THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Food Science)

DEGREE

Food Science | Food Science and Technology

FIELD | **DEPARTMENT**

TITLE: Improving Water Dispersibility of Rice Phytochemicals Concentrated by Molecular Distillation of Rice Bran Oil Deodorizer Distillate

NAME: Miss Pattong Sawadikiat

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Parichat Hongsprabhas, Ph.D.)

THESIS CO-ADVISOR

(Associate Professor Siree Chaiseri, Ph.D.)

THESIS CO-ADVISOR

(Assistant Professor Utai Klinkesorn, Ph.D.)

THESIS CO-ADVISOR

(Professor Harry Wichers, Ph.D.)

DEPARTMENT HEAD

(Assistant Professor Wannee Jirapakkul, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

IMPROVING WATER DISPERSIBILITY OF RICE PHYTOCHEMICALS CONCENTRATED BY MOLECULAR DISTILLATION OF RICE BRAN OIL DEODORIZER DISTILLATE

PATTONG SAWADIKIAT

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Food Science)
Graduate School, Kasetsart University
2014

Pattong Sawadikiat 2014: Improving Water Dispersibility of Rice Phytochemicals Concentrated by Molecular Distillation of Rice Bran Oil Deodorizer Distillate. Doctor of Philosophy (Food Science), Major Field: Food Science, Department of Food Science and Technology. Thesis Advisor: Associate Professor Parichat Hongsprabhas, Ph.D. 135 pages.

This study aimed to use the deodorizer distillate (DD) from physical refining process as the source for the production of health-promoting phytochemicals, namely phytosterols, tocols and γ-oryzanol. This is to enhance the utilization of oil-soluble nutraceuticals in aqueous phase. Physical refining process resulted in the reduction of phytosterols and γ-oryzanol in refined rice bran oil, which was evaporated together with free fatty acids (FFAs) during deodorization and accumulated in the DD. Therefore, DD was further distilled to evaporate out the FFAs and concentrate phytosterols, tocols and γ-oryzanol in the unevaporated fraction (UMD) using a pilot-scale molecular distillation unit (MD). It was found that the MD unit operated at 120, 140 or 160 °C, 0.1 Pa and a flow rate of 10.14 - 10.66 kg/h could concentrate phytosterols from 1,540.8 mg in 100 g DD to 3,990.2 – 4,904.8 mg in 100 g UMDs. Although γ-oryzanol content was increased from 598.9 mg in 100 g DD to 870.0-1,018.1 mg in 100 g UMDs when temperature was raised to 160 °C, such high temperature decreased tocol contents from 2,185.7 mg/100 g DD to 850.5 mg/100 g UMD and antioxidant capacity of UMD measured as 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity. The dispersibility of oil-soluble phytochemicals in UMD was improved by fabricating the vesicles using polyoxyethylene sorbitan monooleate (Tween80). The size of Tween 80/UMD vesicles ranged from 200 nm to 300 nm in phosphate buffered saline (PBS) pH 7.0 suspensions. The filtered sterile vesicle suspensions in PBS were stable within the temperature range of 4 to 37 °C and maintained the size range of 200 – 300 nm for 96 h. Results indicated that the Tween 80/UMD vesicle was able to carry 814 μg phytosterol/mL, 453 μg tocols/mL and 200 μg γ-oryzanol/mL as maximum load without causing phase separation of the oil phase in the PBS after preparation and storage at low temperatures. The suspensions containing Tween 80/UMD vesicle could be uptaken by Caco-2 and THP-1 cells. At high concentration of Tween80/UMD vesicle of 4 mg/mL (130 μg/mL phytosterol, 72 μg/mL tocols and 32 μg/mL γ-oryzanol), the vesicles reduced viability of Caco-2 ($p<0.05$). However, the vesicle showed potential immunomodulation properties in THP-1 macrophages. Overall, this research revealed that a pilot-scale MD unit could be used to evaporate out FFAs, resulting in concentrating rice phytochemicals. The resulting UMD could form vesicle in aqueous phase in the presence of Tween80 that was quite stable at low temperature after filtered sterilization.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

This work could not have been accomplished if it were not for the kindly help and support of many peoples. I would like to take this opportunity to show my deepest appreciation those people.

Firstly, I would like to express my deepest appreciation to my advisor, Assoc. Prof. Parichat Hongsprabhas, for giving me opportunity to study PhD, for her excellent guidance and patience. She has provided encouragement throughout this entire process and valuable suggestion to achieve not only the research work but fulfill the skill in the real life as a good person. Second, I would like to thank Prof. Harry Wichers, and Dr. Jurriaan Mes who served as my supervisor at Wageningen University and Research Centre, encouraging and advise me throughout my time there. I would like to thank my committee members, Assoc. Prof. Siree Chaiseri for her valuable comments and suggestion in research work as well as Asst. Prof. Utai Klinkesorn, for their time and assistance in completion of this work. I would like to thank Asst. Prof. Tanaboon Sajjaanantakul for his generous support, valuable advices and guidance.

I would like to thank Surin Bran Oil Co., Ltd. for kindly provided all samples, useful information and support throughout this research. I would also like to express my gratitude to the Royal Golden Jubilee Ph.D. Program (RGJ) and The Office of Small and Medium Enterprises Promotion (OSMEP) for their financial supports.

A special thanks to my friends and members of the Department of Food Science and Technology at Kasetsart University, who always support me and made my life filled with joy and happiness. Finally, I would like to thank my family for their endless love, empathy and encouragement during the time of my Ph.D. study.

Pattong Sawadikiat

November 2014

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

IMPROVING WATER DISPERASIBILITY OF RICE PHYTOCHEMICALS CONCENTRATED BY MOLECULAR DISTILLATION OF RICE BRAN OIL DEODORIZER DISTILLATE

INTRODUCTION

Between year 2012-2013, Thailand's rice production was about 38-39 million metric tons per year. This produced rice bran as a by-product for approximately 3.8-3.9 million metric tons per year (Office of Agriculture Economic, 2014). High quality rice bran has been used as raw material for rice bran oil production. Rice bran oil (RBO) is well recognized as healthy oil that contains proper ratio of saturated, monounsaturated and polyunsaturated fat. Not only suitable fatty acid profile, RBO is well-known as the source of valuable phytochemicals, especially tocopherols, tocotrienols, phytosterols and γ-oryzanol (Van Hoed *et al*., 2006). However, some of these phytochemicals are lost during refining process. They are separated from triacylglycerols (TAG) and disposed with free fatty acids (FFAs) and other impurities during oil refining process. Consequently, they become concentrated in the by-products, in particular gum, soapstock, deodorizer distillate (DD) and wax, depending on the types of refining process used.

In physical refining process, whereby neutralization step is not included, the DD usually contains tocopherols, tocotrienols and phytosterols depending on the sources of oil and refinery processes (Copeland and Belcher, 2003). Consequently, the DD could be potential source for phytochemicals production. To my knowledge, the uses of DD are limited in Thailand and practical recovery of oil-soluble phytochemicals from DD has not been done at a pilot-scale production. Therefore,

the chemical characteristics and potential use of DD were investigated in this study.

Although the use of DD is limited due to the great extent of FFAs, a number of methods have been proposed to recover and to utilize these phytochemicals. The techniques are crystallization, saponification, esterification, supercritical fluid extraction and molecular distillation (MD) or in combination (Shimada *et al*., 2000; Copeland and Belcher, 2003; Lin and Koseoglu, 2003; Nagesha *et al.,* 2003; Martins *et al*., 2006; Dobbins *et al*., 2008; Yang *et al*., 2010; Yan *et al*., 2012). Nevertheless, DD can undergo thermal degradation during the refining process, causing the undesirable *trans*-fatty acid. Molecular distillation process (MD) is the high vacuum distillation suitable for separation and purification of high molecular weight and thermosensitive materials (Lei *et al*., 2005). The separation principle of MD is depended on the difference of molecular mean free path of materials (Lei *et al*., 2005). Due to the high vacuum condition, oxidation that might occur in the presence of oxygen was reduced (Batistella *et al*., 2002; Martins *et al*., 2006; Liu *et al*., 2008). Many researchers demonstrated that separation efficiency of MD is dependent on many operation factors, i.e. evaporating temperature, feed flow rate and operating pressure (Batistella *et al*., 2002; Martins *et al*., 2006; Posada *et al*., 2007; Liu *et al*., 2008). In this study, molecular distillation (MD) was applied to separate FFAs and concentrate oil-soluble rice phytochemicals. The influence of molecular distillation temperature on the unevaporated fraction after MD (UMDs) was addressed.

