namely campesterol, stigmasterol and β-sitosterol and is summarized in Table 19. The chromatograms of phytosterols were sorted as campesterol at 16.3 min, stigmasterol at 16.6 min and β-sitosterol at 17.5 min as shown in Figures 33 and 34 (HN-SC-CO_2). The concentration of individual component of phytosterols were determined by using calibration curves of standard. For RBO samples extracted by all methods, β-sitosterol was more prevalent than campesterol and stigmasterol. The concentrations of total phytosterols ranged from 8.78–11.43 mg/g oil. RBO samples from HN rice extracted by CPE and SC-CO_2 methods had the highest ($p \leq 0.05$) total phytosterol content whereas RBO sample from KDML 105 rice extracted by SC-CO_2 method showed the lowest ($p \leq 0.05$) value.

The campesterol contents of HN's RBO samples were significantly higher ($p \leq 0.05$) than other rice varieties for all extraction methods. For each rice variety, there were significant differences ($p \leq 0.05$) of campesterol contents in RBO samples extracted using different extraction methods. However, the stigmasterol content of RBO samples from HN rice extracted from all methods did not show any significant difference ($p > 0.05$). The RBO samples from HN rice had significantly higher ($p \leq 0.05$) stigmasterol content than other varieties, except RJM extracted by SC-CO_2 method. No significant difference ($p > 0.05$) in the stigmasterol content was observed for KDML 105 and RJM extracted by CPE and SE. In terms of β-sitosterol content, RBO samples from HN rice extracted by SC-CO_2 method was the highest ($p \leq 0.05$) while those of KDML 105 and RJM extracted by SC-CO_2 method were the lowest ($p \leq 0.05$). RBO samples of KDML 105 and RJM extracted by SE and SC-CO_2 did not show any significant difference ($p > 0.05$) in β-sitosterol content.

As γ-oryzanol is a mixture of ferulic acid esters of triterpene alcohols and sterols, various phytosterols in RBO samples have been reported [122]. This study confirms the occurrence of major phytosterols, campesterol, stigmasterol and β-sitosterol, found in RBO samples. Current researches focus on two aspects in this area, health benefits and analytical techniques. Influences of rice varieties and effects of processing steps on the contents of phytosterols were mostly studied as major bioactive

compounds such as oryzanol and tocopherol. Very limited published papers that investigate deeply to each component are available.

Table 19 Phytosterol contents of refined RBO samples from three rice varieties extracted using different extraction methods

RBO samples	Content (mg/g RBO)			
	Campesterol	Stigmasterol	β-sitosterol	Total phytosterols
KDML 105 (CPE)	2.59±0.10^{e}	1.87±0.05^{bc}	5.46±0.14^{c}	9.93±0.30^{d}
RJM (CPE)	2.57±0.01^{e}	1.81±0.03^{cd}	5.78±0.05^{b}	10.17±0.10^{bcd}
HN (CPE)	3.52±0.03^{a}	2.04±0.01^{a}	5.86±0.04^{b}	11.43±0.00^{a}
KDML 105 (SE)	2.75±0.02^{d}	1.90±0.03^{b}	5.67±0.05^{bc}	10.32±0.06^{bc}
RJM (SE)	2.47±0.05^{f}	1.81±0.00^{cd}	5.70±0.14^{b}	9.99±0.20^{cd}
HN (SE)	3.33±0.03^{b}	1.99±0.00^{a}	5.19±0.03^{d}	10.51±0.06^{b}
KDML 105 (SC-CO_2)	2.03±0.06^{g}	1.77±0.02^{d}	4.97±0.19^{e}	8.78±0.28^{f}
RJM (SC-CO_2)	2.43±0.02^{f}	1.99±0.00^{a}	4.98±0.17^{de}	9.41±0.19^{e}
HN (SC-CO_2)	2.91±0.08^{c}	2.05±0.07^{a}	6.25±0.10^{a}	11.21±0.25^{a}
Molecularly distilled RBO	4.22±0.13	2.38±0.12	6.22±0.28	12.82±0.38

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

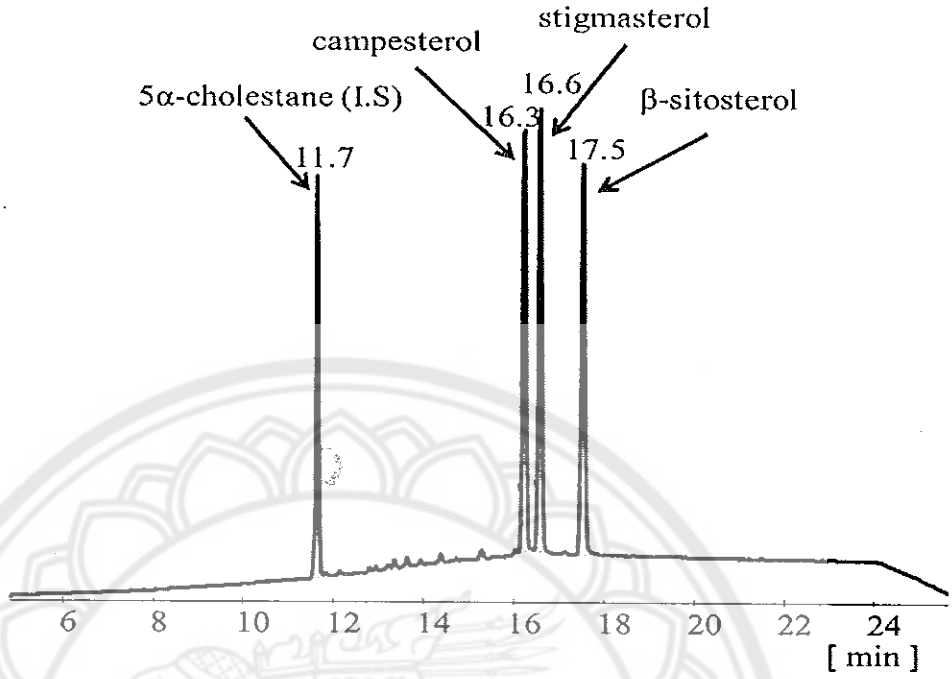


Figure 33 The chromatograms of phytosterols standard

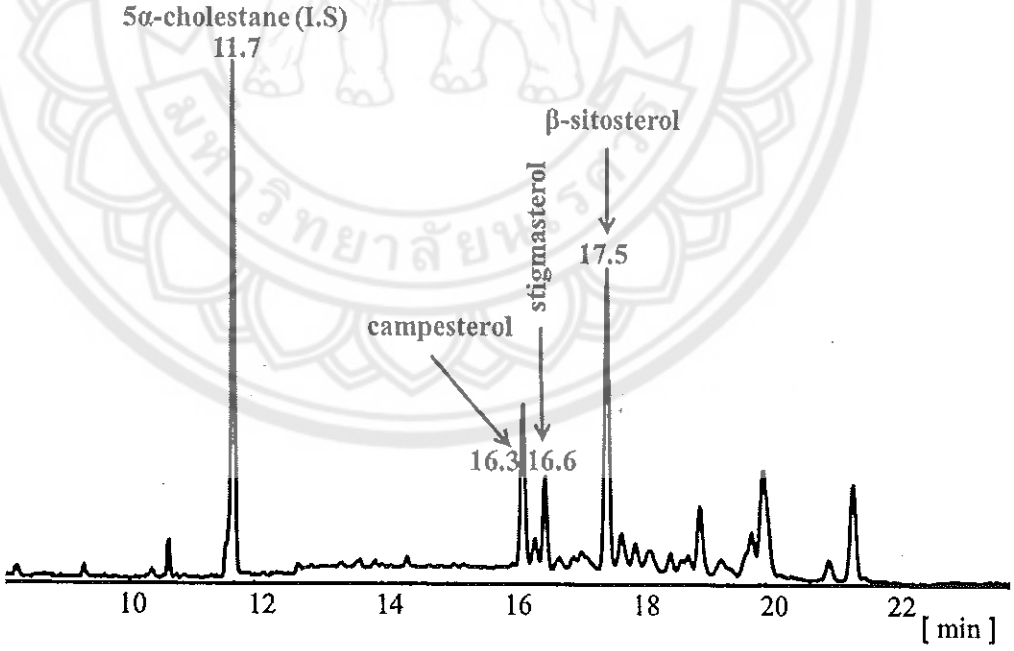


Figure 34 The chromatograms of phytosterols detected in RBO sample obtained from Hom-nin rice extracted by the SC-CO_2 extraction method

7. Fatty acid profile of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company.

The fatty acid profiles of the refined RBO samples are presented in Table 20. There were seven fatty acids found including tetradecanoic (myristic, C14:0), hexadecanoic (palmitic, C16:0), cis-9-hexadecenoic (palmitoleic, C16:1 n7), octadecanoic (stearic, C18:0), cis-9-octadecenoic (oleic, C18:1 n9), cis-9,12-octadecadienoic (linoleic, C18:2 n6) and cis-9,12,15-octadecatrienoic (linolenic, C18:3 n3). The proportion of saturated fatty acid (SFA) 23.30%, monounsaturated fatty acid (MUFA) 44.54% and polyunsaturated fatty acid (PUFA) 32.16% was in accordance with the range described by Susana, Grimaldi and Hense [130]. The three SFA found in the samples were palmitic, stearic and myristic acids with the concentration of 17.87–20.57, 2.18–2.65 and 0.29–0.42 g/100 g oil, respectively. The two MUFA found were oleic and palmitoleic acid with the concentration of 41.51–44.15 and 0–0.21 g/100 g oil, respectively. The two PUFA found were linoleic and linolenic acids with the concentration of 27.03–32.11 and 1.01–1.13 g/100 g oil, respectively. The fatty acid contents of all samples obtained from different extraction methods were significantly different ($p \leq 0.05$). Palmitic, linoleic and oleic acids were the major fatty acids in the RBO samples [131, 132]. The content of unsaturated fatty acids was higher than saturated fatty acids. The result of fatty acid profile was consistent with the IV as shown in Figure 25. Simultaneously, two oil samples from CEO Agrifood Co., Ltd. had fatty acids that were similar to the refined RBO samples from three rice varieties extracted using different extraction methods. The results of fatty acid profiles in this study are in accordance with Oluremi, Solomon and Saheed [133], Yoshie et al. [116] and Rani et al. [113]. High amount of unsaturated fatty acids in the RBO samples could promote nutritional and health benefits since unsaturated fatty acids could prevent diseases that associated with the cholesterol [134, 135].

Table 20 Fatty acid composition of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company

Samples	Fatty acid composition (g/100 g oil)						
	C14:0 (Myristic)	C16:0 (Palmitic)	C16:1 n7 (Plamioleic)	C18:0 (Stearic)	C18:1 n9 (Oleic)	C18:2 n6 (Linoleic)	C18:3 n3 (Linolenic)
KDML 105 (CPE)	0.31±0.00^{d}	20.25±0.05^{c}	0.21±0.00^{a}	2.35±0.00^{c}	43.14±0.03^{bc}	28.42±0.11^{e}	1.03±0.01^{e}
RJM (CPE)	0.29±0.00^{e}	20.51±0.01^{a}	0.20±0.00^{b}	2.39±0.00^{c}	42.85±0.07^{c}	28.31±0.00^{e}	1.13±0.01^{b}
HN (CPE)	0.38±0.01^{b}	17.92±0.09^{e}	0.17±0.00^{c}	2.54±0.07^{b}	41.51±0.34^{e}	32.11±0.38^{a}	1.03±0.02^{de}
KDML 105 (SE)	0.35±0.01^{c}	20.09±0.07^{d}	0.21±0.00^{a}	2.35±0.05^{c}	42.14±0.20^{d}	29.39±0.27^{c}	1.06±0.02^{cd}
RJM (SE)	0.29±0.01^{e}	20.57±0.06^{a}	ND	2.49±0.04^{b}	44.15±0.06^{a}	27.03±0.00^{f}	1.05±0.00^{cde}
HN (SE)	0.42±0.01^{a}	17.87±0.01^{e}	ND	2.65±0.02^{a}	42.24±0.17^{d}	31.43±0.19^{b}	0.97±0.01^{f}
KDML 105 (SC-CO_2)	0.32±0.01^{d}	20.09±0.05^{d}	0.21±0.00^{a}	2.18±0.02^{d}	42.94±0.12^{bc}	28.76±0.07^{d}	1.08±0.01^{c}
RJM (SC-CO_2)	0.31±0.00^{d}	20.35±0.02^{b}	0.20±0.00^{b}	2.24±0.02^{d}	43.19±0.11^{b}	28.11±0.09^{e}	1.17±0.01^{a}
HN (SC-CO_2)	0.41±0.00^{a}	17.56±0.05^{f}	ND	2.69±0.04^{a}	42.88±0.18^{bc}	31.12±0.15^{b}	0.93±0.01^{g}
Molecularly distilled RBO	0.40±0.01	19.90±0.18	ND	2.32±0.18	39.64±0.61	32.10±0.78	1.15±0.06
Refined RBO	0.28±0.02	19.10±0.11	ND	2.34±0.12	44.69±0.45	28.51±0.58	0.69±0.02

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). ND : Not detected.

CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction;

KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

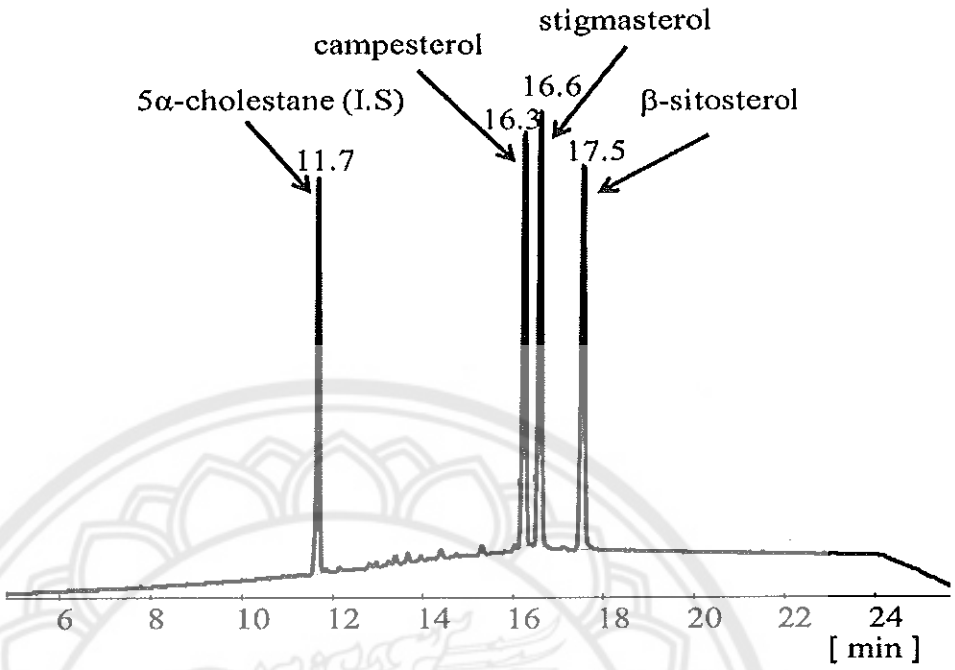


Figure 33 The chromatograms of phytosterols standard

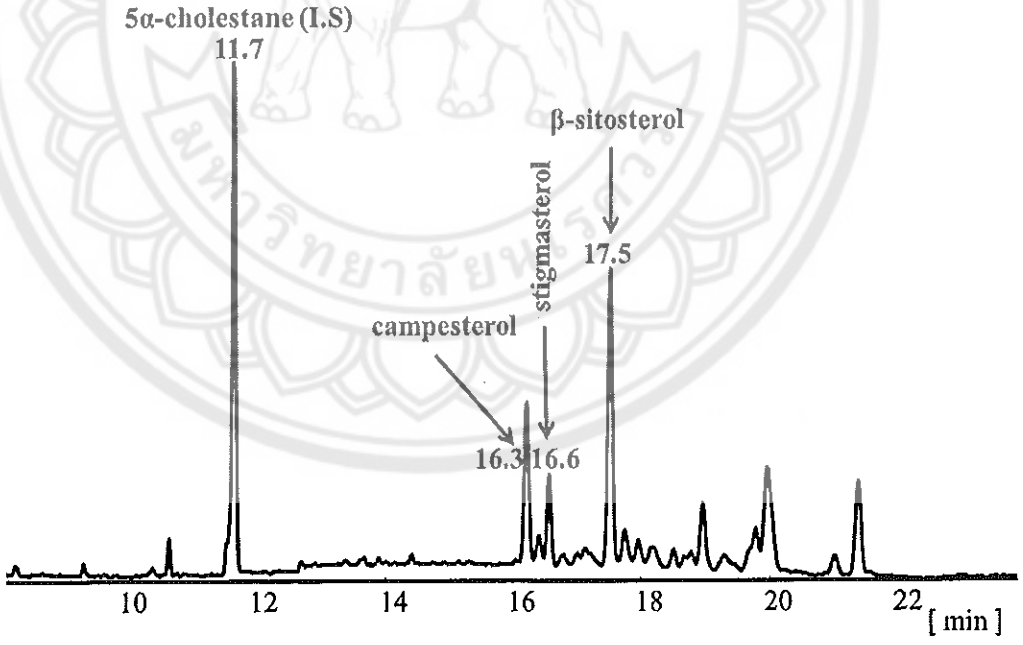


Figure 34 The chromatograms of phytosterols detected in RBO sample obtained from Hom-nin rice extracted by the SC-CO_2 extraction method

Table 21 Antioxidant activity of refined RBO samples from three rice varieties extracted using different extraction methods and two RBO samples from the company

Extraction methods	Samples	DPPH IC_{50} (mg/g)	FRAP (μM TE/g)	ORAC (μM TE/g)
CPE	KDML 105	2.44±0.00^{b}	38.18±0.72^{f}	537.69±5.30^{g}
	RJM	2.11±0.04^{d}	71.72±1.83^{c}	577.99±9.26^{e}
	HN	1.27±0.01^{g}	79.12±1.01^{b}	640.22±8.75^{b}
SE	KDML 105	3.41±0.02^{a}	39.60±0.52^{f}	561.36±8.77^{f}
	RJM	2.28±0.01^{c}	60.38±0.80^{d}	622.78±5.16^{c}
	HN	1.71±0.07^{f}	78.35±4.06^{b}	690.10±7.51^{a}
SC-CO_2	KDML 105	2.28±0.00^{c}	51.78±2.01^{e}	505.20±9.75^{i}
	RJM	1.85±0.01^{e}	57.07±0.34^{d}	523.91±6.02^{h}
	HN	0.93±0.01^{h}	89.76±4.64^{a}	596.03±8.68^{d}
	Molecularly distilled RBO	1.69±0.01	77.00±2.07	639.80±5.29
	Refined RBO	2.98±0.03	63.22±2.85	186.17±7.90

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). CPE: Cold-press extraction; SE: Solvent extraction; SC-CO_2: Supercritical carbon dioxide extraction; KDML 105: Khao Dawk Mali 105; RJM: Red Jasmine; HN: Hom-nin

When considering the amount of antioxidants and antioxidant activity, it was found that RBO from HN rice extracted by SC-CO_2 was the most prominent sample. This could be due to having the highest value of TPC, γ-oryzanol, total Toc and T3 and phytosterol including antioxidant activity in terms of DPPH CI_{50} and FRAP. These results agreed with the study of Balachandran et al. [138], Dunford and King [139] and Perretti et al. [140] who indicated that the SC-CO_2 extraction is an extraction method to obtain antioxidant enriched extracts. Therefore, the HN-SC-CO_2 sample was selected to further study in an animal model (Part 4).