Due to low water dispersion and insolubility of oil-soluble rice phytochemicals, the phytosterols, tocols and γ-oryzanol have limited uses in aqueous food systems. If these lipophilic phytochemicals are entrapped in the structure that can disperse in the aqueous phase, their fortification in foods can be expanded. Vesicles, a closed spherical structure of amphiphilic molecules having bilayer membrane, could be used to incorporate active ingredients (both hydrophiles and lipophiles) inside their structure.

A well-known vesicle structure fabricated by phospholipid is known as liposome. Vesicle has been extensively studied and used as drug delivery system to entrap and protect active compound from processing and storage environment. Crude rice bran oil phytochemicals concentrated from MD process employed to DD contained low molecular weight non-ionic surfactants, mainly mono- and di-oleylglycerol (Nukit *et al*., 2014), that possibly enhanced the formation of vesicles with commercial surfactants and encapsulated rice phytochemicals. This thesis employed the existence of phytosterols and residual mono- and di-acylglycerols in the UMDs to fabricate submicrometer size of structure that can disperse in aqueous phase. The knowledge acquired could help validate their bioactivities as oil-soluble nutraceuticals and expand utilization of rice bran products.

OBJECTIVES

1. To determine the distribution of selected phytosterols contents in commercial edible vegetable oil in Thailand,

2. To characterize the rice phytochemicals in crude rice bran oil, deodorizer distillates and refined rice bran oil,

3. To study the influence of operating temperature during molecular distillation (MD) on the characteristics of unevaporated fraction (UMD),

4. To improve water dispersibility and stability of rice phytochemicals concentrated in UMD in aqueous phase, and

5. To evaluate the influence of UMD entrapped in vesicular structure in viability of Caco-2 and THP-1 cell models.

LITERATURE REVIEW

1. Rice bran oil production

Rice bran oil has been used extensively in rice-growing countries including Thailand. It has received much attention lately due to its positive effects on health. Rice bran, consists of pericarp, seed coat, nucleus, aleurone layer, germ, and part of the subaleurone layer of the starchy endosperm of rice grain, contains about 15-20% fat and is a by-product from rice milling process. The major use of rice bran is in the feed industries, followed by rice bran oil industries. The latter requires much higher quality of bran with low FFAs of less than 10 mg KOH/g (Orthoefer, 2005). Crude rice bran oil is usually extracted by solvent such as hexane or alternative method such as supercritical fluid extraction. After solvent removal, crude rice bran oil would be subjected to refining process to remove the impurities, i.e. pigment, residual bran, wax and FFAs. The refining process includes dewaxing, degumming, neutralizing of FFAs, bleaching to improve color, steam deodorizing and winterizing to remove high melting fat (Orthoefer, 2005).

1.1 Rice bran oil refining process

Crude vegetable oils are rarely used without refining process except for some vegetable oils; i.e. virgin olive oil and virgin coconut oil. For crude rice bran oil, color is varied from dark greenish brown to light yellow depending on the source and conditions of the bran (stabilized or non-stabilized bran by steaming), extraction method, quality and composition of the bran. The color of oil comes from pigments, including carotene, chlorophyll, and Maillard browning products. In order to achieve light yellow color rice bran oil (Lovibond 3.0 R 30Y) and consumer acceptance, crude rice bran oil must be subjected to refining process. The compositions of crude rice bran oil play a crucial role on refining process. Typically, crude rice bran oil is

composed of many impurities such as residual fine bran (<149 mm) up to 0.5% and wax ranging from 0.5 to 5% (Orthoefer, 2005). The purpose of refining process is to remove FFAs along with other undesirable substances.

There are two major refining methods; namely chemical and physical refining processes. Both refining methods are quite similar with the major difference is FFAs removal process. In chemical refining, FFAs, most of the phosphatides, and other impurities are removed by neutralizing with an alkaline solution such as NaOH. While in physical refining, the FFAs are removed by steam distillation during deodorization, as showed in Figure 1. Rice bran oil is currently used either of refining methods or in combination. The refining method selected is depended on the characteristics of crude oil.

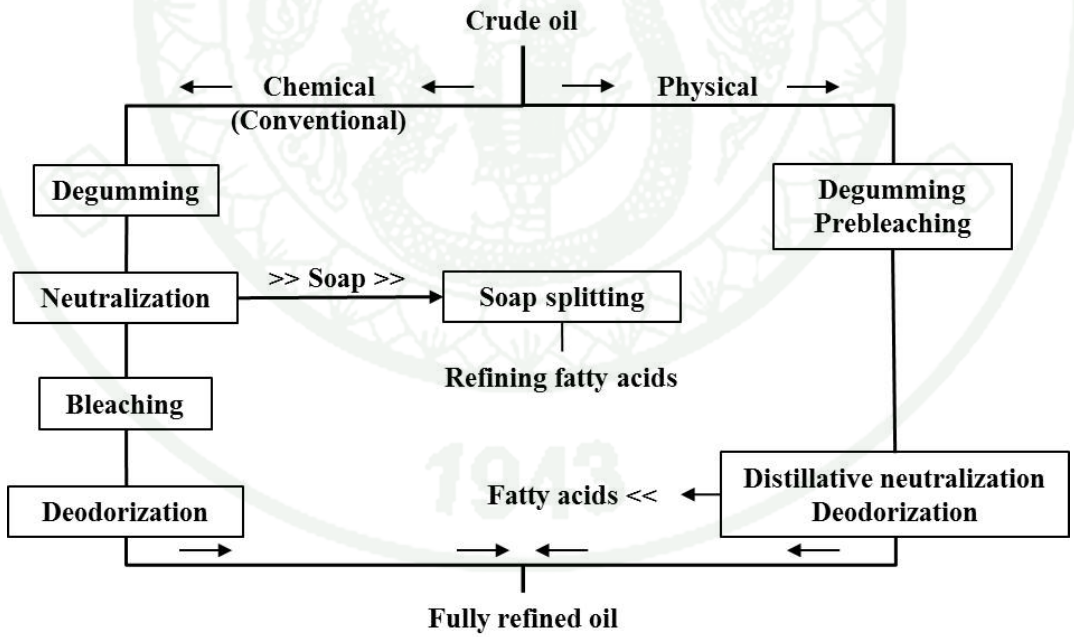


Figure 1 Chemical and physical refining processes.

Source: Modified from Bockisch (1998)

Conventional refining or chemical refining process consists of 4 major operations, i.e. degumming, alkali neutralizing or deacidifying, bleaching, and deodorizing, to produce refined edible oil as showed in Table 1 (Čmolík and Pokorný, 2000; O'Brien *et al.,* 2000). Alkali neutralization of rice bran oil results in soapstock. This by-product is rich in γ-oryzanol and a mixture of sodium salts of fatty acids, neutral oil, water, unused caustic, and other compounds resulted from the reactions of NaOH with various impurities in the oil. Due to the reduction of phytochemicals in finished products and other causes as mentioned, physical refining process has become more attractive choice than chemical process (Čmolík and Pokorný, 2000; O'Brien, 2009). Moreover, chemical refining method has drawbacks due to expensive cost, loss in production yield, time consuming, excessive waste and great loss of nutraceuticals to by-product, i.e. soapstock (Čmolík and Pokorný, 2000; Orthoefer, 2005; O'Brien, 2009).

Table 1 Major refining operation in chemical refining process

Refining operation	Substances removed
Hydration, degumming	Phospholipids, other polar lipids (gums)
Alkali neutralizing, deacidifying	FFAs, residual phospholipids, metals and γ-oryzanol
Bleaching	Pigments, residual soapstock and phospholipids
Deodorizing	Volatile oxidation products, FFAs and unsaponifiable matters, e.g. phytosterols, tocols and squalene

Source: Čmolík and Pokorný (2000), O'Brien *et al.* (2000).