Shelf life of RBO

In this part, the shelf life of RBO samples was studied for six months at room temperature and the AV, FFA content and PV were determined during the shelf life study. The results of AV, FFA and PV are summarized in Figures 35, 36 and 37, respectively. The results shown that, the levels of AV, FFA content and PV increased with time of storage. Due to refined RBO samples contained relatively high levels of unsaturated fatty acids with estimated value of 76.45%, oxidation could occur during the extraction and storage. This results were consistent with Genkawa et al. [141] who reported that the AV was increased from 24.3–59.1 and 68.8 mg KOH/g oil when samples were stored at 15 °C and 25 °C for 6 months. The molecularly distilled RBO sample had the lowest AV and FFA values because the molecular distillation plays a significant role in the deacidification (which is suitable in oil with high FFA value such as RBO) and deodorization of the refining processes [142, 143, 144]. Apart from molecularly distilled RBO sample, all samples had higher AV and FFA content than the standard (0.6 mg KOH/g oil and 0.3%) due probably to long extraction time, lab scale refinery process and package. For PV, all samples including molecularly distilled RBO had higher PV than the standard (10 mg eq/kg oil) after stored for about 120 days. Although the RBO samples have higher PV than the standard, they were not rancid. This result was consistent with Akubugwo and Ugbogu [145] who reported that the oil became rancid when the PV was in the range of 20–40 mg Eqv/kg oil. From the conditions used in this study, its had shelf life for 3–5 months.

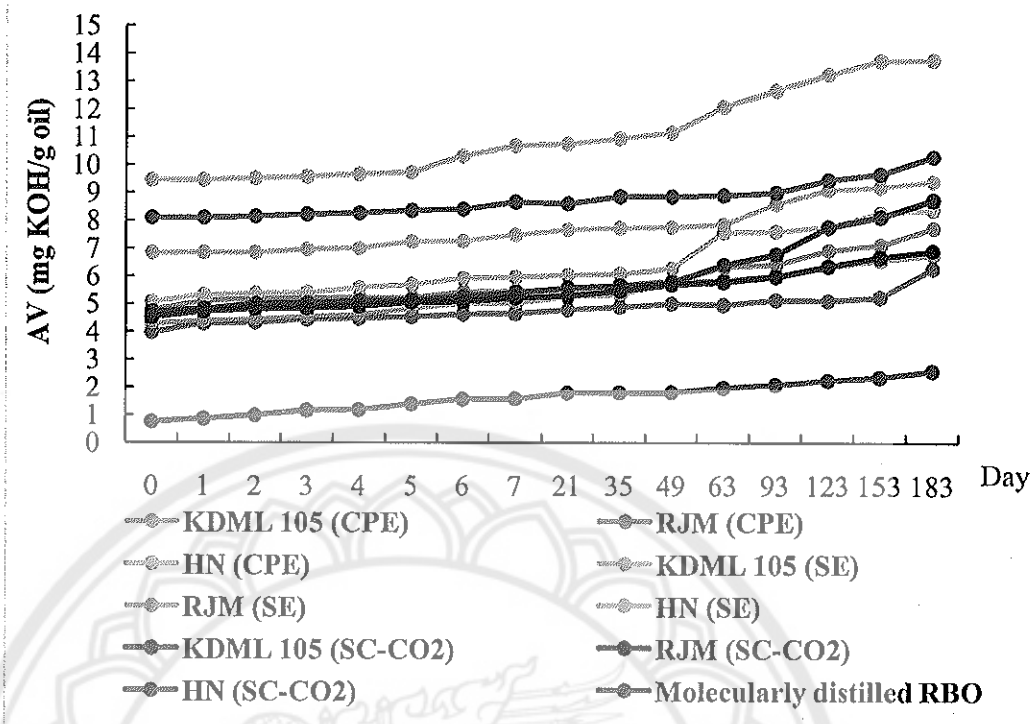


Figure 35 Acid value of RBO samples

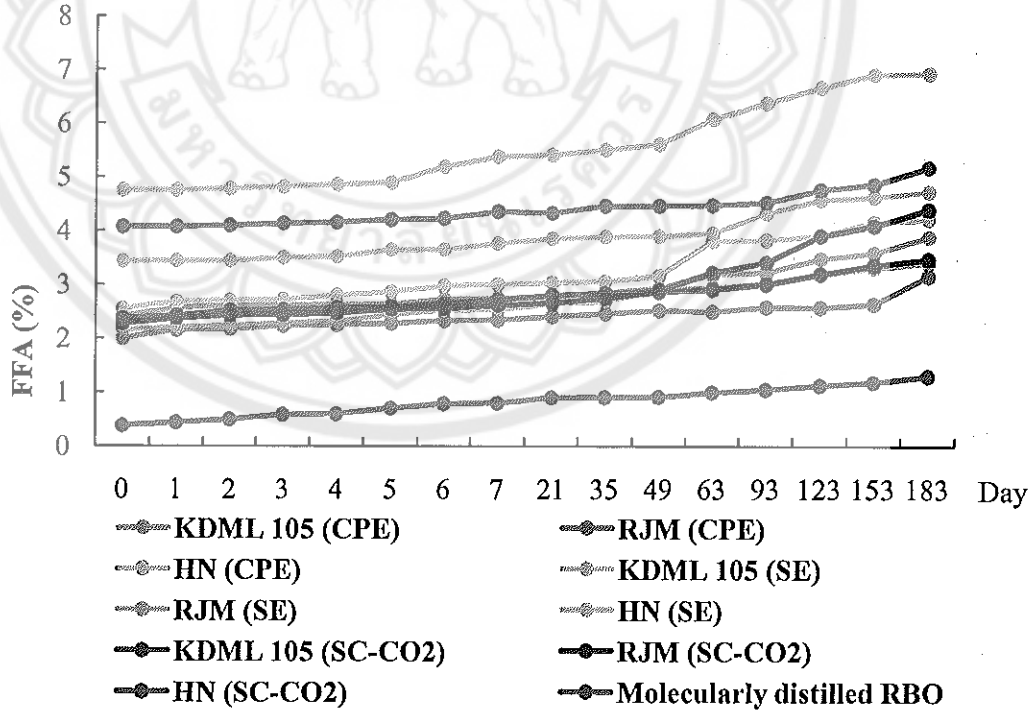


Figure 36 Free fatty acid content of RBO samples

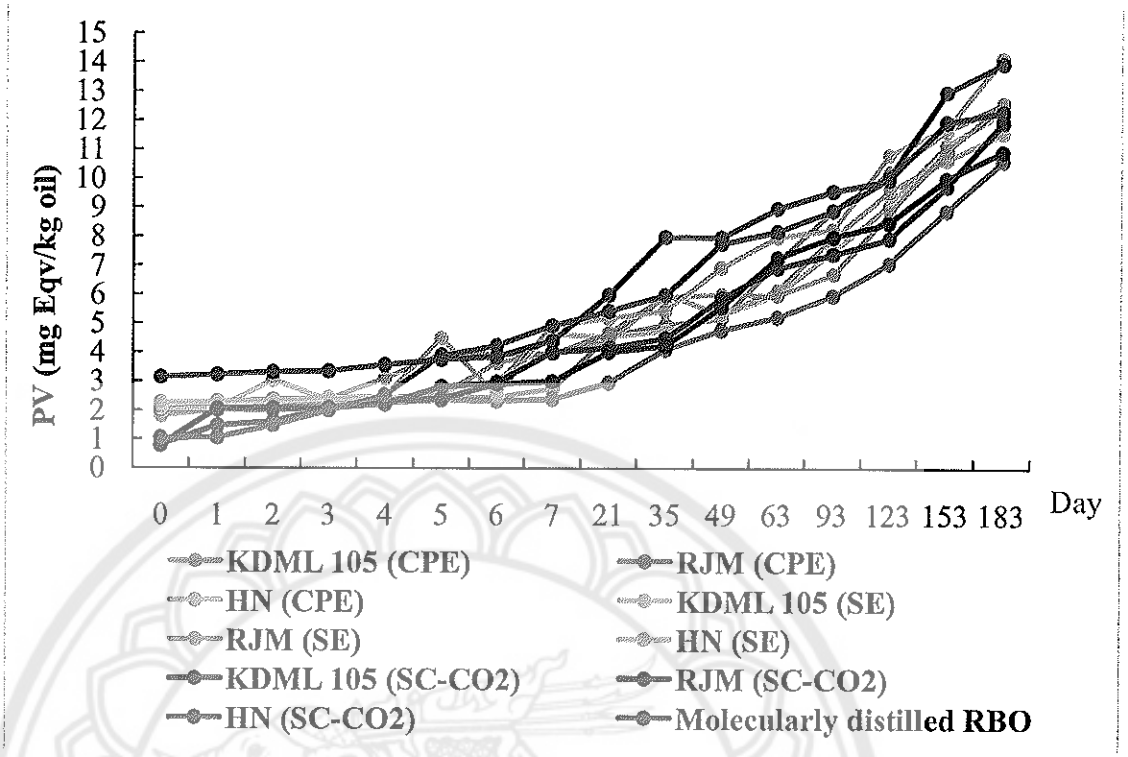


Figure 37 Peroxide value of RBO samples

Efficiency of RBO on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat

This session used the selected RBO from section 4.2 (HN oil extracted by SC-CO_2) and two commercial RBO samples from CEO Agrifood Co., Ltd. (molecularly distilled RBO and refined RBO).

1. Diet development with a mixture of RBO and chemical analysis of the diets

This research has 4 formula diets as well as the standard diet (AIN93M) showed in Table 10 (Chapter III, p. 47) including normal diet, high fat diet, high fat diet mixed with refined RBO and high fat diet mixed with HN-SC-CO_2. The 4 diets were analyzed for the chemical properties, including proximate analysis, type and amount of fatty acid, α-tocopherol, γ-oryzanol, total phenolic and antioxidant activity.

1.1 Proximate analysis of the diets

The results of proximate analysis of the diets are shown in Table 22. The moisture, protein, fiber, ash, fat and carbohydrate contents of the diets were in the

range of 6.56–7.06, 12.99–14.26, 3.66–4.20, 2.75–3.10, 4.22–5.00 and 67.45–68.95 g/100 g, respectively. There were no significant differences ($p > 0.05$) due to all of diets were made using the standard ingredients (AIN93M), except the moisture content that showed significant difference ($p \leq 0.05$). The normal diet had significantly higher ($p \leq 0.05$) moisture content than other samples while high fat diet and high fat diet mixed with HN-SC-CO_2 showed the lowest value ($p \leq 0.05$).

Table 22 Proximate analysis of the diets

Diet	Content (g/100 g)		
	Moisture	Protein ^{ns}	Fiber ^{ns}
Normal diet	7.06±0.12^{a}	14.26±0.94	3.92±0.37
High fat diet	6.63±0.08^{bc}	13.44±0.21	4.20±0.41
High fat diet mixed with refined RBO	6.77±0.09^{b}	13.77±0.51	3.76±0.10
High fat diet mixed with HN-SC-CO_2	6.56±0.11^{c}	12.99±0.72	3.66±0.37
	Ash ^{ns}	Fat ^{ns}	Carbohydrate ^{ns}
Normal diet	3.07±0.15	4.22±0.78	67.45±0.75
High fat diet	2.75±0.21	4.73±0.48	68.23±0.21
High fat diet mixed with refined RBO	3.10±0.35	4.64±0.55	67.94±0.35
High fat diet mixed with HN-SC-CO_2	2.81±0.55	5.00±0.61	68.95±0.59

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).
ns: Not significant

1.2 The physicochemical properties of the diets

The properties of the diets were analyzed in this section including the amount of TPC, total γ-oryzanol, α-tocopherol and fatty acid composition. More details of each components were described as followed.

1.2.1 The total phenolic content (TPC) of the diets

The TPC of the diets were determined following the same method analyzed of refined oil samples [6]. The TPC content of the diet samples for testing in animals showed significant difference in all diets ($p \leq 0.05$), except normal diet and high

fat diet that showed no significant difference ($p > 0.05$). The TPC found in the diets were ranged between 189–382 mg gallic acid/100 g (Table 23). The high fat diet mixed with HN-SC-CO_2 had the highest TPC content, followed by high fat diet mixed with refined RBO, high fat diet and normal diet, respectively.

Table 23 The unsaponifiable maters in the diets

Diet	Content (mg/100 g)		
	TPC	γ-Oryzanol	α-Tocopherol
Normal diet	188.59±2.62^{c}	ND	11.25±0.06^{b}
High fat diet	196.76±5.29^{c}	ND	10.64±0.01^{c}
High fat diet mixed with refined RBO	289.72±3.62^{b}	9.73±0.42^{b}	11.59±0.01^{b}
High fat diet mixed with HN-SC-CO_2	382.24±3.66^{a}	42.66±1.35^{a}	15.55±0.61^{a}

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).
ND: Not detected.

1.2.2 The total γ-oryzanol content of the diets

The total γ-oryzanol content of all diet samples were determined the same method for those analysis in the refined RBO. The normal diet and high fat diet could not detect total γ-oryzanol, due to the ingredients in these diets used soybean oil which had no γ-oryzanol content. High fat diet mixed with refined RBO and high fat diet mixed with HN-SC-CO_2 had total γ-oryzanol content of 9.73 mg/100 g and 42.66 mg/100 g, respectively. It is obvious that the high fat diet mixed with HN-SC-CO_2 had almost 5 times higher concentration of γ-oryzanol than those found in high fat diet mixed with refined RBO (Table 23).

1.2.3 The α-tocopherol content of the diets

The α-tocopherol content of all diets were determined by using HPLC and calculated by calibration curve of the standard. The concentration of α-tocopherol of all diets shows in Table 23. The amount of α-tocopherol of all diets ranged between 10.64–15.55 mg/100 g and showed a significant difference ($p \leq 0.05$). High fat

diet mixed with HN-SC-CO_2 had the highest α-tocopherol content, followed by high fat diet mixed with refined RBO, normal diet and high fat diet ($p \leq 0.05$), respectively. However, normal diet had α-tocopherol content not significant different ($p>0.05$) to the high fat diet mixed with refined RBO. As HN's RBO extracted by SC-CO_2 had higher α-tocopherol content than refined RBO about 4 times (Table 18), it contributed to higher α-tocopherol content in the diet as well.

1.2.4 Fatty acid profile of the diets

The fatty acid profiles of the diet samples are presented in Table 24. There were six fatty acids found in the samples including C14:0, C16:0, C18:0, C18:1 n9, C18:2 n6 and C18: n3 with the concentration of 0.42–0.77, 14.34–21.92, 3.01–5.69, 27.30–43.74, 30.04–48.14 and 0.85–4.73 g/100 g, respectively. The fatty acid contents of all diet samples were significantly different ($p \leq 0.05$). The content of unsaturated fatty acids in the diet samples was higher than saturated fatty acids which was the same trend of those refined RBO samples. The high fat diet mixed with refined RBO and HN-SC-CO_2 oil had higher fatty acid content than normal diet and high fat diet (it has soybean oil in the formula) except the value of C18:0 in normal diet and high fat diet which were higher than those of high fat diet mixed with refined and HN-SC-CO_2 oil. The diet mixed with RBO had higher levels of C16:0, C18:1 n9, C18:2 n6 and C18:3 n3 than the diets without RBO 35%, 38%, 38% and 82%, respectively.

Table 24 Fatty acid composition of the diets

Diets	Fatty acid composition (g/100 g)		
	C14:0	C16:0	C18:0
Normal diet	0.56±0.02^{b}	14.34±0.31^{c}	5.02±0.44^{b}
High fat diet	0.51±0.00^{b}	14.65±0.13^{c}	5.69±0.08^{a}
High fat diet mixed with refined RBO	0.42±0.21^{b}	21.92±0.04^{a}	3.01±0.04^{d}
High fat diet mixed with HN-SC-CO_2	0.77±0.03^{a}	20.85±0.00^{b}	3.70±0.00^{c}

Table 24 (cont.)

Diets	Fatty acid composition (g/100 g)		
	C14:0	C16:0	C18:0
	C18:1 n9	C18:2 n6	C18:3 n3
Normal diet	27.30±0.99^{b}	30.04±0.56^{c}	0.85±0.07^{c}
High fat diet	28.49±0.21^{b}	30.23±0.75^{c}	1.03±0.14^{c}
High fat diet mixed with refined RBO	43.74±0.42^{a}	48.14±0.41^{a}	4.73±0.31^{a}
High fat diet mixed with HN-SC-CO_2	43.42±0.92^{a}	46.24±0.38^{b}	4.37±0.10^{b}

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

1.3 Antioxidant activity of the diets

The antioxidant activity of the diet samples for feeding the rats was evaluated using DPPH, FRAP and ORAC assays and the results are shown in Table 25. The DPPH IC_{50} was found between 505.43–1047.89 mg/g and showed a significantly different ($p \leq 0.05$). The high fat diet mixed with HN-SC-CO_2 had the lowest DPPH IC_{50} (505.43 mg/g), followed by high fat diet mixed with refined RBO (766.93 mg/g), normal diet (1033.64 mg/g) and high fat diet (1047.89 mg/g), respectively. The FRAP was found between 6.09–7.41 μM TE/g and no significantly different ($p > 0.05$) was detected. However, the diet samples containing RBO had slightly higher FRAP value than the diet samples without RBO (normal diet and high fat diet) in the formula. The ORAC was found between 87.26–119.02 μM TE/g and showed a significantly different ($p \leq 0.05$). The diet samples mixed with refined RBO and HN-SC-CO_2 oil had significantly higher ($p \leq 0.05$) ORAC value than the normal diet. This is mainly due to the unsaponifiable matter of RBO, which the high fat diet mixed with HN-SC-CO_2 oil contained higher γ-oryzanol, TPC and α-tocopherol than three other diets (Table 23).

Table 25 Antioxidant activity of the diets

Diet	DPPH IC_{50} (mg/g)	FRAP ^{ns} (μM TE/g)	ORAC (μM TE/g)
Normal diet	1033.64±4.32^{a}	6.54±0.53	87.26±3.52^{b}
High fat diet	1047.89±3.41^{a}	6.09±0.66	93.28±3.39^{b}
High fat diet mixed with refined RBO	766.93±6.30^{b}	7.04±0.48	123.17±4.05^{a}
High fat diet mixed with HN-SC-CO_2	505.43±4.61^{c}	7.41±1.34	119.02±3.26^{a}

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

Na = not significant

2. The efficiency of RBO on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat

The total number of rat in this study were thirty-six rats which were divided by body weight into 2 major groups, six rats were control group and thirty rats were induced to hypercholesterolemia rats for two months. After hypercholesterolemia induction, thirty rats were randomLy divided by the total cholesterol and LDL levels into 5 groups. Hypercholesterolemia rats group consisted of 5 subgroups as 1) six hypercholesterolemia rats fed with high fat diet, 2) six hypercholesterolemia rats fed with high fat diet and 0.1 mL of molecularly distillation RBO (gavage), 3) six hypercholesterolemia rats fed with high fat diet mixed with 4% refined RBO, 4) six hypercholesterolemia rats fed with high fat diet and 0.1 mL of HN-SC-CO_2 oil (gavage) and 5) six hypercholesterolemia rats fed with high fat diet mixed with 4% of HN-SC-CO_2 oil. Throughout the period of experiment, the rats were given diets and water ad libitum. During the experiment, the rats were measured for; 1) body weight, food intake and water intake every 7 days, 2) lipid profile and blood glucose every 1 month, 3) at the end of 4 months-experimental period, the rats were measured for liver and kidney function tests, oxidative stress, antioxidant enzyme levels, inflammation, antioxidant capacity and histological changes.