1.2 Physical refining process

Physical refining process was used as pre-neutralization process of oil with a high initial FFAs content (followed by chemical refinery) in early 1930s. Later on, it has been used to refine palm oil with high FFA contents and low gum content in the 1950s (O'Brien, 2009). Physical refining process involves 3 operations, the first two processes (degumming and bleaching) are similar to chemical refining process; while the third one is deacidification by distillation. FFAs are removed based on the difference in boiling points of the acids and neutral oil by steam distillation. Other methods, such as membrane refining, molecular distillation have been used to replace alkali neutralization in chemical process (Cvengroš, 1995). Degumming by water and bleaching are aimed to remove certain non-volatile impurities that might decrease the qualities of the final product such as darkening in color or other unfavorable alterations; while the volatile and thermo-labile impurities are stripped during steam distillation. Consequently, the complete pretreatment processes (degumming and bleaching) play an important role on physically refined oil's stability. In early period of physical refining process, the quality and oxidative stability of physical refined oil were usually low due to insufficient degumming process (water degumming) that caused high residual phospholipid and heavy metal contents (Čmolík and Pokorný, 2000).

Several degumming processes have been introduced to industries to fulfill requirements for physical refining to obtain high stability and good quality oil. For physical refining process, phospholipid content of the oil must be less than 5 mg/kg phosphorus before stripping and less than 20 mg/kg phosphorus before the bleaching step. This requires the removal of both hydratable phospholipids (usually removed by water degumming) and nonhydratable phospholipids (calcium and magnesium salts of phosphatidyl ethanolamine and phosphatidic acids) (Čmolík and Pokorný, 2000). Physical refining is thus suitable for high FFAs oils with low phosphatide contents such as coconut, palm kernel, palm, lard, tallow, some of the seed oils and rice bran

oil (O'Brien *et al.,* 2000). The advantages and disadvantages of physical refining method are summarized in Table 2.

Table 2 Advantage comparison between chemical and physical refining process

Chemical refining	Physical refining
Advantages	
- Suitable for high phosphatide contents oil - Low formation of *trans* fatty acid - Flexible to treat all kind of oil and quality	- Low capital cost - High nutraceutical retention in refined oil - High production yield and suitable for high FFAs oil - Environmental friendly - Fewer process to operate and maintain
Disadvantages	
- Loss of nutraceuticals especially γ-oryzanol in refined oil - Time consuming - High energy requirements - Great loss of production yield - Large volume of waste and high cost waste management	- Not suitable for high phosphatide content oil - Partial loss of nutraceuticals, i.e. tocols and phytosterol - Formation of *trans* fatty acid - Require pretreatment process, i.e. complete phosphatide removal with water degumming and bleaching

2. Composition of rice bran oil

The major fatty acids in rice bran oil are oleic acid, linoleic acid and palmitic acid that make up more than 90% of the fatty acid component as showed in Table 3. The majority of triglycerides in rice bran oil are glycerol esterified with palmitic-linolenic-oleic, oleic-linoleic-palmitic, palmitic-linoleic-linoleic, linolenic-linoleic-palmitic, and trioleic. The ratio of saturated, monounsaturated and polyunsaturated fatty acid of rice bran oil is close to the ideal ratio of edible oil recommended by many health organizations such as WHO, American Heart Association (AHA) and Japan's Ministry of Health and Welfare (Table 4). Moreover, rice bran oil also has high smoke point, making it suitable for cooking, stir frying and deep frying application (Orthoefer, 2005).

Table 3 Fatty acid composition of rice bran oil

Fatty acid compostion	Percent (%)
Myristic acid (C14:0)	0.6
Palmitic acid (C16:0)	21.5
Stearic acid (C18:0)	2.9
Oleic acid (C18:1)	38.4
Linoleic acid (C18:2)	34.4
Linolenic acid (C18:3)	2.2

Source: Orthoefer (2005)

Table 4 Fatty acid ratios among saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) of common edible oil and recommendations from health organizations

Name of oil	Fatty acid ratio		
	SFA	MUFA	PUFA
Palm	49	37	9
Sunflower	10	45	40
Corn	13	28	55
Soybean	16	23	58
Olive	14	73	11
Rice bran	20	39	35
World Health Organization	28.6	42.8	28.6
American Heart Association	33.3	33.3	33.3
Japan Ministry of Health and Welfare	28.6	42.8	28.6

Source: U.S. Department of Agriculture, Agricultural Research Service (2013)

Not only the fatty acid composition, but also its phytochemical components having health-promoting effects, that make rice bran oil recognized as healthy oil. Many reports emphasized the positive health effects of rice bran oil for its cholesterol-lowering properties in both animal and human trials (Sharma and Rukmini, 1986; Rajnarayana *et al*., 2001; Frank, *et al*., 2005; Most *et al*., 2005; Orthoefer, 2005; Yean *et al.,* 2008). It has been identified that minor components of rice bran oil such as

phytosterols, tocotrienols, squalene and γ-oryzanol are responsible for these actions (Rukmini and Raghuram, 1991; Vissers *et al*., 2000; Wilson *et al*., 2000; Cicero and Gaddi, 2001; Huang *et al*., 2009; Wilson *et al*., 2007).

3. Deodorizer distillate (DD)

Deodorization or steam stripping process is used to remove FFAs and undesirable odors resulting from peroxides, aldehydes, and ketones, as well as characteristic odors and flavors of rice bran oil (Orthoefer, 2005). The deodorizer distillate (DD) is a by-product obtained from the steam deodorization process of vegetable oil refinery. Its compositions and characteristics depend on a number of factors, including the type of oil being processed, methods of pretreatment (chemical *vs.* physical refining) and operating conditions during the refining process (temperature, pressure, time) etc. (Verleyen *et al.,* 2001a). The DD mainly consists of volatile compounds that are distilled during the deodorization process. As a result, FFAs are major constituents in DD. Moreover, FFAs composition in DD may differ from that in refined oils; e.g. DD from soybean and sunflower oils had lower linoleic acid content than their refined oil (Čmolík and Pokorný, 2000). Fatty acids in DD are generally ranged between 30-50% and 80-90% for chemical and for physical refining process, respectively (Verleyen *et al.,* 2001a). Besides FFAs, DD from rice bran oil refining process also contains other valuable health-promoting phytochemicals as minor components, including tocopherols, tocotrienols, phytosterols, γ-oryzanol, squalene, carotenoids etc. (Table 5) (Van Hoed *et al*., 2006). Typical DD is composed of 1-8% tocopherol, 5-30% glyceride, 0-5% steryl ester and 2-15% free sterols, dependent on the refining conditions (Fernandes and Cabral, 2007). Consequently, DD becomes one of the principal commercial sources of phytosterols, tocopherols and tocotrienols (Martins *et al.,* 2006; Dobbins *et al*., 2008). Selected phytosterol and tocopherol contents in vegetable oil DD are showed in Table 6.

Phytosterol and tocopherol contents of DD from chemical refining process were 50% higher than their corresponding oil from physical refining process (Čmolík and Pokorný, 2000; Verleyen *et al*., 2001a). This is because in chemical refining process, most of FFAs were neutralized and washed out during alkali neutralization step before deodorization. As a result, DD obtained from chemical refining process has much lower FFA contents and higher unsaponifiable matter contents than their corresponding oil from physical refining process.

Table 5 Composition of rice bran oil deodorizer distillate (DD) from chemical refining process

Components	g/100g
FFAs[a]	32.9
Monoglyceride	5.8
Diglyceride	6.1
Unsaponifiable matter	37.9
Squalene	1.9
Total tocols [b]	3.2
α-tocoperol	0.6
γ-tocoperol	0.5
α-tocotrienol	0.1
γ-tocotrienol	1.9
δ-tocotrienol	0.0

[a]Expressed as oleic acid. [b]Tocols, sum of tocopherols and tocotrienols. [c]This includes the sum of three different isomers eluted before 24-methylenecycloartanol.