2.1 Body weight, food intake and water intake

The initial body weight of the rats were about 298–303 g as shown in Table 26. After that, the rats were acclimated for 1 week. After acclimation, the rats had increased in body weight to about 34–45 g for all groups when compared to the initial weight but these were no significantly different ($p > 0.05$). After 2 months of inducement, the rats were treated with RBO samples. The results shown that the body weight of the rats in all groups continuously increased (Figure 38). This trend continued until the end of the studying period with an average weight of 578–608 g. However, there were no significantly different ($p > 0.05$) in the body weight throughout the studying period. The result of this can be concluded that the rats used in the experiment were healthy, not sick and did not have anorexia during testing.

Food intake, the rats of all groups ate diets about 30–35 g/day. The results are shown in Figure 39 and Table 27. However, no significant different were found in food intake ($p > 0.05$), except for 84 and 105 days of the experiment where there were significantly different ($p \leq 0.05$).

Water intake, the rats of all groups eat water about 20–35 mL/day and there were no significant different ($p > 0.05$) in the water intake throughout the experimental period (Table 28 and Figure 40). Both food intake and water intake in the period of experiment have inconsistent trend.

Table 26 Body weight of rats for studies its efficiency on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat

Group	Body weight (g)						
	Beginning	After acclimation	Day0 ns	Day7 ns	Day14 ns	Day21 ns	Day28 ns
C	303.14±10.06	342.57±11.21	348.50±11.91	383.50±10.94	418.16±12.20	440.66±11.72	455.16±14.93
HC	299.67±4.84	343.17±10.13	247.83±9.72	384.16±14.27	416.00±14.68	437.83±10.81	457.16±13.97
HMDG	299.57±4.64	334.43±5.08	343.50±15.47	376.50±15.54	408.16±14.67	429.50±17.56	445.83±12.76
HRFF	297.71±7.67	366.14±10.02	339.16±8.99	374.16±7.88	405.00±8.43	425.50±8.11	444.33±11.41
HHNG	302.57±10.03	344.57±9.57	348.00±10.35	383.33±10.80	418.83±13.68	440.83±15.30	457.33±15.38
HHNF	300.86±7.31	342.43±8.32	349.66±5.46	386.00±6.03	417.66±8.31	439.66±14.10	458.66±14.93
Group	**Day35 ns**	**Day42 ns**	**Day49 ns**	**Day56 ns**	**Day63 ns**	**Day70 ns**	**Day77 ns**
C	476.66±12.14	497.50±12.86	513.66±17.22	526.33±12.12	529.50±13.92	540.50±12.49	554.83±12.26
HC	475.83±17.29	497.33±19.66	516.50±17.49	529.50±17.66	538.33±18.42	550.00±12.38	562.16±11.17
HMDG	459.33±11.42	484.00±12.11	505.16±19.74	516.66±12.57	520.33±12.24	534.83±13.53	542.83±13.87
HRFF	463.50±11.99	489.00±14.91	505.83±17.73	520.66±12.65	522.33±13.21	536.16±17.03	550.00±14.19
HHNG	471.33±14.85	499.66±15.01	518.16±14.53	532.00±12.21	534.16±15.77	549.66±12.16	559.00±12.75
HHNF	478.16±14.46	501.83±16.36	517.16±18.12	532.83±11.02	535.16±19.99	549.50±12.92	561.66±12.41

Table 26 (cont.)

Group	Body weight (g)				
	Day84 ns	Day91 ns	Day98 ns	Day105 ns	Day112 ns
C	567.00±12.30	571.16±12.15	583.33±13.19	584.50±13.58	592.33±11.99
HC	569.66±10.62	576.16±12.83	594.00±12.26	600.50±12.38	607.50±10.03
HMDG	549.16±11.18	555.33±13.49	565.66±13.17	572.50±13.71	577.66±13.57
HRFF	561.00±11.70	566.00±10.36	578.33±11.40	580.33±12.22	586.66±12.14
HHNG	567.83±14.94	572.66±14.54	587.33±12.34	596.33±10.51	605.50±17.96
HHNF	569.00±13.86	570.66±10.48	580.83±11.21	586.33±10.78	593.83±12.32

Note: ns: Not significant C: normal control, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

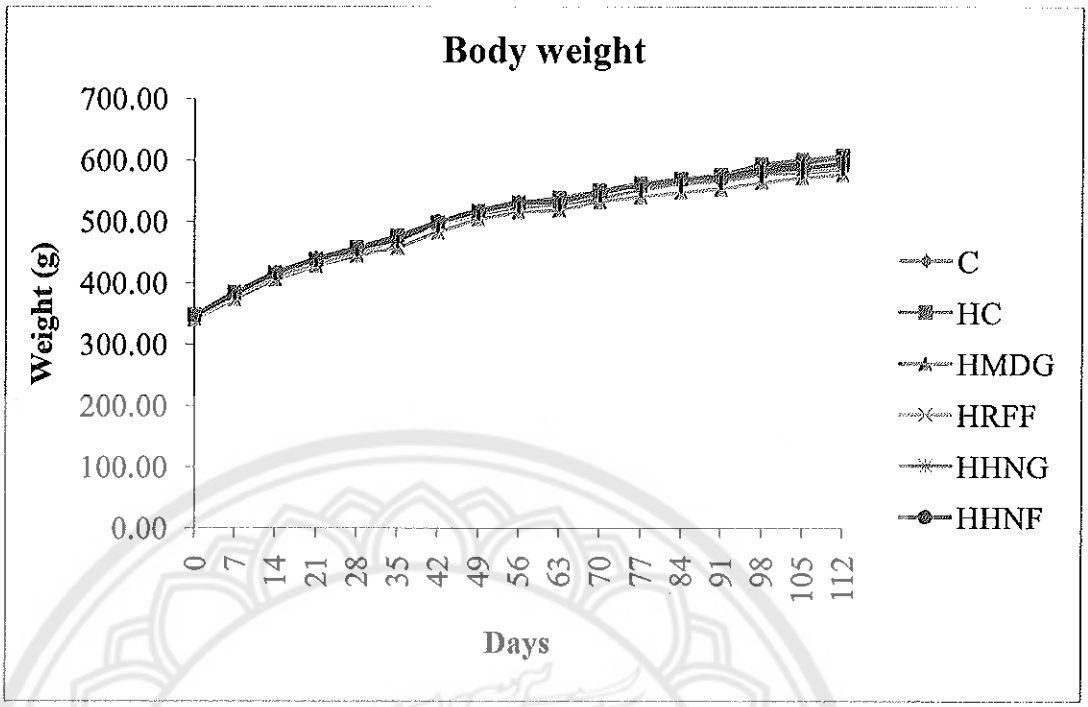


Figure 38 Body weight of hypercholesterolemia rats

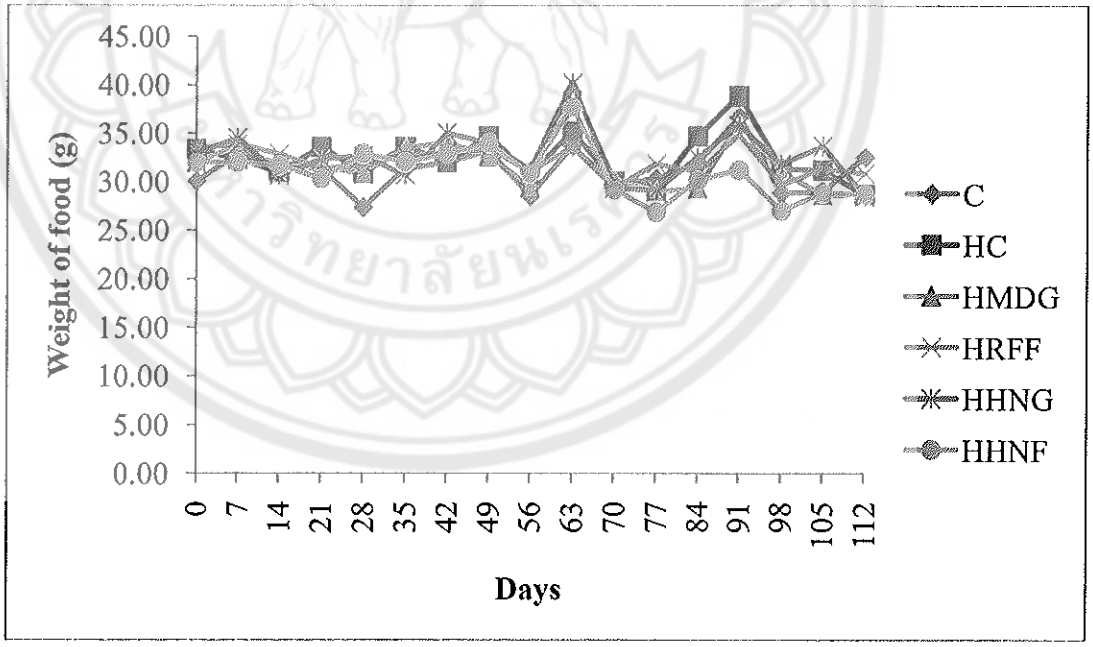


Figure 39 Food consumption of hypercholesterolemia rats

Table 27 Food intake of rats for studies its efficiency on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat

Group	Food intake (g)					
	Day0 ns	Day7 ns	Day14 ns	Day21 ns	Day28 ns	Day35 ns
C	30.01±1.90	32.83±1.72	31.83±2.63	31.33±3.50	27.33±4.88	31.33±2.80
HC	33.30±2.87	32.33±2.94	31.00±1.78	33.50±1.37	31.00±5.35	33.50±2.42
HMDG	32.01±4.88	33.66±3.61	31.50±4.54	31.50±3.93	30.83±5.77	33.50±1.87
HRFF	33.40±2.25	33.83±2.56	32.83±3.76	31.33±1.50	31.66±2.50	32.66±3.20
HHNG	32.91±2.01	34.50±3.20	30.66±2.06	32.33±2.65	32.66±4.84	30.66±3.72
HHNF	31.91±4.59	32.00±4.81	31.66±3.61	30.30±4.03	32.83±3.31	32.00±3.74
Group	**Day42 ns**	**Day49 ns**	**Day56 ns**	**Day63 ns**	**Day70 ns**	**Day77 ns**
C	32.00±4.42	33.16±3.54	28.50±3.14	33.66±5.62	29.66±2.33	30.33±3.26
HC	32.00±3.40	34.66±2.80	30.66±2.42	35.16±1.72	30.00±1.41	29.33±2.87
HMDG	34.00±3.03	32.66±4.76	29.50±5.20	39.83±5.52	30.16±6.14	29.00±5.09
HRFF	33.00±2.75	33.33±3.55	31.00±3.25	33.50±4.84	29.33±2.94	31.83±3.31
HHNG	35.00±3.03	34.00±3.16	31.16±2.78	40.16±6.76	29.33±2.94	29.16±3.06
HHNF	32.83±1.94	34.00±4.93	30.83±3.18	37.66±2.62	29.16±4.71	26.83±5.26

Table 27 (cont.)

Group	Body weight (g)				
	Day84	Day91 ns	Day98 ns	Day105	Day112 ns
C	32.00±3.22^{ab}	36.66±4.07	28.83±4.53	29.00±1.89^{ab}	32.50±2.34
HC	34.66±2.65^{a}	38.83±5.74	31.33±5.42	31.16±2.99^{ab}	28.66±3.50
HMDG	29.33±4.41^{b}	36.00±5.44	31.00±4.97	28.66±3.20^{b}	28.66±4.03
HRFF	31.00±5.21^{ab}	34.83±5.03	29.66±3.44	31.33±4.54^{ab}	30.83±5.77
HHNG	32.50±1.22^{ab}	35.83±2.50	31.83±3.06	33.66±5.57^{a}	28.50±3.61
HHNF	30.16±2.31^{b}	31.16±5.98	27.00±2.00	28.83±4.30^{b}	28.66±2.50

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). ns: Not significant

C: normal control, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

Table 28 Water intake of rats for studies its efficiency on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat

Group	Water intake (g)					
	Day0 ns	Day7 ns	Day14 ns	Day21 ns	Day28 ns	Day35 ns
C	24.83±5.74	28.66±4.52	28.50±4.38	34.50±5.25	29.33±4.06	36.00±3.66
HC	22.16±5.77	24.00±3.88	23.16±4.57	24.16±7.77	25.16±2.80	28.50±3.87
HMDG	23.66±3.61	29.16±4.53	22.83±4.35	24.83±3.31	32.66±2.13	34.83±2.89
HRFF	17.83±5.45	20.83±2.08	24.66±5.98	26.83±2.73	29.66±4.86	28.66±4.88
HHNG	20.66±4.96	27.66±3.97	21.83±4.07	29.66±4.58	28.83±2.17	31.16±4.61
HHNF	19.33±4.03	23.66±4.16	23.50±3.93	24.83±4.35	26.16±2.92	29.50±4.32
Group	**Day42 ns**	**Day49 ns**	**Day56**	**Day63 ns**	**Day70 ns**	**Day77 ns**
C	31.16±4.51	25.66±6.05	33.50±2.64[a]	25.33±5.63	28.33±4.24	28.33±5.42
HC	25.16±5.63	33.00±3.08	23.16±2.07[b]	19.66±2.86	26.50±3.97	27.50±2.18
HMDG	26.16±4.99	33.00±4.65	32.66±4.25[ab]	23.33±3.77	23.16±3.12	30.83±8.10
HRFF	30.16±2.88	33.00±4.98	29.16±5.23[ab]	28.50±4.49	25.16±5.67	31.16±3.08
HHNG	30.50±3.68	26.33±3.26	26.33±3.88[ab]	19.00±3.51	22.00±2.33	27.83±4.70
HHNF	29.66±5.50	23.16±5.79	27.33±1.21[ab]	23.66±4.08	24.00±5.86	27.00±4.29

Table 28 (cont.)

Group	Water intake (g)				
	Day84	Day91 ns	Day98	Day105 ns	Day112 ns
C	25.66±4.71^{ab}	23.33±5.94	25.83±6.73^{ab}	35.00±2.72	32.50±2.84
HC	24.50±4.32^{ab}	22.33±7.09	26.50±6.18^{ab}	31.00±2.18	25.66±3.58
HMDG	26.33±4.13^{ab}	23.50±7.96	26.16±7.44^{ab}	28.83±5.83	28.83±5.65
HRFF	32.16±2.25^{a}	24.66±5.01	35.00±4.98^{a}	36.00±5.06	33.66±2.59
HHNG	27.33±5.61^{ab}	22.00±5.29	29.500±3.92^{ab}	24.16±5.23	28.00±3.78
HHNF	21.16±2.13^{b}	17.83±4.75	23.33±3.77^{b}	26.33±6.32	26.00±4.00

Note: Means with different letters within a column are significantly different ($p \leq 0.05$). ns: Not significant

C: normal control, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

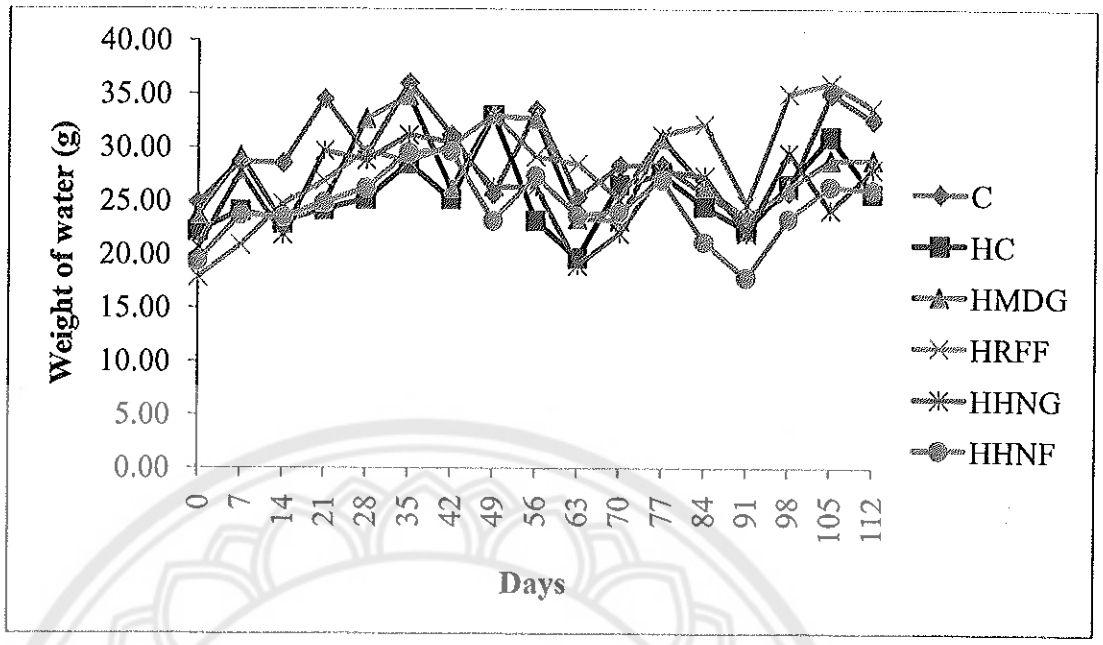


Figure 40 Water consumption of hypercholesterolemia rats

2.2 Effect of RBO on hypercholesterolemia rats

The efficiency of RBO on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat was studied. During the experiment, the rats were treated with the prepared diets as shown in Table 10 (Chapter III, p. 47) and measured for the clinics plasma. The clinics plasma consisted of lipid profile (total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG)) and glucose levels were measured every month. At the end of the 4 months-experimental period, the rats were measured for the liver function tests (alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)), kidney function tests (blood urea nitrogen (BUN) and creatinine (CRE)), oxidative stress levels (malondialdehyde of plasma and liver), antioxidant enzyme levels (superoxide dismutase (SOD)), inflammation (tumor necrosis factor-alpha (TNF-α)), antioxidant capacity (ORAC plasma) and histological changes of liver, kidney and aorta. The results of each measurement are discussed below.

2.2.1 Lipid profiles

Lipid profiles composed of total cholesterol, LDL-C, HDL-C and TG. The levels of total cholesterol, LDL-C, HDL-C and TG of hypercholesterolemia rats are presented in Tables 29, 30, 31 and 32, respectively.

1) Total cholesterol

The result of total cholesterol is shown in Table 29 and Figure 41. After the rats were induced on hypercholesterolemia by 2% cholesterol for 1 month, the levels of total cholesterol showed between 90–100 mg/dL, while the normal group had 87 mg/dL with no significantly different ($p > 0.05$). After 2 months of induction, the levels of total cholesterol increased in normal group. The levels were increased to 137–166 mg/dL in hypercholesterolemia rats and 97 mg/dL in normal rats. The normal rats showed significantly different ($p \leq 0.05$) from the hypercholesterolemia rats. The rats in the normal group had higher levels of total cholesterol due to age of the rats became older. Many studies confirmed that the total cholesterol levels increase with age [146, 147, 148]. After 2 months of inducement, the rats in HMDG, HRFF, HHNG and HHNF were treated with RBO samples. After treatment with RBO samples, the levels of total cholesterol were increased for all groups in particular, the group that fed with a high fat diet (HC). This could be due to the age and weight of the rats. The same observations were also found in the control group. After treated with RBO for 1 month, hypercholesterolemia groups shown no significantly different ($p > 0.05$) but after 2 months these were significantly different ($p \leq 0.05$), especially the rats that fed with HN-SC-CO_2 both gavage and mixed in diet, HHNG and HHNF group, respectively. At the end of the experiment, the total cholesterol levels found the rats that were treated with RBO had decreasing trend, especially in HHNG group decreased 33.42% (Figure 41) that fed with HN-SC-CO_2 by gavage and was significantly lower ($p \leq 0.05$) than the HC group. The cholesterol levels of HHNG group was not significantly different ($p > 0.05$) to the control group. This results were consistent with Tae-Youl et al. [149], who reported that consuming RBO as a dietary supplement can reduce cholesterol. This was due to the rats of HMDG and HHNG groups were treated with RBO in the form of supplements. In contrast, the rats in HMDG group were treated with RBO in the form of supplements but the levels of total cholesterol was not significantly different ($p > 0.05$) to the HC group. This could probably due to the diet contained HN oil

extracted by SC-CO_2 had unsaponifiable matters more than the diet contained molecularly distilled RBO (Table 23).