Source: Van Hoed *et al*. (2006)

Table 5 (Continued)

Components	g/100g
Total phytosterol	14.83
Campesterol + Campestanol	2.11
Stigmasterol	1.80
β-Sitosterol	6.16
Sitostanol + Δ^5-Avenasterol	0.48
Δ^5(24)-Stigmastadienol	0.51
Gramisterol	0.12
Δ^7-Sitosterol + cycloartenol	0.65
Δ^7-Avenasterol	0.06
Isomer 24-methylenecycloartanol[c]	0.51
24-Methylenecycloartanol	1.31
Citrostadienol	0.56
Isomer 24-methylenecycloartanol	0.54

[a]Expressed as oleic acid. [b]Tocols, sum of tocopherols and tocotrienols. [c]This includes the sum of three different isomers eluted before 24-methylenecycloartanol.

Source: Van Hoed *et al*. (2006)

Table 6 The contents of selected phytosterol and tocopherol contents in vegetable oil deodorizer distillate (mg/100g).

DD	Phytosterol contents (g/100 g)			Tocopherol contents (mg/100 g)			
	Campesterol	Stigmasterol	Sitosterol	δ	β	γ	α
Soybean[P]	1.91	1.38	3.03	2.01	0	4.96	0.54
Soybean	5.06-5.66	4.10-4.81	7.9-8.34	4.41-5.59	0.36-0.52	10.73-11.26	0.82-0.81
Corn[P]	0.84-1.67	0.19- 0.37	1.68-3.38	0.12	0.06-0.08	1.09-2.75	0.15-0.36
Sunflower[P]	0.45	0.62	2.6	Nd	Nd	0.07	1.21
Sunflower	1.58	2.04	8.6	Nd	Nd	0.03	4.76
Rapeseed	4.37	Nd	6.24	0.18	0.18	2.48	0.14
RBO*	2.11	1.80	6.16	-	-	0.50	0.60

Note: P is physical refining

Source: Verleyen *et al*. (2001a), *is from Van Hoed *et al*. (2006)

The high contents of phytosterols and tocols in DD make it an attractive source for their use as raw materials for nutraceuticals production at commercial scale. However, the recovery of phytosterols and tocols from deodorizer distillate faces 3 major problems: (a) DD contains great amount of FFAs, (b) thermal degradation of phytosterols and tocols are prone to take place if DD were to expose to high temperature, at which phytosterols and tocols could be vaporized or degraded for extended period of time and (c) boiling point of phytosterols and tocols are in the same range, making difficulties in purification of each compound.

4. Oil-soluble rice phytochemicals

4.1 Phytosterols

Phytosterols are triterpenes having molecular structure and function similar to those of cholesterol. However, cholesterol and phytosterol differ by their side chain. Cholesterol has eight carbon atoms, whereas phytosterol has extra methyl or ethyl group on C-24 in their side chain (Figure 2). Phytosterols regulate plant cell membrane fluidity. Each type of phytosterol has different impact on structure and membrane stability. In addition, phytosterols are more hydrophobic than cholesterols. Phytosterols have low solubility in both water and oil phases (Rozner and Garti, 2006). More than 200 types of phytosterol have been reported in plant species (Piironen *et al*., 2000; Lagarda *et al*., 2006). The most abundant are β-sitosterol (24α-ethylcholest-5en-3β-ol), campesterol (24α-methyl-5-cholesten-3β-ol) and stigmasterol (5,22-cholestadien-24α-ethyl-3β-ol) (Figure 2). Normén *et al.* (2007) reported the compositions of phytosterol in fatty foods in Sweden and the Netherlands, which covered over eighty food items. They found that β-sitosterol was the most abundant phytosterol, representing over 60 % of total phytosterol, followed by campesterol (24 %), stigmasterol (7 %) and 5-avenasterol (5 %), brassicasterol (3 %), β-sitostanol (1 %) and campestanol (0.1%), respectively.

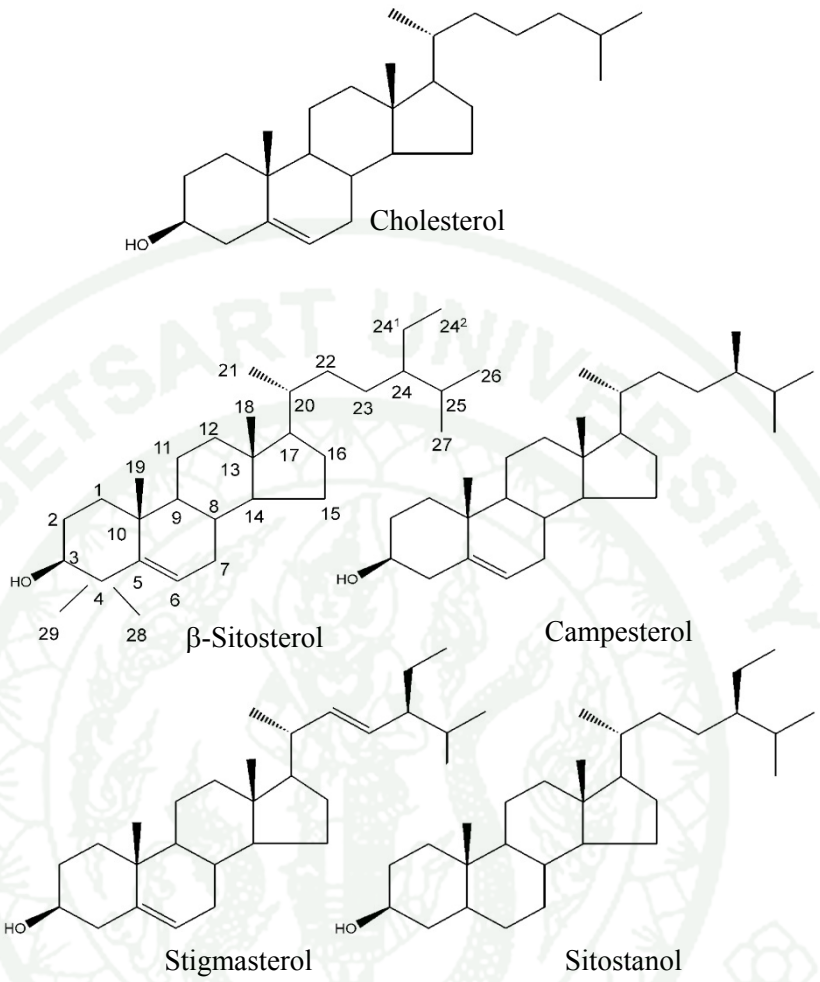


Figure 2 Structure of the most common sterols.

Source: Moreau *et al*. (2002)

The word "phytosterols" is sometimes referred as phytosterol and their saturated form "phytostanol". Phytostanols are saturated phytosterols, occurred only in trace amount in certain natural sources such as grain (corn, rye and wheat) and coniferous tree (pine). Most of the commercial phytostanol are from hydrogenation process of phytosterols (Moreau *et al*., 2002). Phytosterols are well known as a cholesterol-lowering nutraceuticals. In 1950s, sitosterol was used commercially as

supplement and drug for the first time, namely Cytellin, after the cholesterol-lowering effect of phytosterols was evident (Moreau *et al*., 2002).

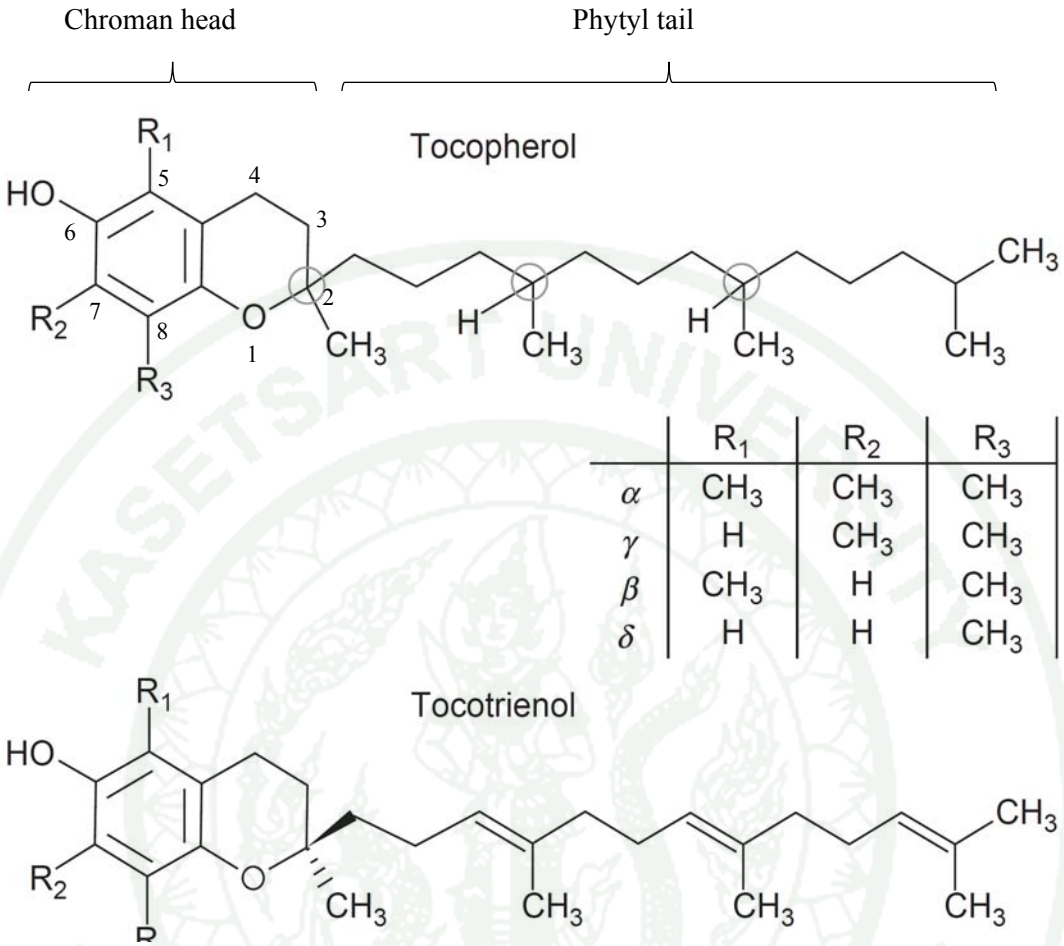
Nevertheless, due to poor solubility, inconsistent efficiency and high doses required, phytosterols was replaced by drug call Statin (Moreau *et al*., 2002). In order to accomplish the lower dosage of phytosterols while maintaining high bio-efficacy, the method to improve phytosterol solubility was developed. In early 1970s, Procter & Gamble enhanced solubility of phytosterols by esterifying phytosterol with fatty acids. However, their technique was not commercially practiced. In mid 1990s, phytosterols faced their turning point. A group of scientists at Raisio Group in Finland esterified phytostanol with fatty acid in order to improve their solubility in lipid and used them in fat-based food such as margarines. Commercial phytostanyl ester-containing margarines had consistent effect in cholesterol-lowering in clinical studies at low dosages (2–3 g/day).

The esterified phytosterol are highly soluble in oil phase, making it possible to be incorporated in fat-based food products (Rozner and Garti, 2006). Another method to increase solubility of phytosterol is suspending free or esterified phytosterol microcrystalline in liquid medium. Christiansen *et al*. (2002) and Bonsdorff-Nikander *et al*. (2003) produced a microcrystalline β-sitosterol suspension in medium-chain triacylglycerol (TAG) by recrystallization to avoid coarse-grained structure of β-sitosterol. According to their process, β-sitosterol in TAG suspension can be stored for 16 weeks at 4 ^{o}C and the dose could be increased up to 30% of TAG suspension without any changes in crystal size and behavior. Engel and Schubert (2005) successfully formulated phytosterol emulsions by creating supersaturating phytosterols with lecithin or monoacylglycerol as crystallization inhibitor in the oil phase of oil-in-water (o/w) emulsion. The recent method is the entrapment of the phytosterols by fabricating in microemulsions (Rozner and Garti, 2006).

Phytosterols naturally occur in plant and are found in most plant-based food products such as vegetable oils, cereals and their products, vegetables, fruits, berries, nuts, seeds and legumes. Consequently, phytosterols assimilation in human is only from diet. Consumption of phytosterols can reduce absorption of dietary cholesterol. This results in the reduction of both low density lipoprotein (LDL) and total cholesterols but had no effect on high density lipoprotein (HDL) cholesterol. Since September 2000, the US FDA has allowed a health claim for phytosterols in reducing the risk of coronary heart diseases in foods containing plant stanyl and steryl esters, as long as the foods were low in saturated fat, low in cholesterol, and contain plant steryl and stanyl esters of at least 1.3 and 3.4 g/day, respectively (Moreau *et al*., 2002). The recommendation includes stanyl esters in spreads, salad dressings, snack bars, and dietary supplements (soft gels). However, it includes steryl esters in only two of these applications; i.e. spreads and salad dressings. Stanyl and steryl esters are now listed as Generally Recoginzed as Safe (GRAS) by the US FDA. According to Moreau *et al*. (2002), many clinical trials on over 2400 subjects and dose up to 25 g/day showed no adverse effect. The supplement Cytellin (marketed by Eli Lilly Company) sold over the last 50 years had an excellent safety record (Moreau *et al.,* 2002). Many studies conducted over the last 10 years have indicated a complete lack of toxicity in animal and human models, except for individuals with phytosterolemia (sitosterolemia) (Moreau *et al*., 2002).

4.2 Vitamin E

Vitamin E is a generic term describing 8 lipophilic compounds, which includes α, β, γ and δ isoforms of tocopherol and tocotrienol. Tocopherol has saturated side chain at position 2 of chromanol ring whereas tocotrienols are unsaturated at 3, 7 and 11 prime position of the side chain (Figure 3). Vitamin E is well known for its chain-breaking antioxidant activity that could prevent cyclic propagation of lipid peroxidation (Mustacich *et al.* 2007).



	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
γ	H	CH_3	CH_3
β	CH_3	H	CH_3
δ	H	H	CH_3

Figure 3 Structure of tocopherols and tocotrienols

Source: Mustacich *et al*. (2007)

Tocopherol naturally occurs as a free form, while tocotrienol could be found in their esterified form (Kamal-Eldin and Appelqvist, 1996). Tocopherols are found in oil seed and green parts of higher plant, while tocotrienols are mostly found in bran and germ fractions of certain seeds and cereals.

Lipid autoxidation is a chain reaction that involves with 3 phases of reaction, i.e. initiation, propagation and termination steps. In the initiation phase, C-centered alkyl radicals ($R^{\bullet}$) are generated from unsaturated fatty acid (*RH*) (Equation 1). The initiation reaction rate is dependent on initiator (*I*) and can be catalyzed by heat, light, trace metals and/or certain enzymes (Kamal-Eldin and Appelqvist, 1996)

In propagation phase, C-centered alkyl radicals ($R^{\bullet}$) from initiation phase quickly react with oxygen (O_2) and form peroxyl radical ($ROO^{\bullet}$) (Equation 2) that are able to react with another unsaturated fatty acid (*RH*) (Equation 3). The peroxyl radical from initiation phase is converted to hydroperoxide (*ROOH*) and generate new C-centered radical, which rapidly converts to another peroxyl radical. The propagation process will continue until almost all of unoxidized unsaturated fatty acids are consumed. The radicals ($ROO^{\bullet}$ and $R^{\bullet}$) are dimerized and form inactive product in termination phase (Equations 4 and 5).