Table 29 Effect of RBO samples on total cholesterol levels in hypercholesterolemia rats

Groups	Total cholesterol levels (mg/dL)				Decrease (%)
	Inducement		Treatments		
	1st month ns	2nd month	1st month	2nd month	
C	87.00±6.71	96.83±6.85^{b}	105.16±9.45^{b}	121.80±5.91^{c}	-
HC	100.33±8.32	153.16±5.50^{a}	183.00±5.84^{a}	228.60±4.73^{a}	-
HMDG	100.83±4.30	165.50±3.36^{a}	161.33±3.93^{a}	184.20±4.93^{ab}	19.42
HRFF	97.83±5.53	157.50±4.76^{a}	150.66±3.53^{a}	185.80±4.46^{ab}	18.72
HHNG	90.33±3.24	136.66±2.67^{ab}	160.00±3.14^{a}	152.20±2.78^{bc}	33.42
HHNF	95.33±4.22	142.50±2.66^{a}	168.16±3.97^{a}	179.00±7.16^{b}	21.69

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

ns: Not significant. C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO.

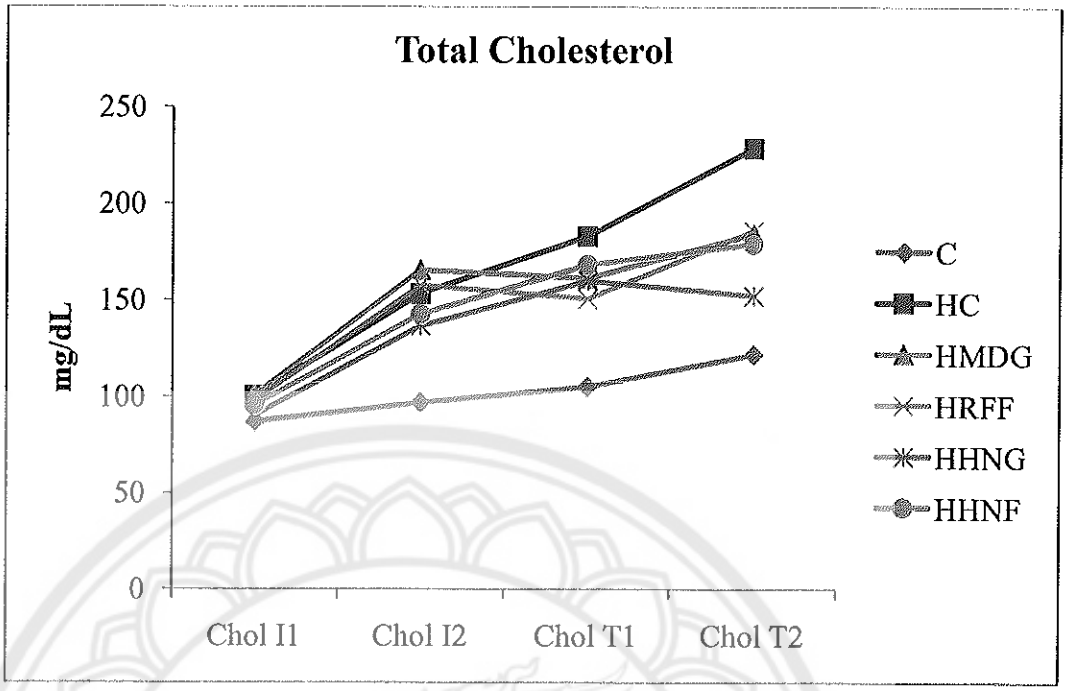


Figure 41 Total cholesterol levels of hypercholesterolemia rats

Note: I1: Inducement with 2% cholesterol of 1st month, I2: Inducement with 2% cholesterol of 2nd month, T1: Treatment with RBO of 1st month, T2: Treatment with RBO of 2nd month

2) LDL-C level

Table 30 and Figure 42 show the results of LDL-C levels. The LDL-C levels were decreased when treated the rats with RBO samples for 2 months for all groups, especially the rats of HHNG group ($p \leq 0.05$). HHNG had LDL-C of 87.60 mg/dLthat decreased 39.90% when compared with HC group (Table 30). When considering rats of HMDG, HRFF and HHNF were decreased 25.66 %, 9.99 % and 18.08%, respectively, which was consistent with several studies [150–152]. However, no significant differences ($p > 0.05$) were found in the LDL-C in those rats that fed with RBO.

Table 30 Effect of RBO samples on LDL-C levels in hypercholesterolemia rats

Groups	LDL-C levels (mg/dL)				Decrease (%)
	Inducement		Treatments		
	1st month	2nd month	1st month	2nd month	
C	17.65±4.55^{b}	19.40±2.85^{b}	24.18±5.21^{b}	29.08±3.81^{c}	-
HC	67.16±2.04^{a}	109.35±2.07^{a}	145.98±5.13^{a}	145.78±5.61^{a}	-
HMDG	68.48±5.86^{a}	114.55±3.83^{a}	135.68±4.97^{a}	108.36±4.92^{ab}	25.66
HRFF	64.46±6.01^{a}	110.46±4.71^{a}	137.58±6.98^{a}	131.21±3.42^{ab}	9.99
HHNG	55.51±3.06^{a}	113.43±4.69^{a}	134.06±6.75^{a}	87.60±4.42^{b}	39.90
HHNF	60.98±2.67^{a}	112.55±3.29^{a}	121.91±3.06^{a}	119.41±4.95^{ab}	18.08

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

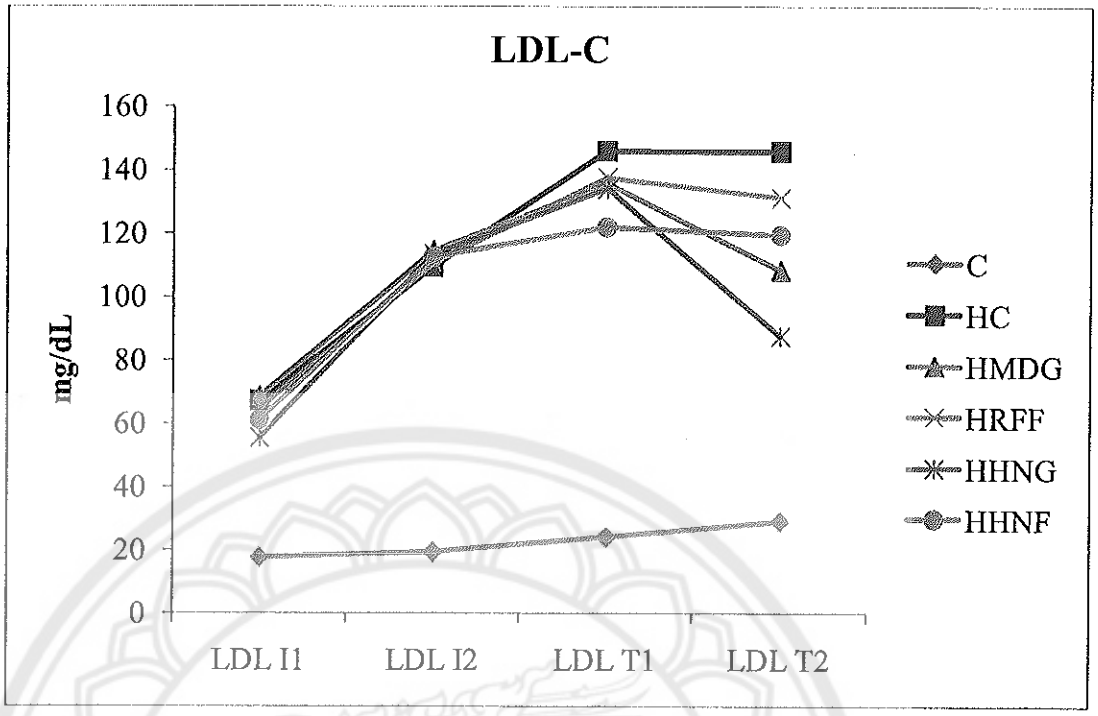


Figure 42 LDL-C levels of hypercholesterolemia rats

Note: I1: Inducement with 2% cholesterol of 1st month, I2: Inducement with 2% cholesterol of 2nd month, T1: Treatment with RBO of 1st month, T2: Treatment with RBO of 2nd month

3) HDL-C level

The Table 31 and Figure 43 show the results of HDL-C levels. The rats fed with different RBO samples in the first month were not significantly different ($p > 0.05$) in HDL-C level when compared with the HC group but were found an increasing trend. After treated with different RBO for 2 months, the HDL-C concentration were significantly different ($p \leq 0.05$) to the HC group. It is also noteworthy that the greatest effect on HDL-C level was the rats fed with the oil extracted by SC-CO_2 (HHNG and HHNF). Because of the increasing of levels from the first month fed with RBO about 25.64–31.50%. Especially HHNG group had HDL-C levels higher than the HC group of 31.50%. However, the rats fed with RBO did not show significant different ($p > 0.05$).

Table 31 Effect of RBO samples on HDL-C levels in hypercholesterolemia rats

Groups	HDL-C levels (mg/dL)				Increase (%)
	Inducement		Treatments		
	1st month	2nd month	1st month	2nd month	
C	72.66±4.96[a]	81.33±4.22[a]	83.83±9.96[a]	87.50±5.89[a]	-
HC	53.50±4.84[b]	53.66±7.22[b]	54.16±5.38[b]	54.60±6.16[c]	-
HMDG	51.83±5.90[b]	56.33±3.26[b]	63.00±9.39[b]	68.66±6.50[b]	25.57
HRFF	50.00±6.26[b]	58.00±9.18[b]	61.00±6.69[b]	64.00±6.74[b]	17.21
HHNG	53.16±3.23[b]	57.83±7.25[b]	63.50±9.39[b]	71.80±9.71[b]	31.50
HHNF	54.33±5.71[b]	54.00±6.84[b]	58.66±4.88[b]	68.60±7.55[b]	25.64

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

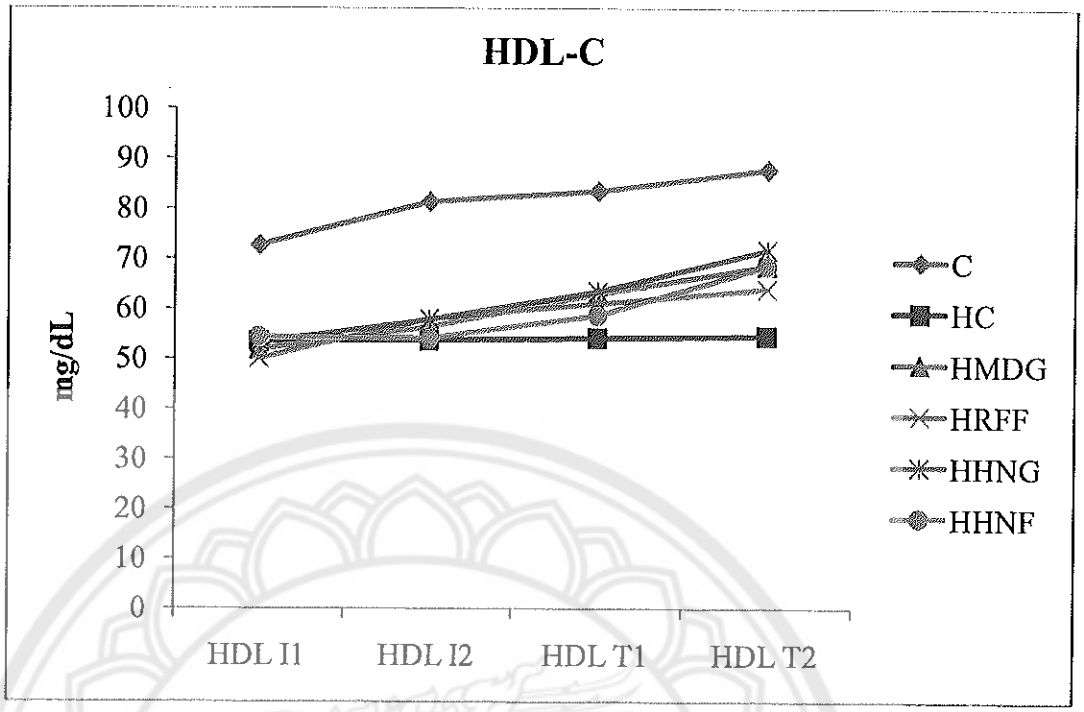


Figure 43 HDL-C levels of hypercholesterolemia rats

Note: I1: Inducement with 2% cholesterol of 1st month, I2: Inducement with 2% cholesterol of 2nd month, T1: Treatment with RBO of 1st month, T2: Treatment with RBO of 2nd month

4) Plasma TG level

The plasma TG concentration results are shown in Table 32 and Figure 44. After induction with a high fat diet containing 2% cholesterol, the triglyceride value showed in the range of 81–107 mg/dL. After the first month fed with RBO, the values of TG decreased (59–74 mg/dL) and increased in the second month. This result could be due to the age of the rats increased and the rats had been adaptation. As a result, it is noteworthy that the short-term consumption of RBO offers beneficial for triglycerides values.

Table 32 Effect of RBO samples on TG levels in hypercholesterolemia rats

Groups	TG levels (mg/dL)			
	Inducement		Treatments	
	1st month ns	2nd month ns	1st month	2nd month
C	97.00±2.57	83.83±3.42	82.33±5.43^{a}	97.83±3.70^{b}
HC	74.16±2.52	86.50±4.32	85.00±4.92^{a}	125.83±3.41^{a}
HMDG	83.83±1.28	107.16±2.92	59.00±8.83^{b}	107.66±2.94^{ab}
HRFF	88.16±1.15	81.16±9.60	74.33±5.20^{ab}	122.00±9.61^{ab}
HHNG	77.66±2.63	90.33±8.95	72.66±6.41^{ab}	102.16±6.35^{ab}
HHNF	70.83±1.78	95.50±8.49	73.66±3.39^{ab}	118.83±4.47^{ab}

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO.

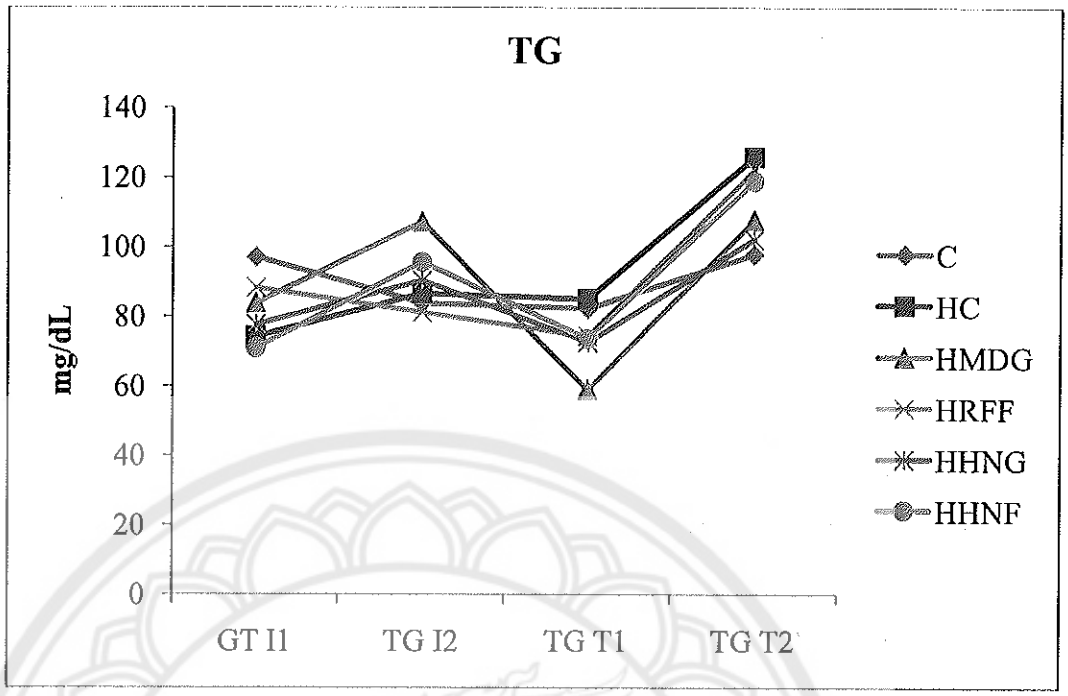


Figure 44 TG levels of hypercholesterolemia rats

Note: I1: Inducement with 2% cholesterol of 1st month, I2: Inducement with 2% cholesterol of 2nd month, T1: Treatment with RBO of 1st month, T2: Treatment with RBO of 2nd month

The overall efficiency of different RBO samples on the plasma lipid profile, in this study showed a decreasing trend of total cholesterol level for the rats fed with diets containing RBO and RBO by gavage, especially HHNG group. LDL levels were found a decreasing trend after 2 months of RBO treatment, while HDL levels shown increasing trend. These occurs could be associated with the unsaponifiable matter in RBO, especially γ-oryzanol. Several studies indicated that an effect of RBO to decrease TC by inhibiting lipid absorption from intestine in both human and animal [67, 153, 154]. Its γ-oryzanol, that increased exertion of bile acids and its combination with cholesterol so decreases the intestinal absorption of cholesterol and increases its excretion in the feces [83]. This is consistent with the result of the physicochemical properties of the diets that showed the high fat diet mixed with HN-SC-CO_2 had the highest content of antioxidant compounds (γ-oryzanol, TPC and α-tocopherol) (Table 19, p. 75). The oil of HN-SC-CO_2 showed the

highest level of phytosterol, too. It is well known that γ-oryzanol comprises a mixture of ferulic acid esters and phytosterols, both of these compounds have the property of lowering cholesterol levels. The body can metabolize γ-oryzanol to ferulic acid and phytosterol, where the structure of phytosterol is closely to cholesterol (Figure 45), so if consuming large amounts of phytosterol, it can help lower cholesterol. The molecular structure of phytosterol is very similar to that of cholesterol. The lipid metabolism process misunderstands due to both substances compete for membrane permeability. In addition, LDL is responsible for transporting cholesterol, misunderstood that phytosterol is a cholesterol. So transfer this substance passes into the bloodstream, resulting in lower cholesterol levels [155, 156, 157]. Other researchers have reported that ferulic acid is effective in reducing cholesterol and also anti-inflammation [158, 159, 160]. Moreover, there are reports on tocotrienol that could be shown to inhibit the HMG CoA reductase activity and subsequently reduced hepatic cholesterol synthesis, resulting in hypocholesterolemia [67, 161, 162, 163].