Initiation	$RH + I \rightarrow R^{\bullet} + IH$	.....Equation (1)
Propagation	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$	.....Equation (2)
	$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$	.....Equation (3)
Termination	$ROO^{\bullet} + ROO^{\bullet} \rightarrow$ inactive product	.....Equation (4)
	$R^{\bullet} + R^{\bullet} \rightarrow$ inactive product	.....Equation (5)

Source: Burton and Traber (1990); Kamal-Eldin and Appelqvist (1996)

The chain-breaking antioxidant activity of tocols is involved by inhibiting of propagation phase in lipid oxidation. Tocols can react with peroxyl radical ($ROO^{\bullet}$) faster than unsaturated fatty acid. First, chromanol head group of tocols (*TOH*) donates phenolic hydrogen to lipid peroxyl radical ($ROO^{\bullet}$), resulting in the formation of resonance-stabilized chromanoxyl (chroman-6-oxyl) radical ($TO^{\bullet}$)

(Figure 4) and hydroperoxide product (Equation 6). The unpaired electron delocalization also induces radical sites on the *ortho*- and *para*- position. The chromanoxyl radical (*TO*•) is very reactive towards alkyl (*R*•) and alkylperoxyl radical (*ROO*•) (Kamal-Eldin and Appelqvist, 1996). The C-centered alkyl radical generally adds to phenoxyl oxygen, while O-centered radical prefer to add to the *ortho*- and *para*- position of phenoxyl radical. The differences in methyl substitution at *otho*-position 5 of α- and β- tocols and their γ- and δ- isomer result in different oxidation pathway of each tocols isomer. The *ortho*-position 7 is sterically hindered. As a result, *ortho*-position 5 is a primary site for radical-radical coupling reaction (termination reaction). The α- and β-chromanoxyl radicals are arranged to chromanol radicals at position 8a, while γ- and δ-chromanoxyl radicals rearrange to radicals that can capture lipid peroxyl radicals at position 5 as showed in Figure 5 (Kamal-Eldin and Appelqvist, 1996). The tocoperoxyl radical (*TO*•) could react with another peroxyl radical and form inactive non-radical product that would be removed from oxidation cycle (Equation 7) (Burton and Traber, 1990).

Inhibition $\quad \alpha\text{-TOH} + ROO^{\bullet} \rightarrow \alpha\text{-}TO^{\bullet} + ROOH$ .....Equation. (6)

$\alpha\text{-}TO^{\bullet} + ROO^{\bullet} \rightarrow$ inactive products .....Equation. (7)

Source: Burton and Traber (1990).

4.2.1 Tocotrienols

Tocotrienols are oil-soluble vitamin belonging to the family of vitamin E-active substances. Tocotrienols consist of α, β, γ, δ- forms and well recognized for their antioxidant properties. In general, antioxidants are known to associate with reduction of chronic diseases such as cardiovascular disease (CVD) and cancer. Not only exhibiting as excellent antioxidant compared to tocopherol, tocotrienols also show cholesterol-lowering, anti-cancer and neuroprotective properties not reported for tocopherols (Sen *et al*., 2007). Rice bran and palm oil are

two major natural sources of tocotrienols. Tocotrienols have been found in significant amount in rice bran oil; i.e. 93 mg/100 g of crude oil and about 50 mg/100g of refined rice bran oil as showed in Table 7 (Orthoefer, 2005).

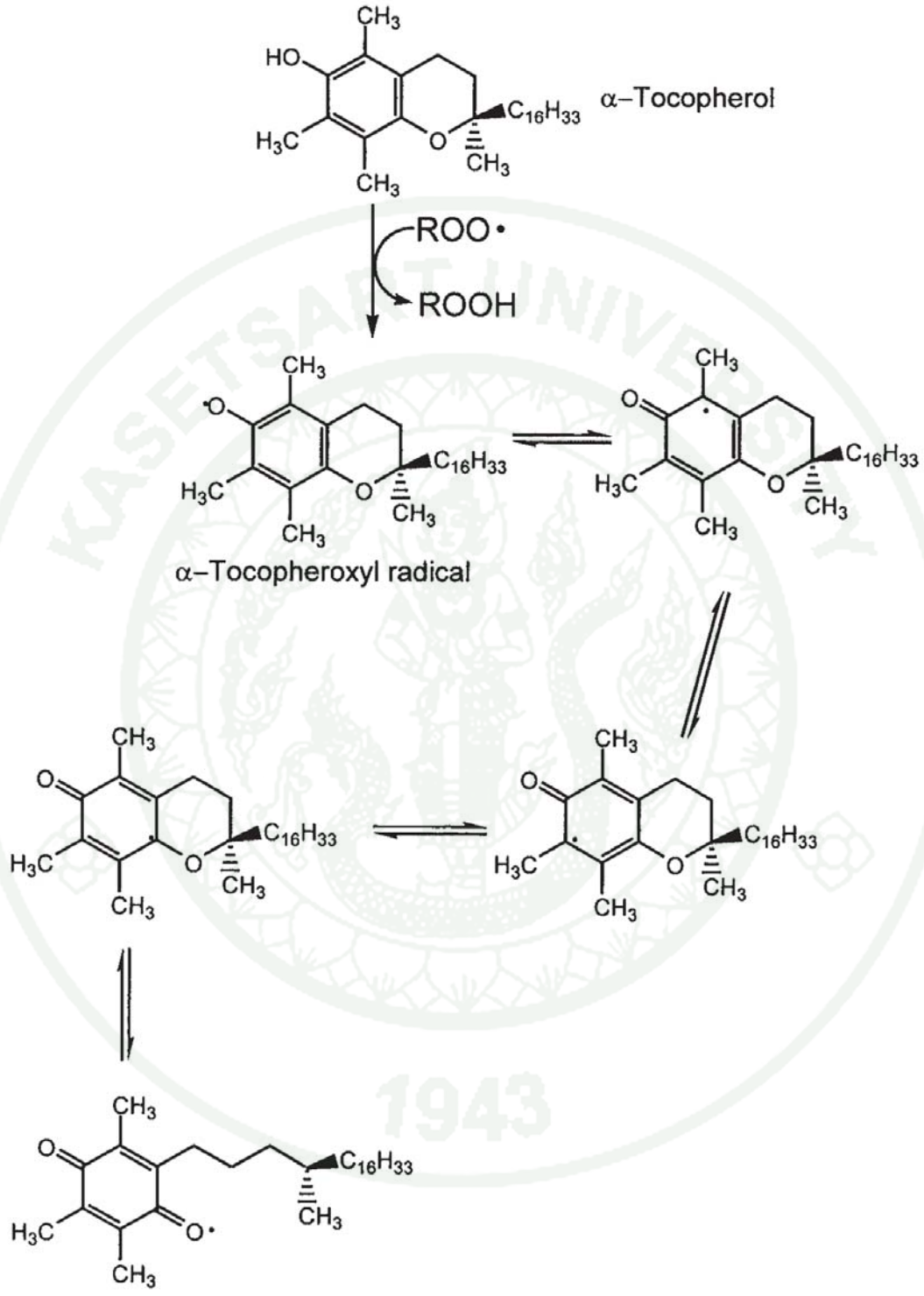


Figure 4 Resonance stabilization of α-tocopherol.

Source: Eitenmiller and Lee (2004)

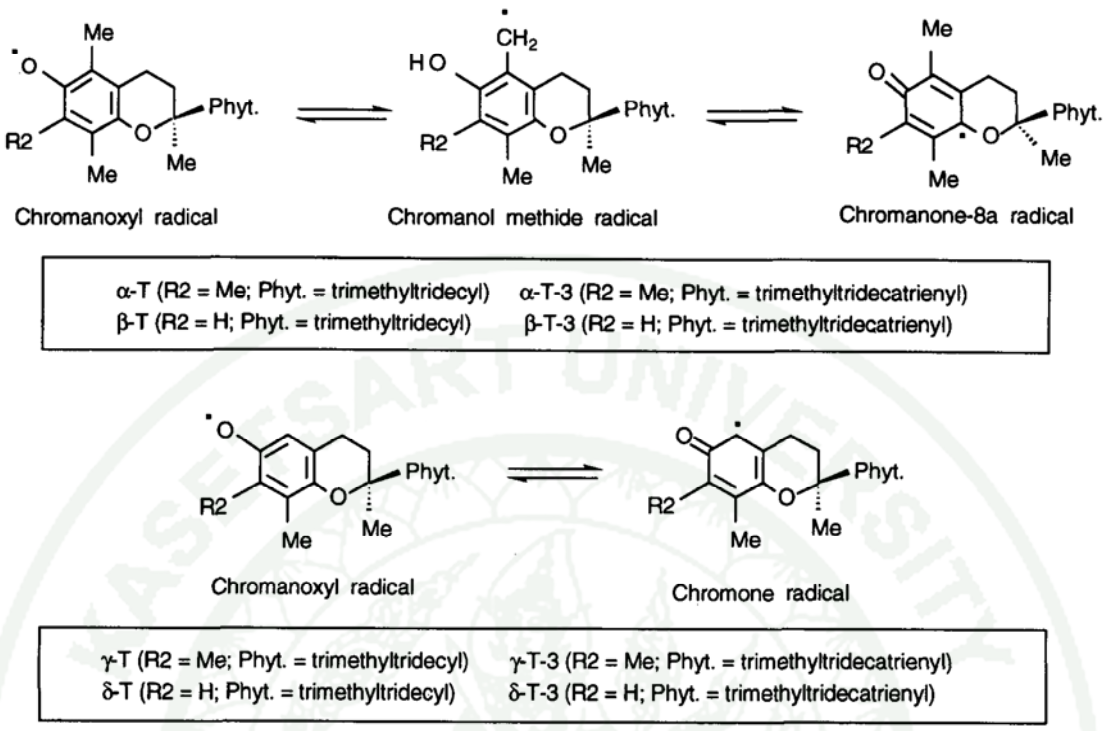


Figure 5 The rasonance forms of the chromanoxyl radicals, tocopherol (T), tocotrienol (T-3) and phytyl (Phyt).

Source: Kamal-Eldin and Appelqvist (1996)

Table 7 Tocols expressed as tocopherol (T) and tocotrienol (T3) contents (mg/100g) in brown rice and rice products.

Source	Tocopherols				Tocotrienols		
	α-T	β-T	γ-T	δ-T	α-T3	γ-T3	δ-T3
Brown rice	0.63	0.09	0.32	0.02	0.38	1.2	0.07
Rice bran	6.3	0.9	3.2	0.2	3.8	12.0	0.7
Crude oil	31.5	4.50	16.0	1.0	19.0	60.0	3.5
Refined oil	8.2	n/a	12.8	1.3	2.1	42.9	3.5

Source: Orthoefer (2005)

4.3 γ–Oryzanol

γ–Oryzanol is a minor component of rice bran oil that has promising health benefits. γ–Oryzanol was first isolated from soapstock, a by-product from chemical refining process of rice bran oil. γ–Oryzanol was originally identified as a single compound; however, it was discovered later and identified as a mixture of steryl and triterpenyl esters of ferulic acid (Figure 6). The major component of γ-oryzanol are ferulic acid esters of 4,4-dimethylsterols (cycloartenol and 24-methylenecycloartanol) and of 4-desmethylsterols (campesterol, β-sitosterol and campestanol) (Orthoefer, 2005; Miller and Engel, 2006). Rice bran oil is composed of 1.5-2.9% γ–oryzanol, depending on rice grain varieties and sources (Orthoefer, 2005).

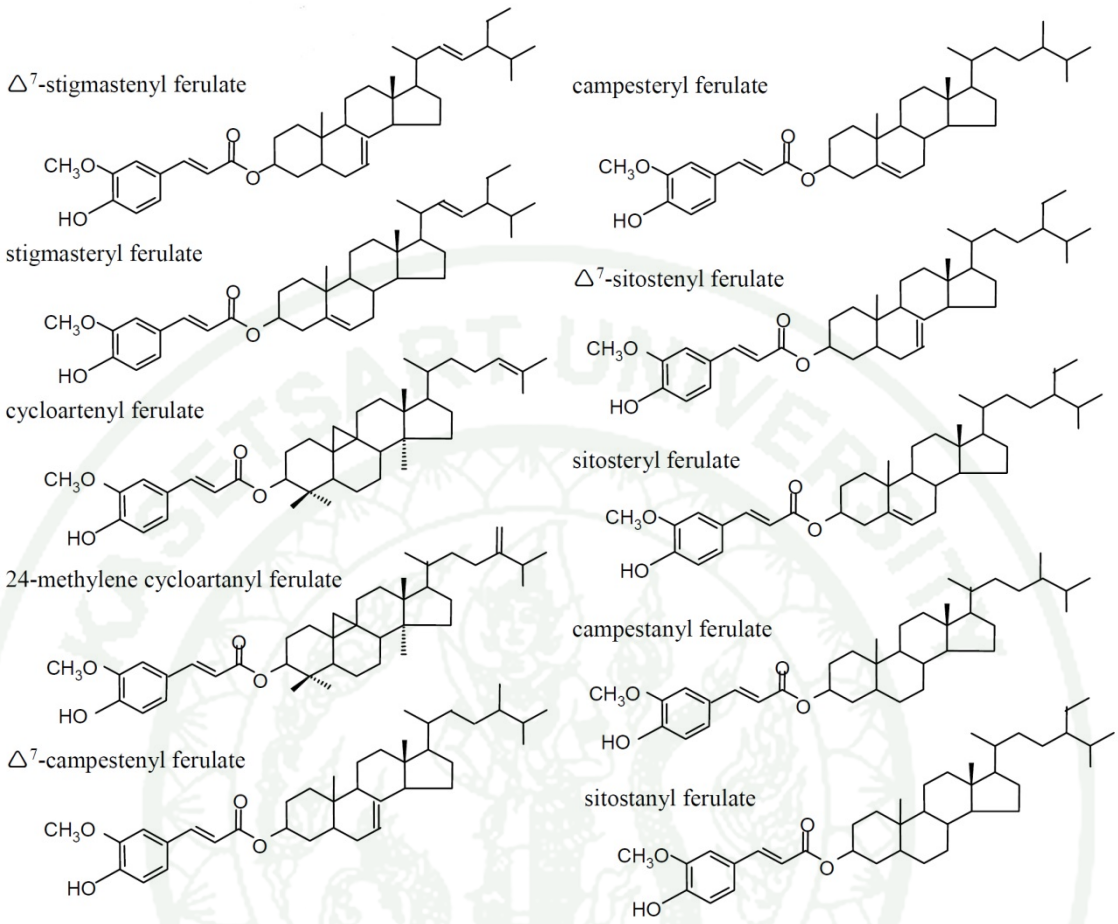


Figure 6 Structure of γ-oryzanol components

Source: Huang (2003)

γ-Oryzanol has showed promising antioxidant activity and antipolymerization of oil during high temperature application. Wang *et al*. (2002) found that sitostanyl ferulate showed antiopolymerization of heated oil when used at high concentration and more effective than γ-oryzanol. Juliano *et al*. (2005) also demonstrated that γ-oryzanol was able to prevent 2,2-azobis(2,4-dimethylvaleronitrile) (AMVN)-triggered lipoperoxidation and improved the oxidative stability of oils containing high polyunsaturated fatty acids. According to

Nyström *et al*. (2007), sitostanyl ferulate was a capable antioxidant for high temperature applications. However, antioxidant activity of γ-oryzanol is influenced by its composition. 24-Methylenecycloartanyl ferulate and campesteryl ferulate were more effective antioxidants than cycloartenyl ferulate for linoleic acid under UV; while 24-methylenecycloartanyl ferulate demonstrated stronger antioxidative activity against autoxidation of linoleic acid in the dark than did cycloartenyl ferulate (Miller and Engel, 2006). According to Xu *et al*. (2001), 24-methylenecycloartanyl ferulate was more effective in inhibiting oxidation of the cholesterol than cycloartenyl ferulate or campesteryl ferulate.

Another important property of γ-oryzanol is cholesterol-lowering effect, a similar health effect close to phytosterol. Wilson *et al*. (2007) reported that γ-oryzanol reduced plasma lipid and lipoprotein cholesterol concentrations in hypercholesterolemic hamsters. Moreover, the cholesterol-lowering activity of γ-oryzanol may also be influenced by its composition. 4-Desmethylsterols liberated from γ-oryzanol in the gut may be responsible for cholesterol-lowering effect (Miller and Engel, 2006).