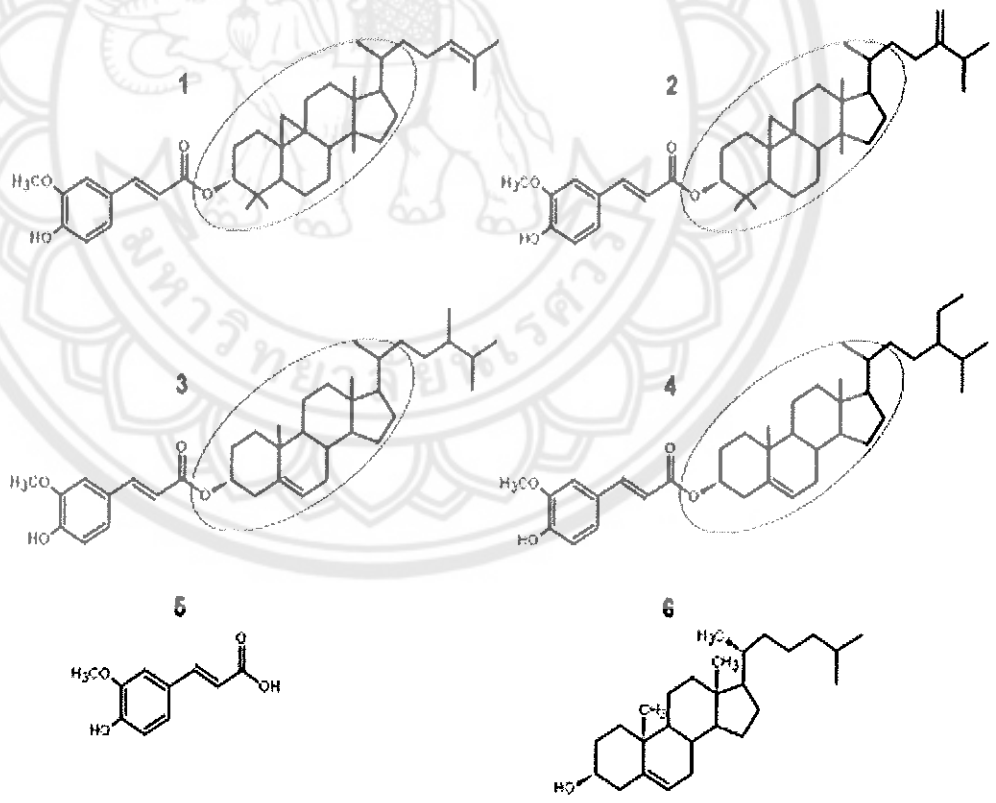


Figure 45 Molecular structures of the four main γ-oryzanol's components

Note: (1–4). Chemical structures are composed by ferulic acid and phytosterol (gray background). (1) cycloartenyl ferulate; (2) 24-methylenecycloartanyl ferulate; (3) campesteryl ferulate; and (4) sitosteryl ferulate. In the human body, γ-oryzanol can be metabolized to (5) ferulic acid, and steryl ferulates closely similar to (6) cholesterol [154]

The results of this present study may not be as effective as other researches because the oil was produced for more than a year before commencing the animal model. For this reason, the antioxidant accumulated in RBO could be reduced. This is consistent with the study of Zhou et al. [164] who indicated that the concentration of antioxidants become low following the storage at room temperature for long term.

5) Glucose plasma level

Table 33 and Figure 46 present the plasma glucose levels (GUL). All groups of the samples, including the control group, had elevated blood glucose levels throughout the trial period. These results agreed with the study of Ohara, Tabuchi and Onai [165] who indicated that feeding modified rice bran to STZ-induced diabetes rats for 2 months failed to improve blood glucose but showed reducing TC and TG. However, these results were contrast with many researchers who reported that RBO can improve blood glucose in diabetic rat [154, 166, 167].

Table 33 Effect of RBO samples on glucose plasma levels in hypercholesterolemia rats

Groups	Glucose plasma levels (mg/dL)			
	Inducement		Treatments	
	1st month ns	2nd month	1st month	2nd month ns
C	145.33±7.91	119.00±3.45^{a}	127.83±8.70^{ab}	237.50±6.36
HC	126.00±6.32	106.50±3.95^{ab}	132.66±3.24^{a}	261.50±5.43
HMDG	125.16±9.68	118.33±5.01^{a}	127.16±2.99^{ab}	288.00±3.40
HRFF	123.83±6.79	105.50±4.48^{ab}	111.50±8.01^{bc}	272.50±2.59

Table 33 (cont.)

Groups	Glucose plasma levels (mg/dL)			
	Inducement		Treatments	
	1st month ns	2nd month	1st month	2nd month ns
HHNG	120.16±4.25	101.00±6.63[b]	107.16±7.06[c]	274.50±5.31
HHNF	125.66±8.40	109.16±6.58[ab]	124.66±8.48[abc]	288.16±6.19

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

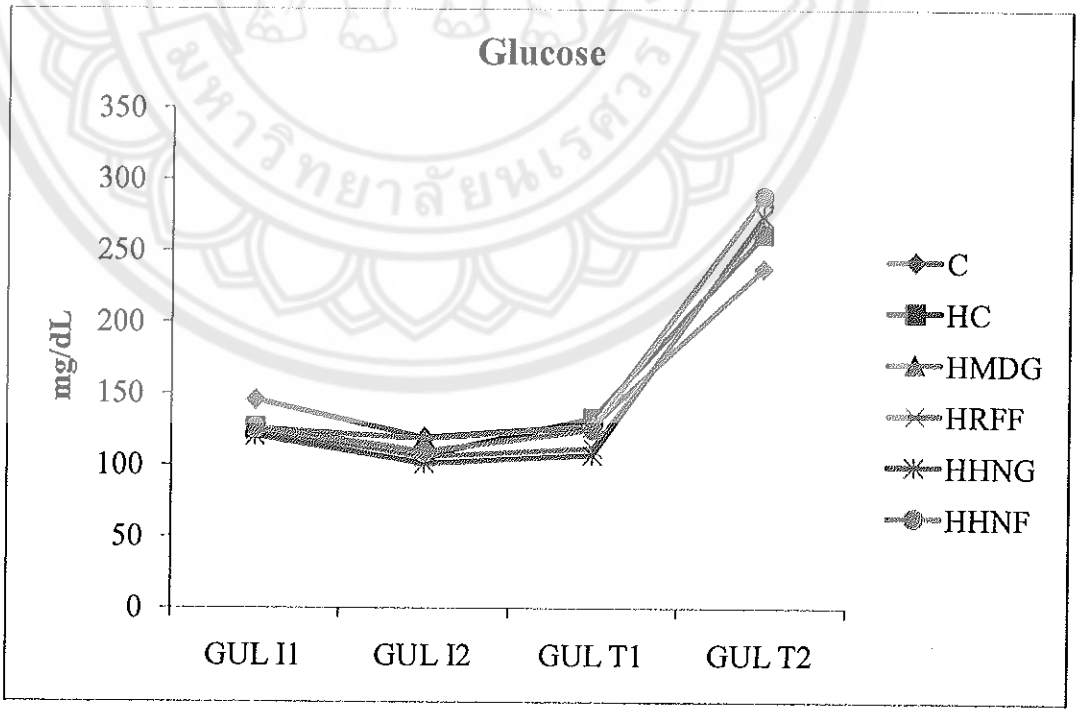


Figure 46 Glucose plasma levels in hypercholesterolemia rats

Note: I1: Inducement with 2% cholesterol of 1st month, I2: Inducement with 2% cholesterol of 2nd month, T1: Treatment with RBO of 1st month, T2: Treatment with RBO of 2nd month

2.2.2 Liver and kidney function tests

The values for the activities of alanine transaminase (ALT), aspartase aminotransferase (AST) and alkaline phosphatase (ALP) are biochemical parameters of damage in liver function while blood urea nitrogen (BUN) and creatinine (CRE) are parameters of damage in kidney function. The results are presented in Table 34. The hypercholesterolemia rats had higher liver functional test than the control group around 2–3 times. The hypercholesterolemia rats were fed with the diet containing RBO and RBO samples (Gavage) had ALT levels lower than the hypercholesterolemia rats fed with high fat diet without RBO (HC group). It was shown that the levels of ALT decreased 16.05–41.79% but no significantly different ($p > 0.05$) was found except HHNG group that showed significantly higher ALT ($p \leq 0.05$) than the HC group. The AST values were slightly reduced from the HC group but had no significantly different ($p > 0.05$). Similar trends were found for the ALP.

For the kidney function, the BUN levels in all RBO treated groups were significantly lower ($p \leq 0.05$) than the HC group. The CRE levels were not significantly different ($p > 0.05$) in all groups.

It could be concluded that RBO from Hom-nin rice extracted by SC-CO_2 method and RBO sample from CEO Agrifood Co., Ltd. could prevent damage of liver and kidney.

Table 34 Effect of RBO samples on liver and kidney function tests in hypercholesterolemia rats

Groups	Liver			Kidney	
	ALT (U/L)	AST (U/L)	ALP (U/L)	BUN (mg/dL)	CRE ^{ns} (mg/dL)
C	25.40±5.46^{c}	59.40±5.90^{b}	52.5±7.42^{b}	15.73±1.90^{b}	0.40±0.00
HC	71.00±4.32^{a}	100.60±5.04^{a}	88.33±4.84^{a}	20.88±4.17^{a}	0.41±0.04
HMDG	58.60±2.26^{ab}	90.60±4.45^{a}	88.00±6.22^{a}	17.30±2.52^{b}	0.410.04
HRFF	59.60±3.27^{ab}	91.20±5.67^{a}	90.33±3.57^{a}	16.80±1.25^{b}	0.38±0.07
HHNG	41.20±2.81^{bc}	82.20±2.64^{ab}	88.33±2.82^{a}	16.01±1.25^{b}	0.40±0.00
HHNF	59.58±6.29^{ab}	91.80±3.18^{a}	93.50±1.11^{a}	18.30±1.24^{b}	0.35±0.08

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

ns: Not significant. C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

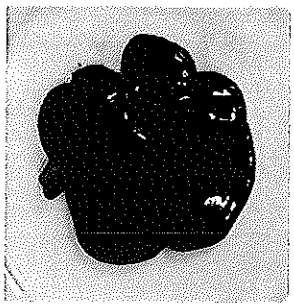
The organ weight of rats are shown in Table 35. It was found that the liver weight of hypercholesterolemia rats was significantly higher ($p \leq 0.05$) than that of the control group. The apparent size of the liver is shown in Figure 47, both have different colors, the yellow color was found in the liver of hypercholesterolemia rats due to the fat accumulation in the liver cells.

Table 35 Organ weight of rats for study of its efficiency on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rats

Groups	Kidney (L) (g)	Kidney (R) ^{ns} (g)	Liver (g)	Relative weight
C	1.49±0.06^{b}	1.48±0.11	14.16±1.15^{b}	0.024±0.00^{c}
HC	1.63±0.09^{a}	1.60±0.10	21.18±2.35^{a}	0.035±0.00^{ab}
HMDG	1.48±0.15^{b}	1.49±0.11	21.57±2.34^{a}	0.037±0.00^{a}
HRFF	1.48±0.07^{b}	1.59±0.09	19.87±1.15^{a}	0.034±0.00^{c}
HHNG	1.53±0.07^{ab}	1..56±0.11	20.77±2.17^{a}	0.035±0.00^{ab}
HHNF	1.50±0.15^{ab}	1.50±0.13	21.31±1.68^{a}	0.036±0.00^{ab}

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

ns: Not significant. C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO



Characteristic of rat liver (control)

Characteristic of rat liver (hypercholesterolemia)

Figure 47 The characteristic rat liver

2.2.3 Oxidative stress

Plasma malondialdehyde (plasma MDA) and liver malondialdehyde (liver MDA) were used to investigate the oxidative stress levels. MDA is some kind of toxin that can destroy beta cell in the pancreas and cause the tissue damage. Therefore, the high levels of MDA indicates the high oxidative stress levels. The results of the plasma MDA and liver MDA are shown in Table 36. The oxidative stress status in both plasma and liver of all RBO treated groups were significantly lower ($p \leq 0.05$) than the HC group about 40–55 % and 13–24 %, respectively. Especially, the MDA levels of HMDG and HHNG groups (gavage group) showed the levels close to that of the control group (normal rats). This result shows that RBO is effective in reducing oxidative stress.

Table 36 Effect of RBO samples on oxidative stress in hypercholesterolemia rats

Groups	Plasma MDA (μM)	Decrease of plasma MDA (%)	Liver MDA (μM)	Decrease of liver MDA (%)
C	14.17±2.34^{b}	-	33.86±2.50^{b}	-
HC	36.66±2.80^{a}	-	44.68±4.56^{a}	-
HMDG	17.46±3.93^{b}	52.37	35.34±3.74^{b}	20.90
HRFF	21.39±4.31^{b}	41.65	38.60±4.41^{b}	13.60
HHNG	16.40±4.70^{b}	55.26	33.76±2.45^{b}	24.44
HHNF	21.95±5.18^{b}	40.12	38.05±4.25^{b}	14.83

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

ns: Not significant. C: normal control, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

2.2.4 Enzymatic antioxidant

Superoxide dismutase (SOD) is the primary defense mechanism against free radical of body which converted superoxide radical to hydrogen peroxide and ground state oxygen. The results of SOD are shown in Table 37. It was found that there were no significant different ($p > 0.05$) in SOD levels for all groups. The SOD levels were 38.92–45.72% inhibition rate. However, it was found that, the rats treated with RBO showed an increase in SOD level. It can be concluded that RBO is effective in increasing the enzymatic antioxidant.

Table 37 Effect of RBOs samples on enzymatic antioxidant in hypercholesterolemia rats

Groups	SOD ^{ns} (inhibition rate %)	Increase of SOD (%)
C	45.28±5.66	-
HC	38.92±5.32	-
HMDG	44.09±2.88	13.28
HRFF	44.18±3.08	13.51
HHNG	45.72±3.83	17.47
HHNF	42.82±3.79	10.02

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

ns: Not significant. C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

2.2.5 Inflammation

The tumor necrosis factor-alpha (TNF-α) is a bioactive cytokine that is an important indicator of the inflammation. If the levels of TNF-α is high, it reflects the high inflammation. The results of TNF-α are shown in Table 38. The TNF-α of HMDG and HRFF groups were significantly lower ($p \leq 0.05$) than the HC group. The groups that treated with RBO showed a decreased level about 39.08–55.49%. The RBO could have a prevention effect of inflammation. This results are in accordance with the other studies [168, 169] where the researchers presented that the unsaponifiable matter of RBO reduced inflammatory effects [170, 171, 172].

Table 38 Effect of RBO samples on inflammation in hypercholesterolemia rats

Groups	TNF-α (pg/mL)	Decrease of TNF-α (%)
C	0.54 ± 0.47^{c}	-
HC	12.74 ± 3.62^{a}	-
HMDG	5.67 ± 1.45^{bc}	55.49
HRFF	6.07 ± 2.30^{bc}	52.35
HHNG	7.40 ± 2.15^{ab}	41.91
HHNF	7.76 ± 2.29^{ab}	39.08

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).

ns: Not significant. C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

2.2.6 Antioxidant capacity

Oxygen radical absorbance capacity (ORAC) was investigated for antioxidant capacity. ORAC assay is available to estimate the ability of body to combat damage caused by free radical. ORAC plasma was used for studying antioxidant capacity because it was contained the key targets of oxidative damage. The results of ORAC are shown in Table 39. All RBO treated groups had significantly higher ORAC ($p \leq 0.05$) than the HC group. The ORAC levels in RBO treated groups were 399.40–428.19 μM TE/100 mL and showed increase levels 21.68–30.45% when compared with the HC group. The HMDG, HHNG and HHNF had the ORAC levels not significantly different ($p > 0.05$) to the control group (431.22 μM TE/100 mL). Positive correlation between unsaponifiable matter in RBO and antioxidant enzyme (SOD) was also supported this finding [173, 174].

Table 39 Effect of RBO samples on antioxidant capacity in hypercholesterolemia rats

Groups	Plasma ORAC (μM TE/100 mL)	Increase of plasma ORAC (%)
C	431.22±20.86[a]	-
HC	328.22±18.86[c]	-
HMDG	417.09±22.31[ab]	27.07
HRFF	399.40±18.53[b]	21.68
HHNG	428.19±22.22[a]	30.45
HHNF	421.54±26.80[ab]	28.43

Note: Means with different letters within a column are significantly different ($p \leq 0.05$).
ns: Not significant. C: Control group, HC: Hypercholesterolemia fed with high fat diet, HMDG: Hypercholesterolemia fed with high fat diet and 0.1 mL of molecularly distilled RBO (gavage), HRFF: Hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO, HHNG: Hypercholesterolemia fed with high fat diet and 0.1 mL of selected RBO (gavage) and HHNF: Hypercholesterolemia fed with high fat diet mixed with 4% of selected RBO

2.2.7 Histological changes

The study of histological changes was conducted by studying the tissues of the liver, kidneys and aorta.

1) Effect of RBO on histopathology of liver

The histopathology of liver sections in control and hypercholesterolemia rats are presented in Figures 48–53. The hepatocyte of all hypercholesterolemia groups showed abnormal of hepatic cord (H) and there were fat cells (F) in cytoplasm of hepatocyte. The sinusoid (S) almost disappeared in all hypercholesterolemia groups compared with the control group (C). There were inflammatory cells in central vein (CV) in all hypercholesterolemia groups except HRFF

group. These results showed that the hypercholesterolemia condition induced rats liver inflammation. However, the groups treated with RBO alleviate it.

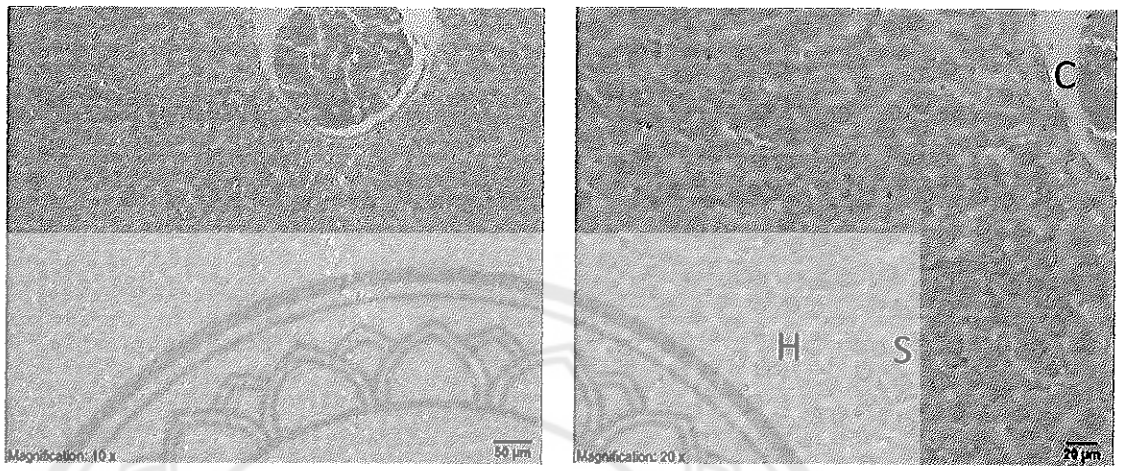


Figure 48 The liver of normal control rats

Note: CV: Central vein, H: Hepatocyte, S: Sinusoid

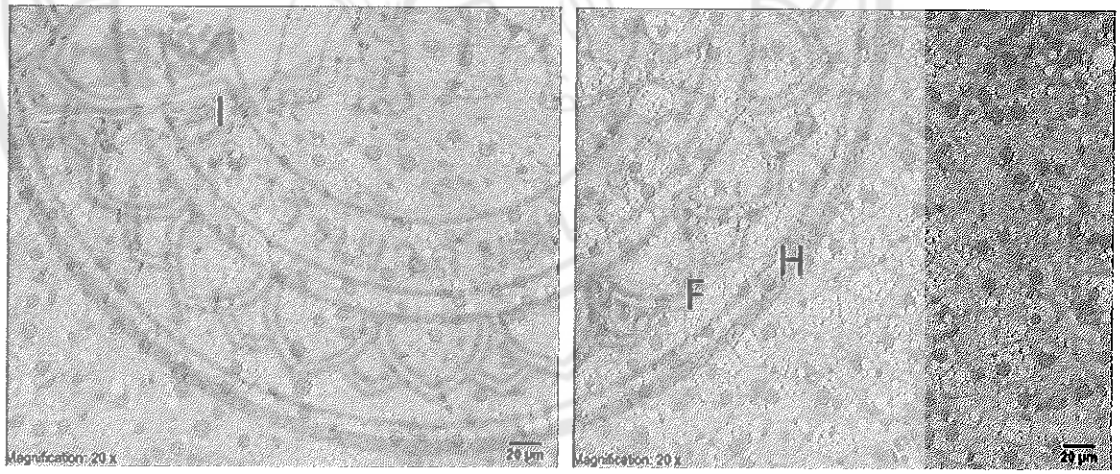


Figure 49 The liver of hypercholesterolemia rats fed with high fat diet (HC)

Note: I: Inflammatory cells, F: Fat cells, H: Hepatocyte

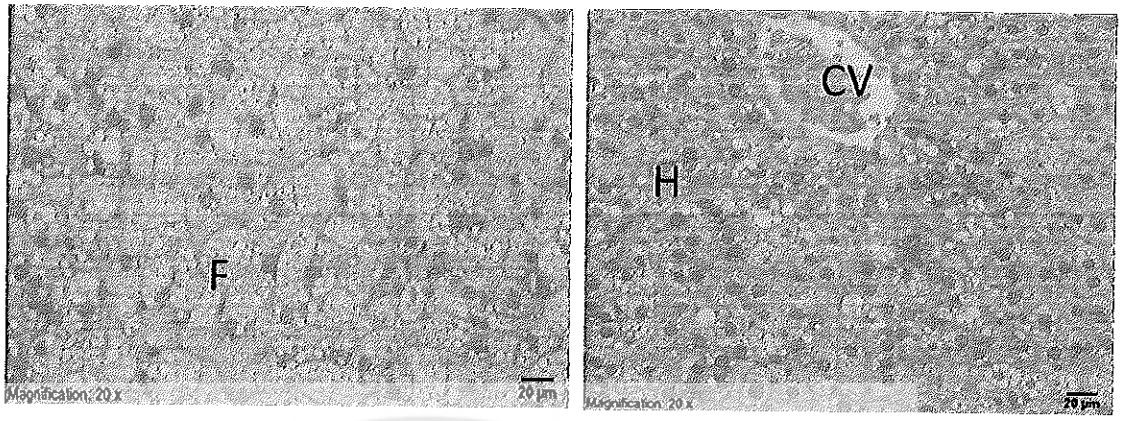


Figure 50 The liver of hypercholesterolemia rats fed with high fat diet and 0.1 mL of molecularly distilled RBO (HMDG, gavage)

Note: CV: Central vein, H: Hepatic cord, F: Fat cells

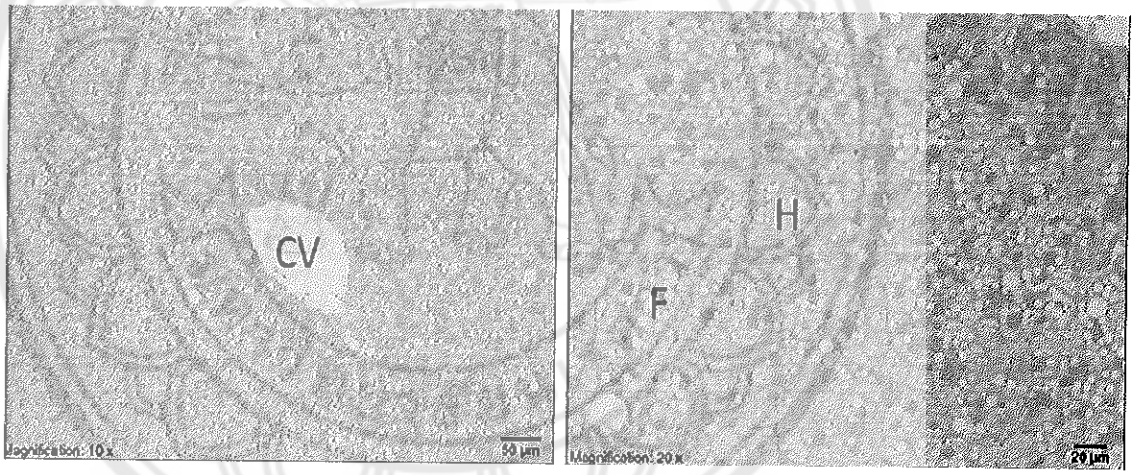


Figure 51 The liver of hypercholesterolemia fed with high fat diet mixed with 4% of refined RBO (HRFF)

Note: CV: Central vein, H: Hepatic cord, F: Fat cells

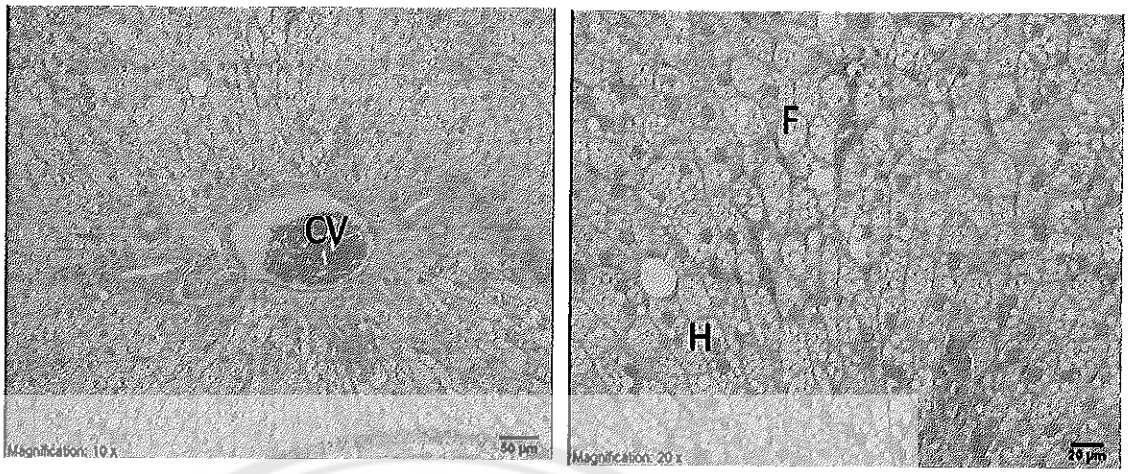


Figure 52 The liver of hypercholesterolemia rats fed with high fat diet and 0.1 mL of selected RBO (gavage, HHNG)

Note: CV: Central vein, H: Hepatic cord, F: Fat cells

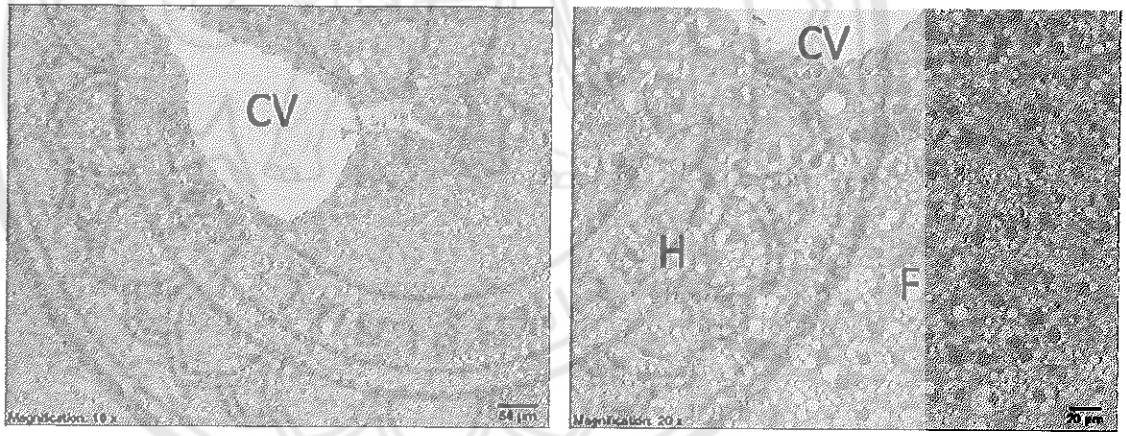


Figure 53 The liver of hypercholesterolemia rats fed with high fat diet mixed with 4% of selected RBO (HHNF)

Note: CV: Central vein, H: Hepatic cord, F: Fat cells

2) Effect of RBO on histopathology of kidney

The histopathology of kidney sections in control and hypercholesterolemia rats were presented in Figure 54. Fat cells (f) were found in kidneys of every hypercholesterolemia groups. Glomerulus (G) was bigger in all hypercholesterolemia groups. These results showed that hypercholesterolemia condition induced the abnormal of kidney. The RBO treatment alleviate these symptoms due to the glomerulus was decreased in size and the bowman's capsule was bigger than the HC group.

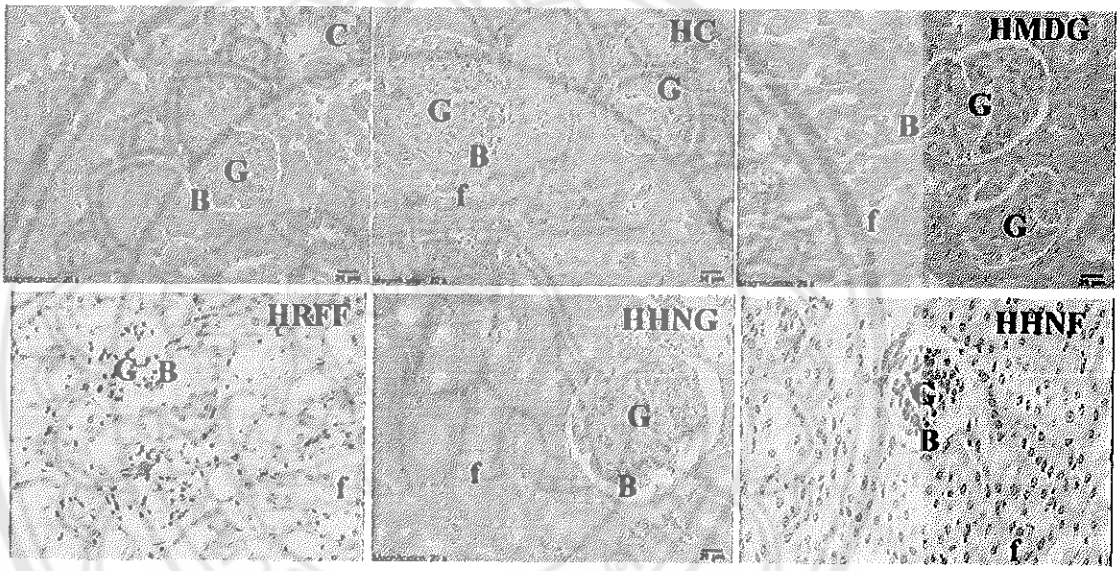


Figure 54 The kidney of control and hypercholesterolemia rats

Note: G: Glomerulus, B: Bowmann's capsule, f: Fat cell

3) Effect of RBO on histopathology of aorta

The histopathology of aorta sections in control and hypercholesterolemia rats were presented in Figure 55. There were adipocytes accumulations inside of lumen in HC, HRFF and HHNF. This result demonstrated that treated RBO in HMDG and HHNG groups decreased adipocytes in aorta of hypercholesterolemia rats.

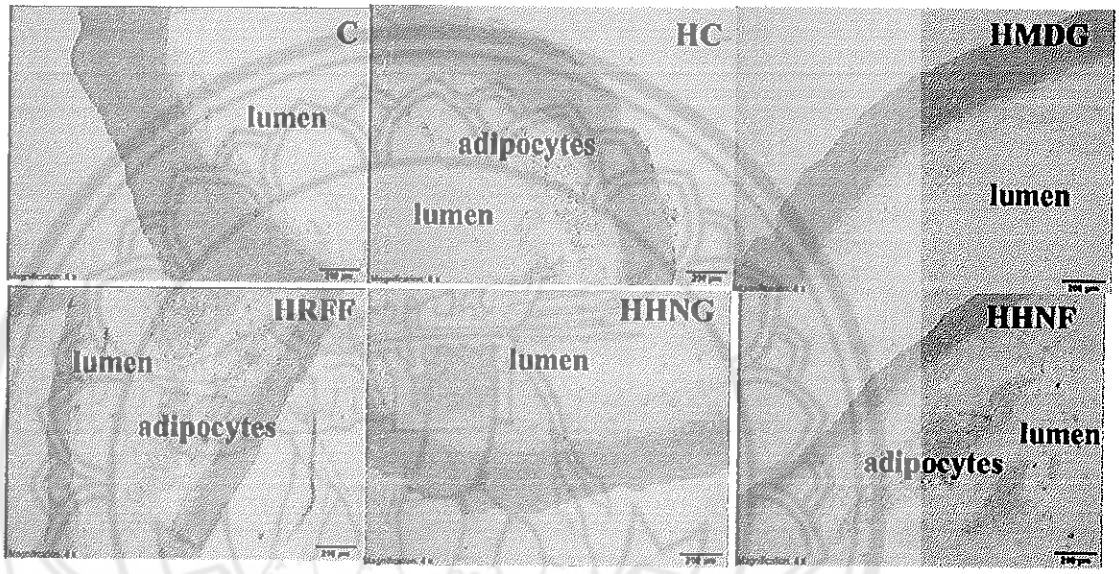


Figure 55 The aorta of control and hypercholesterolemia rats

The results of histological change of liver, kidney and aorta tissues, reviewed RBO is effectively improved inflammation of tissues and reduced fat in aorta.

CHARPTER V

CONCLUSION AND RECOMENEDATIONS

Conclusion

This study investigated the chemical properties and shelf life of RBO from three varieties of rice (KDML 105, RJM and HN) using three extraction methods. Physicochemical and antioxidant properties of RBO produced from colored rice using different extraction methods were determined. The best RBO sample was selected and its efficiency on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat was studied and compared with the RBO from CEO Agifood Co., Ltd.

This study reveals that the RBO from bran of pigmented rice samples presented higher amount of antioxidant and antioxidant activity than those of white rice sample. In terms of extraction methods, SC-CO_2 was the best extraction method as evidenced by their physicochemical and antioxidant properties. With regard to the chemical properties and antioxidant activity, it was found that RBO from HN rice extracted by SC-CO_2 extraction method (HN-SC-CO_2) was the best oil agent due to having the highest values of TPC, γ-oryzanol, total Toc and T3 and phytosterol as well as antioxidant activity in terms of DPPH CI50 and ORAC. Therefore, it was selected for the animal studies.

For the shelf life study of the RBO samples kept at room temperature for six months, the results showed that the levels of AV, FFA content and PV increased during the storage period but all the RBO samples were not rancid. The shelf life of the RBO samples were 3-5 months.

In terms of animal studies, the results showed that both RBO from HN-SC-CO_2 and CEO Agrifood Co., Ltd. had beneficial health effects in hypercholesterolemia rat since it showed a decrease trend in total cholesterol, LDL-C and triglyceride when compared with the untreated with RBO group (HC group), especially the HHNG group that showed a positive result. The oxidative stress status in both plasma and liver of all RBO treated groups were significantly decreased when compared with the HC group,

and the levels of gavage groups (supplement form) were closed to those of the control group. In addition, the results of inflammation (TNF-α), enzyme antioxidant (SOD) and antioxidant capacity (plasma ORAC value) were found improving when compared with the HC group.

It can be concluded that the pigmented rice (HN) extracted by SC-CO_2 method had high antioxidant and activity which is beneficial to health and should be promoted for further uses in food, pharmaceutical and nutraceutical applications.

Recommendations

In this study, the researcher has three main issues for recommendation.

1. For improvement

1.1 The refinery process of crude RBO by using the alkaline solution in the neutralization step in order to reduce the AV and FFA content to conform to the standard.

1.2 The RBO sample used for the study of its efficiency on cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat should not be kept for too long. The researcher recommended that it should not be kept for longer than 3 months after production. This is due to the longer the shelf life the more increasing in the AV, FFA content and PV and decreasing of the antioxidant agents.

2. For the next study

The suitable dose of RBO sample that reduces the cholesterol, triglyceride, inflammation and oxidative stress in hypercholesterolemia rat.

3. For utilization

As a result, pigmented rice has a nutritional value and antioxidant activity than non-pigmented rice. It should be encouraged for the SME manufacturing.

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APPENDIXES

ใบรับรองโครงการวิจัยในสัตว์ทดลอง

คณะกรรมการกำกับดูแลการเลี้ยงและการใช้สัตว์ทดลอง

ศูนย์สัตว์ทดลองแห่งชาติ มหาวิทยาลัยมหิดล

รหัสโครงการ #.... NLAC-MU Protocol No. RA 2016-16.....

ชื่อข้อเสนอการวิจัย .....การศึกษาประสิทธิภาพของน้ำมันรำข้าวในการลดคอเลสเตอรอล ไตรกลีเซอไรด์ **ความดันเลือด** การอักเสบและออกซิเดทีฟสเตรสในหนูที่มีภาวะคอเลสเตอรอลและความดันสูง.....

ชื่อ-สกุล ผู้เสนอข้อเสนอการวิจัย .....ว่าที่ร้อยตรีหญิงขวัญจิต ชัยมงคลนุกูล.....

หน่วยงานที่สังกัด (คณะ/กอง) ..... ศูนย์สัตว์ทดลองแห่งชาติ.....

(มหาวิทยาลัย/กรม) .....มหาวิทยาลัยมหิดล.....

(กระทรวง) .....กระทรวงศึกษาธิการ.....

ข้อเสนอการวิจัยนี้ได้ผ่านการพิจารณาจากคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์ทดลอง**แล้ว จึงเห็นสมควร**ให้ดำเนินการเลี้ยงและใช้สัตว์ตามข้อเสนอการวิจัยนี้ได้

ลงนาม .....

(นางระพี อินปั่นแก้ว)

ตำแหน่ง ประธานคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์

วัน/เดือน/ปี .....3 ต. ค. 57.....

ลงนาม .....

(รองศาสตราจารย์ นายแพทย์ประ**ดิษฐ์** สมประกิจ)

ตำแหน่ง รองอธิการบดีฝ่ายการคลังและแผนงาน

รักษาการแทนผู้อำนวยการศูนย์สัตว์ทดลองแห่งชาติ

วัน/เดือน/ปี .....

11 ต.ค. 2559

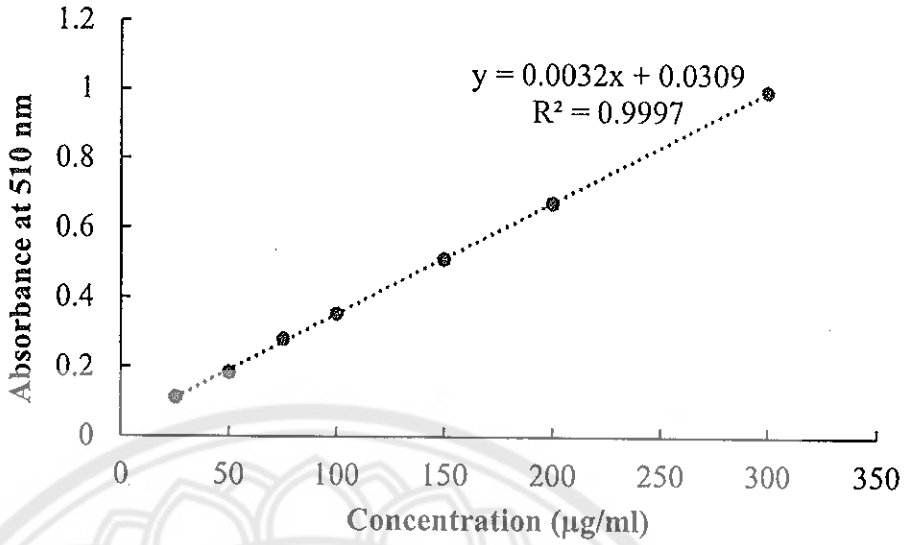

Figure 56 Calibration curve for standard gallic acid

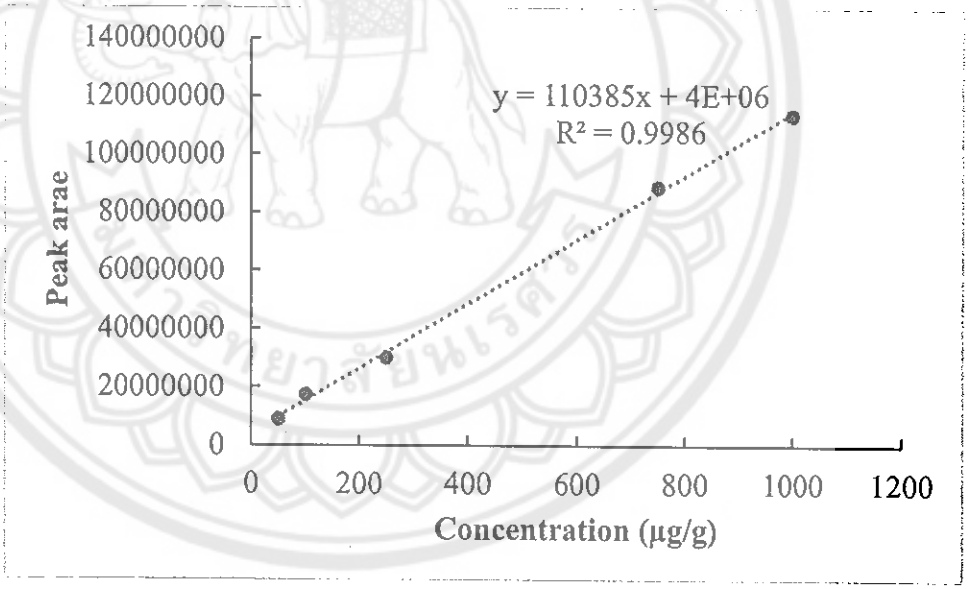

Figure 57 Calibration curve for standard of γ-oryzanol

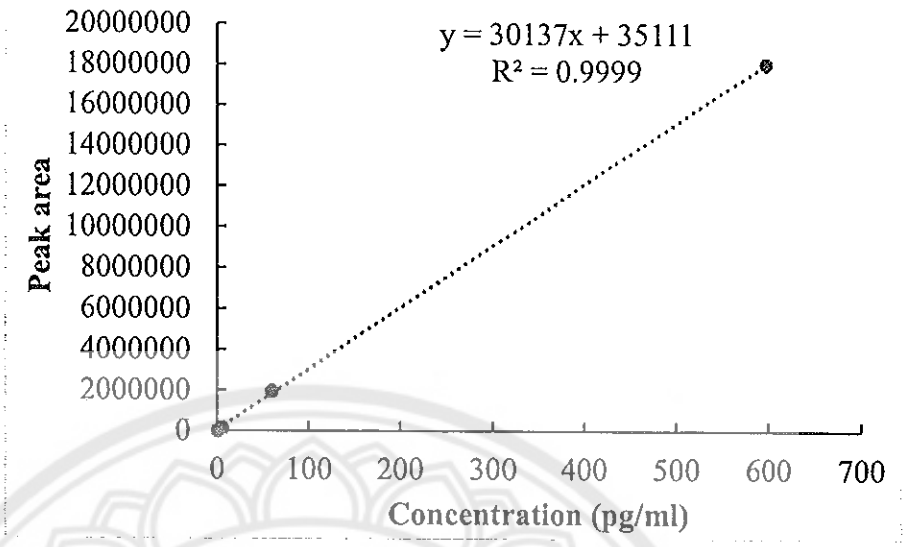

Figure 58 Calibration curve of CAFA standard

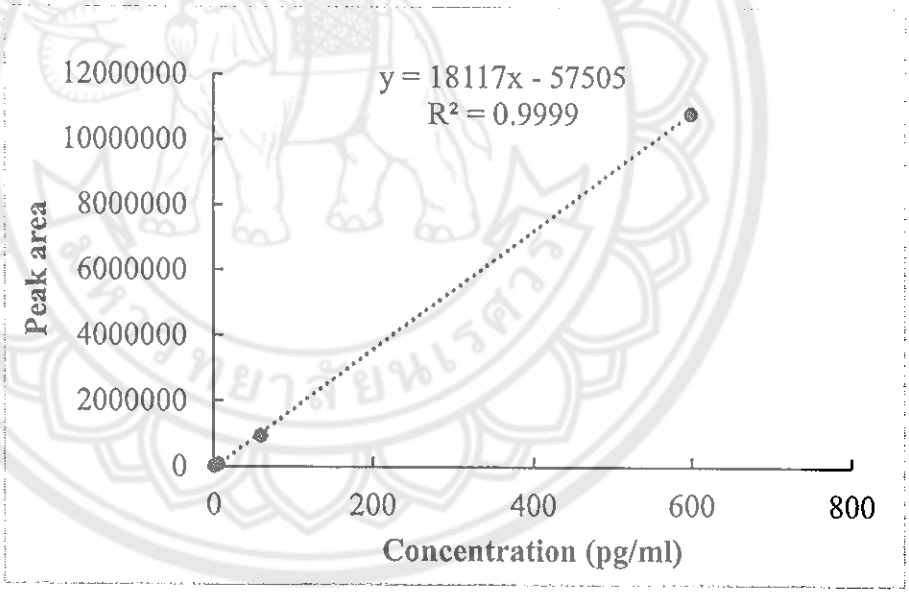

Figure 59 Calibration curve of 24MCAFA standard

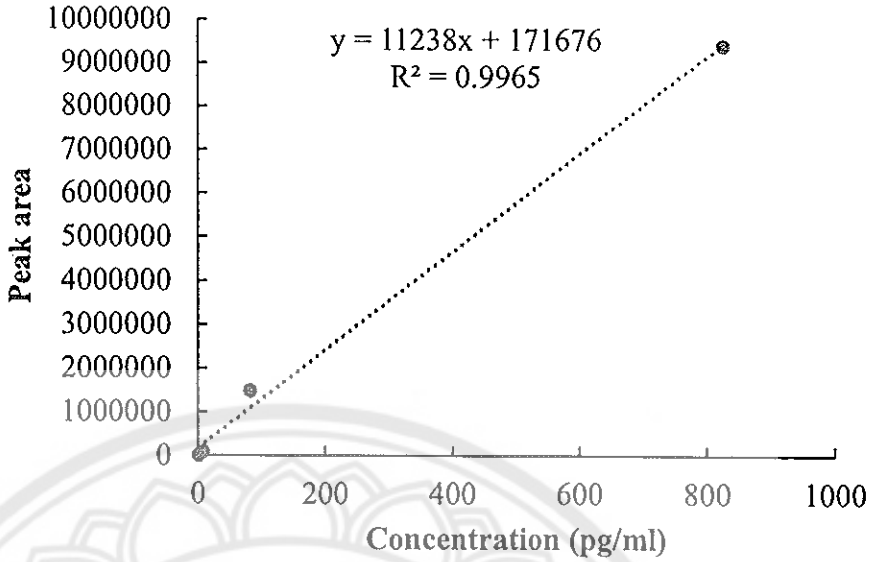

Figure 60 Calibration curve of CampFA standard

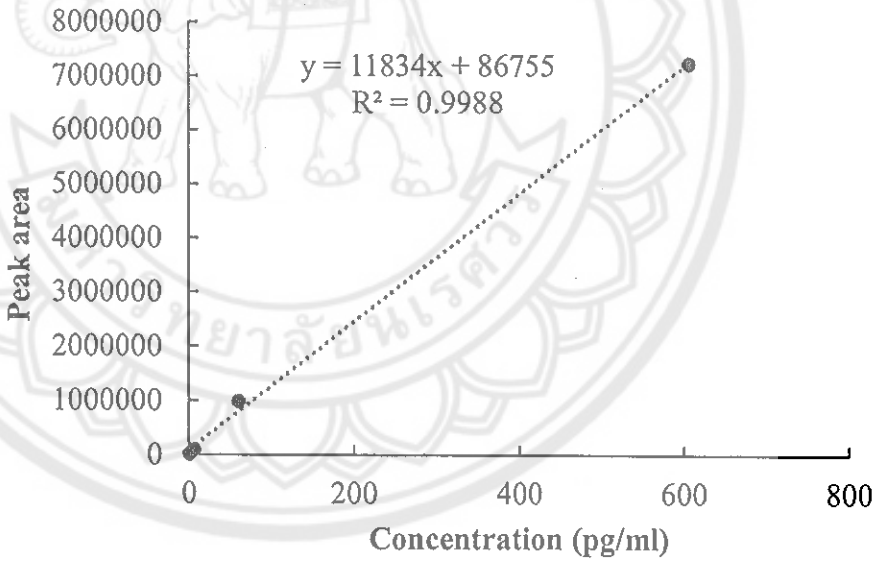

Figure 61 Calibration curve of SitoFA standard

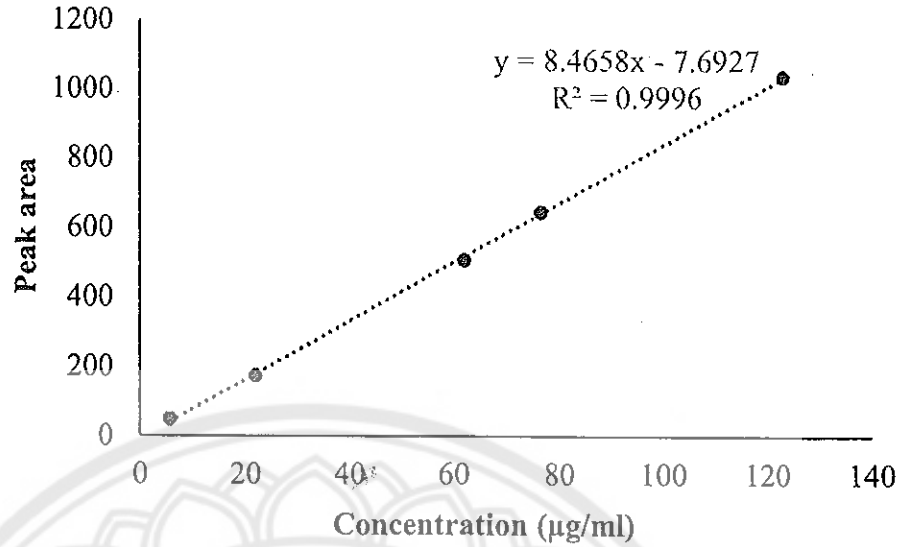

Figure 62 Calibration curve for standard of α-tocopherol

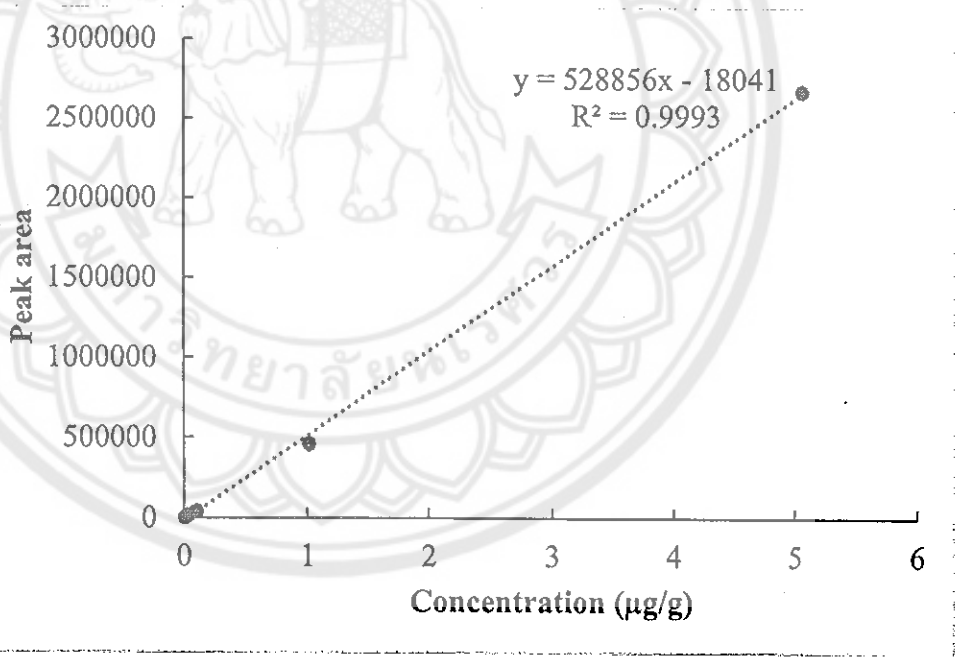

Figure 63 Calibration curve for standard of α-tocopherol measured by HPLC MS/MS

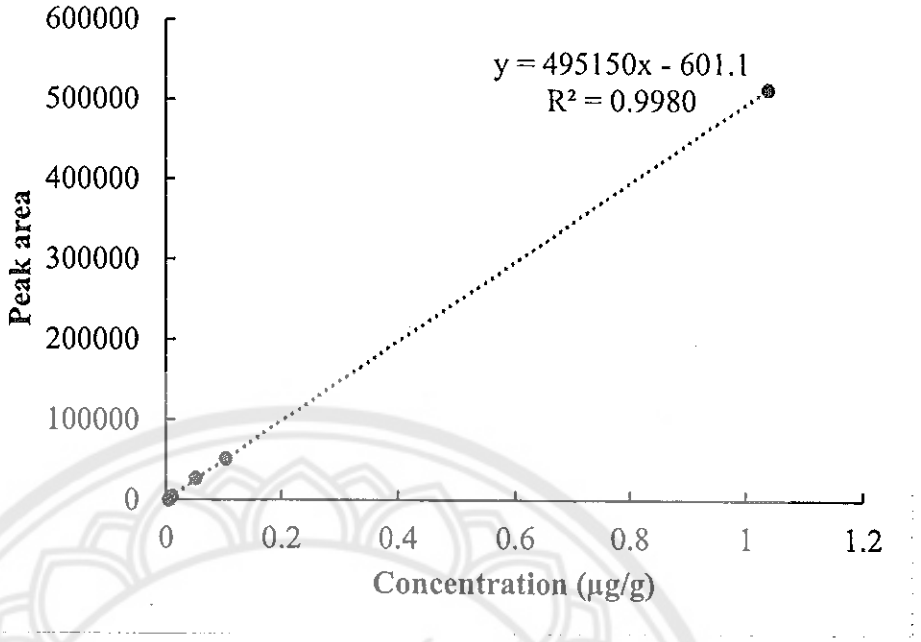

Figure 64 Calibration curve for standard of β-tocopherol measured by HPLC MS/MS

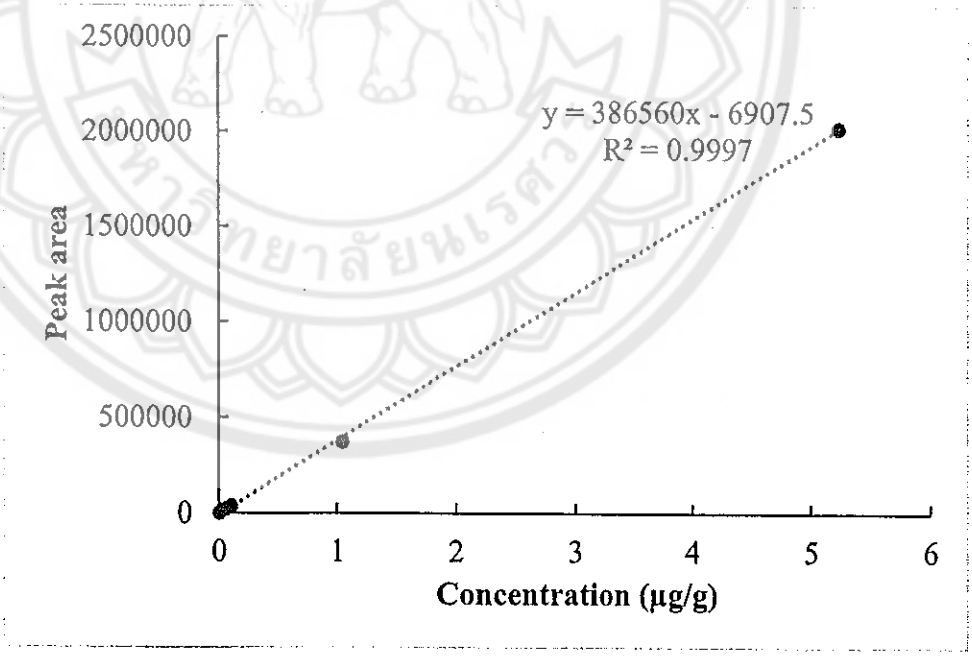

Figure 65 Calibration curve for standard of γ-tocopherol measured by HPLC MS/MS

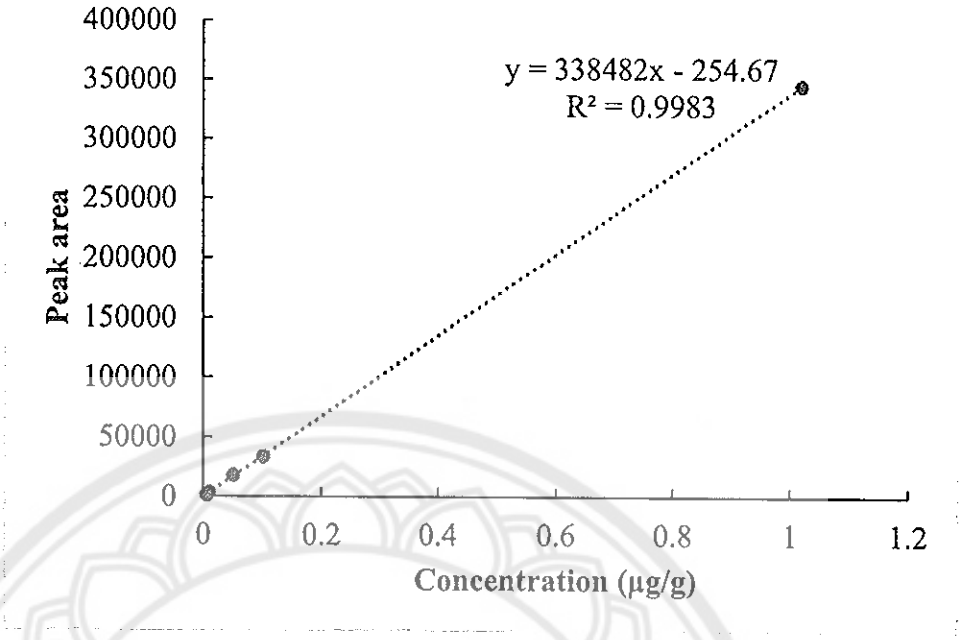

Figure 66 Calibration curve for standard of δ-tocopherol measured by HPLC MS/MS

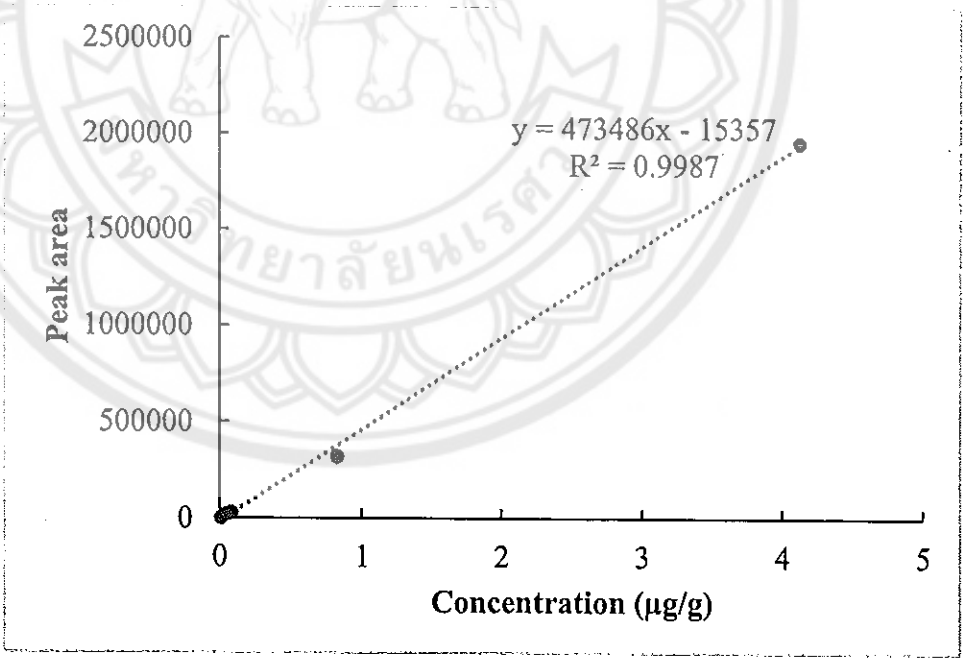

Figure 67 Calibration curve for standard of α-tocotrienol measured by HPLC MS/MS

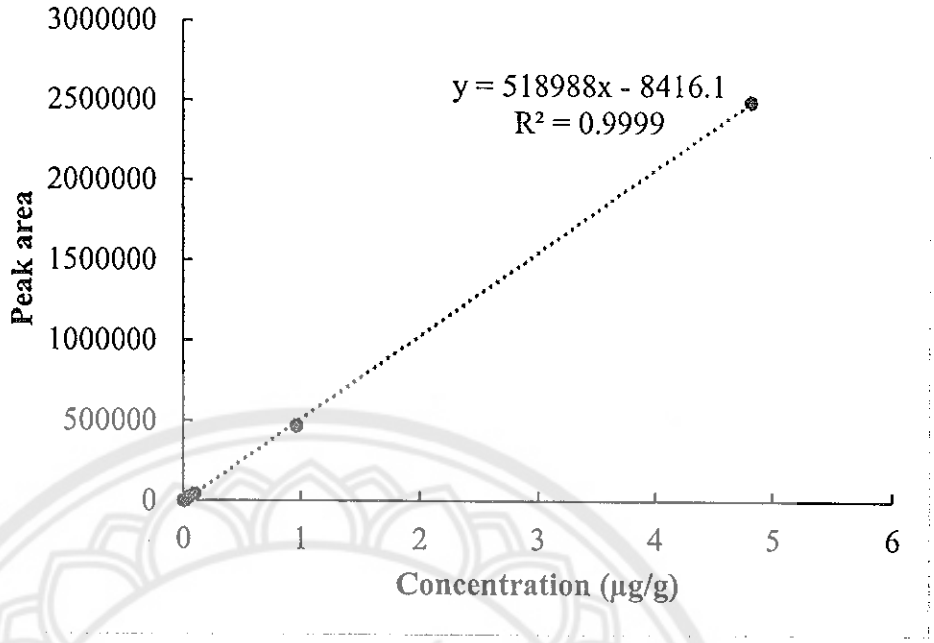

Figure 68 Calibration curve for standard of β-tocotrienol measured by HPLC MS/MS

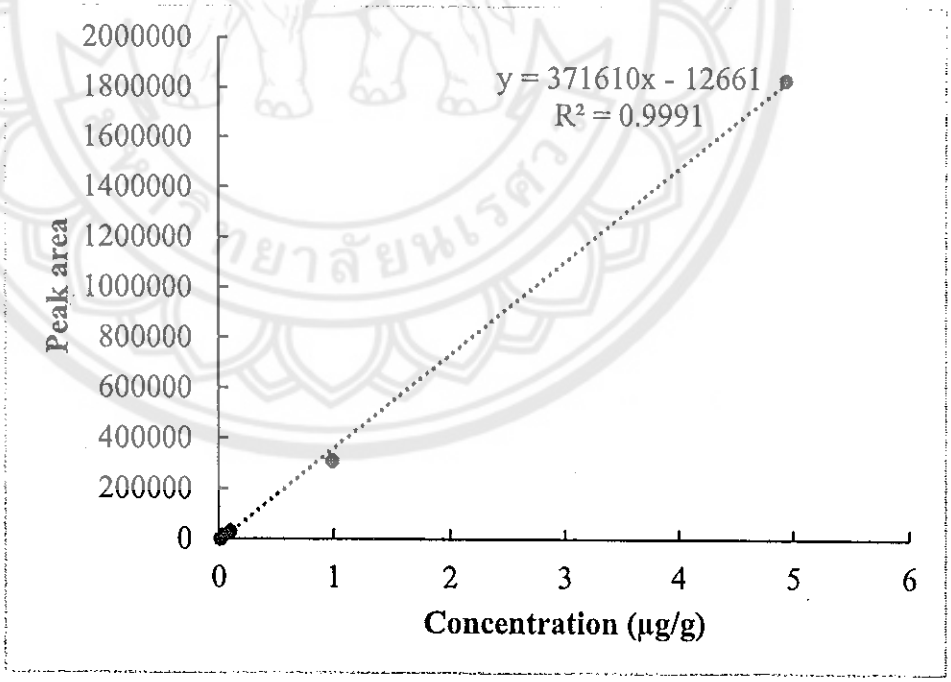

Figure 69 Calibration curve for standard of γ-tocotrienol measured by HPLC MS/MS

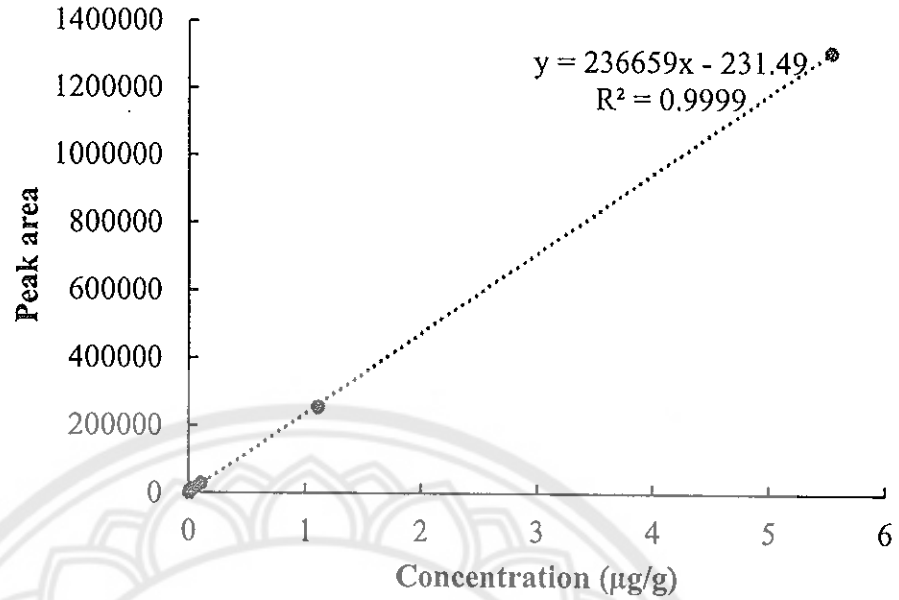

Figure 70 Calibration curve for standard of δ-tocotrienol measured by HPLC MS/MS

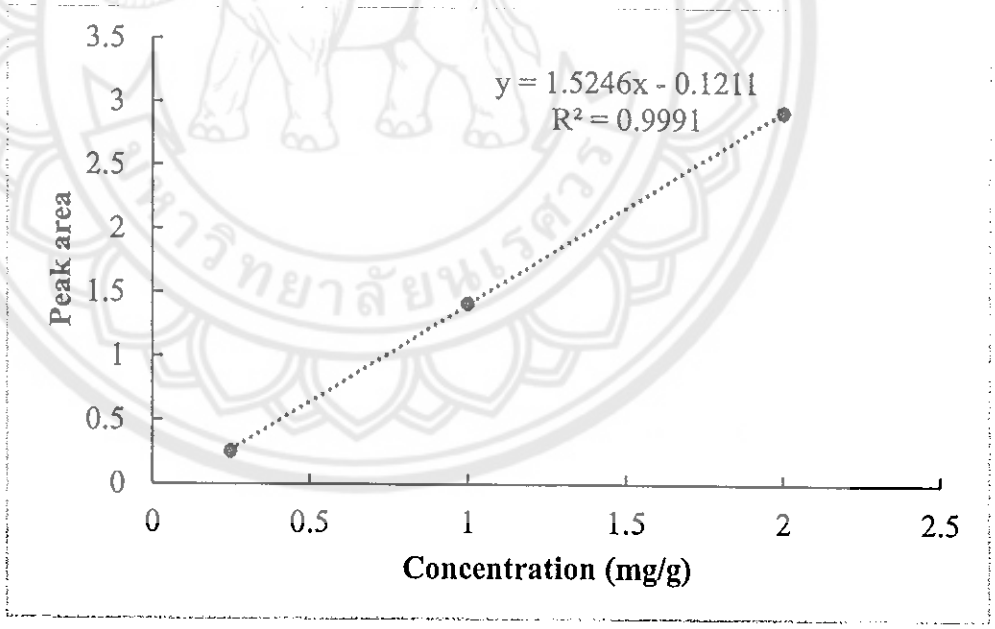

Figure 71 Calibration curve for standard of campesterol

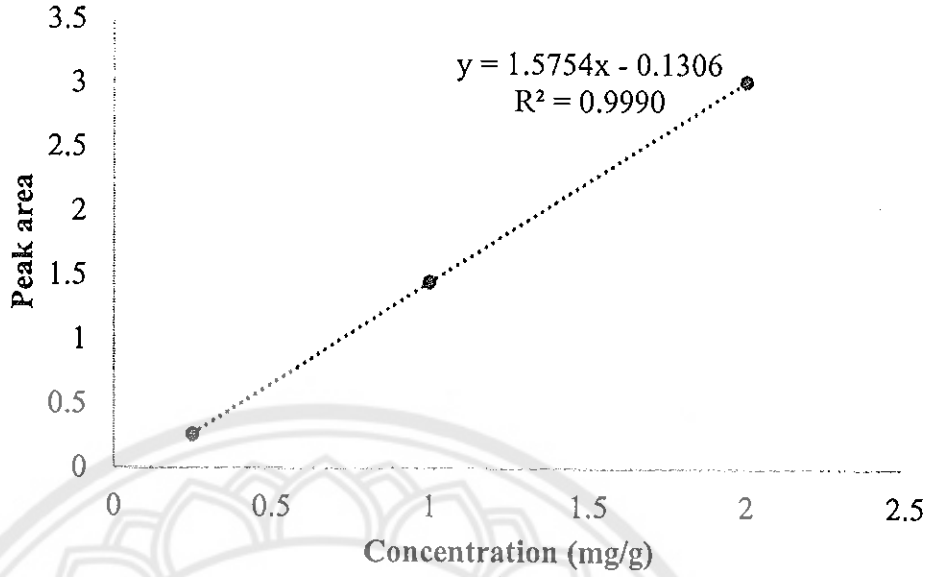

Figure 72 Calibration curve for standard of stigmasterol

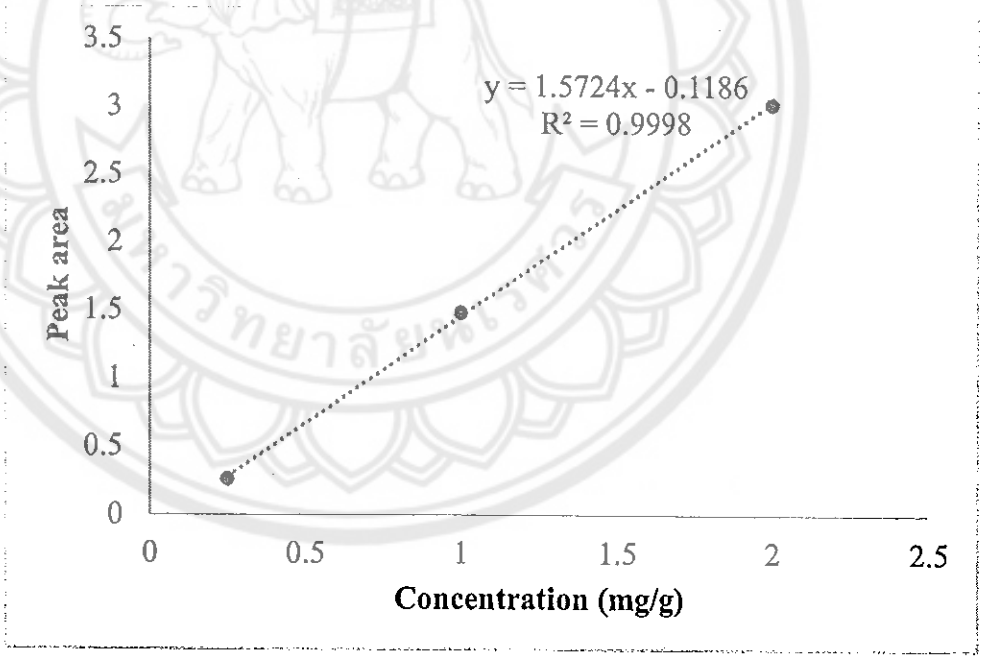

Figure 73 Calibration curve for standard of β-sitosterol

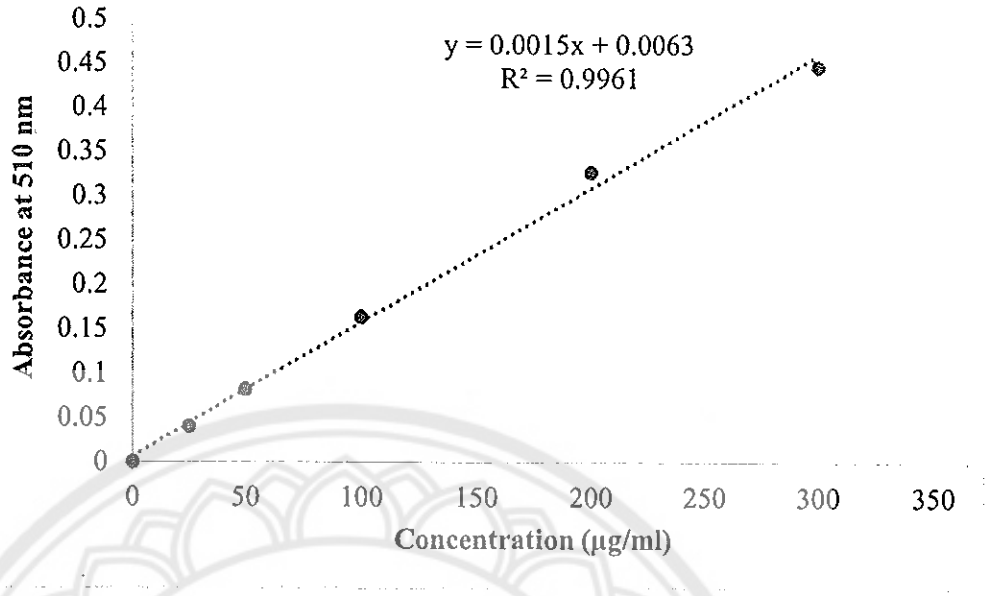

Figure 74 Calibration curve for standard gallic acid of the diets

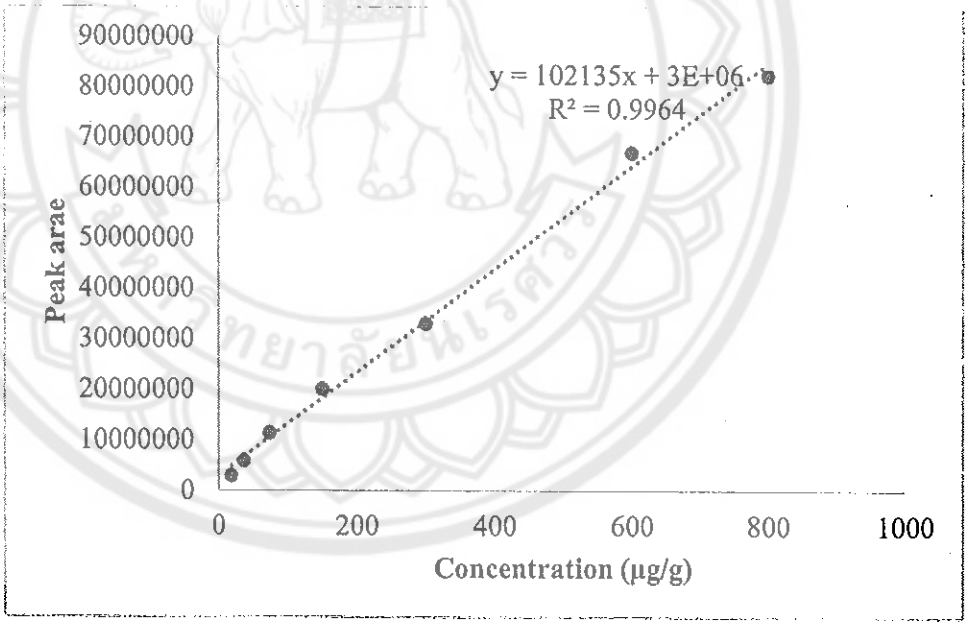

Figure 75 Calibration curve for γ-oryzanol standard of the diets

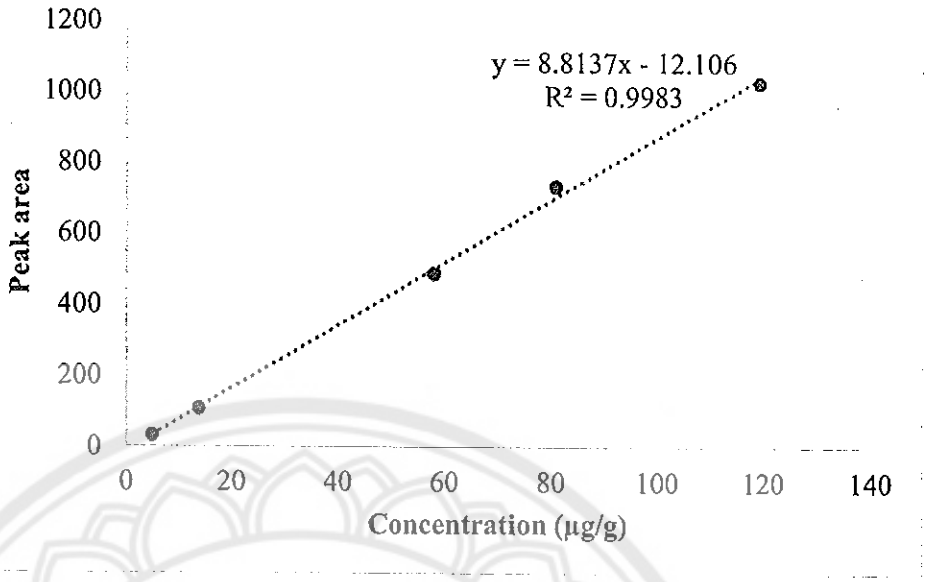

Figure 76 Calibration curve for standard of α-tocopherol