5. Improving water dispersibility of oil-soluble phytochemical

5.1 Vesicle encapsulation

Vesicles are closed uni- or multilamellar spherical or ellipsoidal structures of bilayer membranes that enclosed a number of aqueous or liquid compartments (Mollet and Grubenmann, 2001; Pegg and Shahidi, 2007). The vesicles are formed when amphiphilic molecules such as surfactants and polymers that have both polar and non-polar characteristic are dispersed in polar solvent. As a result, hydrophobic interactions cause them to spontaneously self-assemble into a rich array of thermodynamically stable, lyotropic, liquid crystalline phases with

characteristic length scales in the nanometer range (Pegg and Shahidi, 2007). Vesicles based on amphiphilic lipids made from biological substances like phospholipid are known as liposomes (Mollet and Grubenmann, 2001); whereas vesicles that form from self-assembled non-ionic surfactants are called niosome. Mahale and colleagues (2012) categorized vesicle systems into 5 types according to the major component used in vesicle preparation as showed in Table 8. Vesicles are thus able to encapsulate a wide range of functional or bioactive ingredients, both water soluble and lipid soluble compounds, by incorporating them into their hydrophilic core or hydrophobic bilayer.

Table 8 Different vesicular systems and their principal components

Type	Vesicle system	Principal components
1	Liposomes	Phospholipid (natural or synthetic)
2	Niosomes	Nonionic surfactant + lipids
3	Ethosomes	Phospholipids + ethanol
4	Transferosomes	Phospholipids + single chain surfactants
5	Bilosomes	Phospholipids + nonionic surfactant + bile salts

Source: Mahale *et al.* (2012)

5.1.1 Liposome (phospholipid vesicle)

Liposome structure is depended on its preparation technique. Liposomes are classified by their membrane structure and diameter size. Liposomes that contain a single bilayer membrane are called unilamellar vesicles. They can be divided into small (SUVs), large (LUVs) and giant (GUVs) unilamellar vesicles with diameter of ≤ 50 nm, 100 nm-10 μm and ≥ 10 μm, respectively (Liu, 2008). When liposomes are formed by more than one bilayer, they are called oligolamellar (LOVs) or multilamellar vesicles (MLVs) (Mollet and Grubenmann, 2001; Bagatolli, 2009). Multilamellar liposomes can be prepared by directly swelling and shaking dried phospholipid film in aqueous solvent. This resulted in broad size distribution ranging

from hundreds of nanometers to several micrometers of vesicle diameters (Bagatolli, 2009).

Liposome preparation methods have been categorized by Bagatolli (2009) into 4 groups, i.e. prepared from dry lipid films, micelle-forming detergents (detergent depletion method), mixing non-aqueous lipid solution with aqueous solution (solvent injection method) and emulsion methods. The simplified diagram is showed in Figure 7.

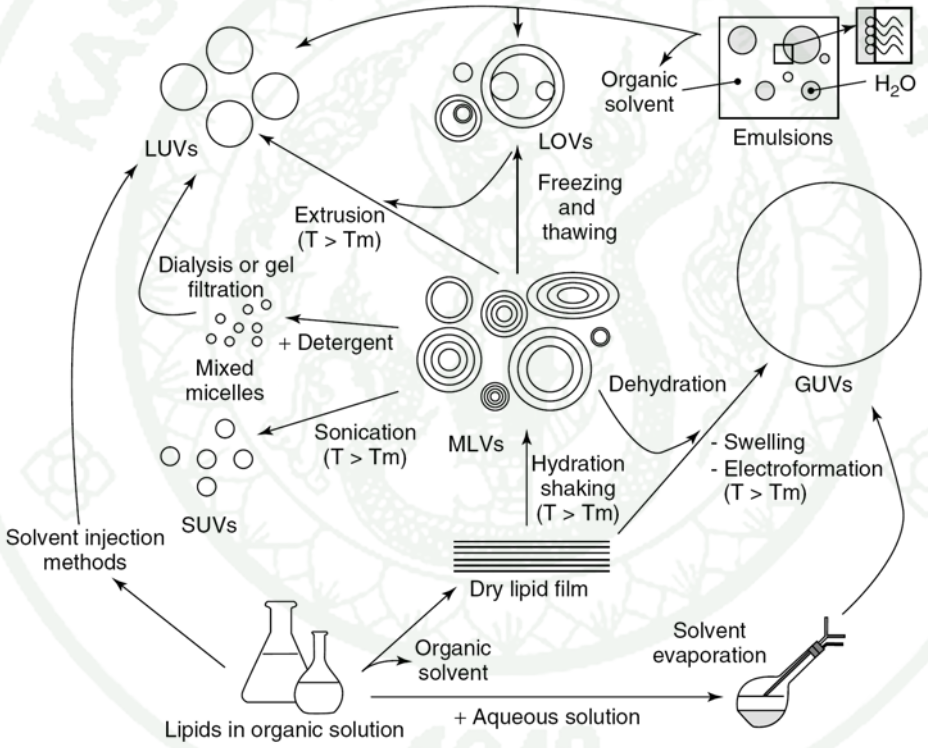


Figure 7 Liposomes preparation method diagram. SUVs: small unilamellar vesicles; LUVs: large unilamellar vesicles; LOVs: large oligolamellar vesicles; MLVs: multilamellar vesicles and GUVs: giant unilamellar vesicles

Source: Bagatolli (2009)

5.1.2 Niosome (non-ionic surfactant vesicle)

Unlike liposome, niosome is formed in the presence of non-ionic surfactant as a result from self-assembled hydrated non-ionic surfactant in aqueous media. The closed bilayer structure is structurally and functionally identical to phospholipid vesicle or liposome (Uchegbu and Florence, 1995; Uchegbu and Vyas; 1998). The closed bilayer structure is rarely spontaneous and usually required energy input such as physical agitation or heat (Uchegbu and Vyas; 1998). Niosome have been extensively studied as an alternative to liposome due to its many advantages, e.g. relatively low toxic, higher stability, longer shelf-life, wide variety of surfactant choice, and cost effective characteristics (Uchegbu and Vyas, 1998; Kazi *et al.*, 2010; Mahale *et al.*, 2012).

Factors influencing the success of niosome formation and the structure of niosome include nonionic surfactant structure and concentration, cholesterol concentration, nature of encapsulated drug, pH of hydration medium, hydration temperature and preparation technique (Kazi *et al.*, 2010; Kumar and Rajeshwarrao, 2011). The nature of nonionic surfactant greatly influenced on niosome formation. The hydrophobic tail of surfactant could consist of one or two alkyl or perfluoroalkyl group or single steroidal group (Uchegbu and Vyas, 1998). Surfactants with alkyl chain length from C12-C18 are suitable for niosome formation. Polyhedral vesicle can be prepared from poly-oxyethylene cetyl ether ($C_{16}EO_5$) or poly-oxyethylene steryl ether ($C_{18}EO_5$) (Biswal *et al.*, 2008). Moreover, vesicle forming ability of surfactant could be indicated by its hydrophile-lipophile-balance (HLB). HLB between 4-8 are suitable for sorbitan monostearate (span) vesicle formation (Uchegbu and Vyas, 1998). HLB value of surfactant also affected entrapment efficiency of niosome. For example, surfactants having HLB value of 8.6 (Span20) resulted in high entrapment efficiency niosome, where those having HLB of 14-17 (Tween20, Tween60 and Tween80) are not suitable for niosome formation by Bangham method with cholesterol (Shahiwala and Misra, 2002). Shahiwala and Misra (2002) explained that hydrophilic surfactants that have high aqueous solubility

or hydration do not reach a state of concentrated systems in order to allow free hydrated units to aggregate and coalesce to form lamella structure.

The structure of surfactant also affected geometry of vesicle. The critical packing parameter (CPP) is greatly related to surfactant structure as explained by Figure 8 and following Equation 8:

$$CPP = V / l_c a_0 \qquad \text{....Equation (8)}$$

Where V = hydrophobic group volume

l_c = the critical hydrophobic group length

a_0 = The area of hydrophilic head group

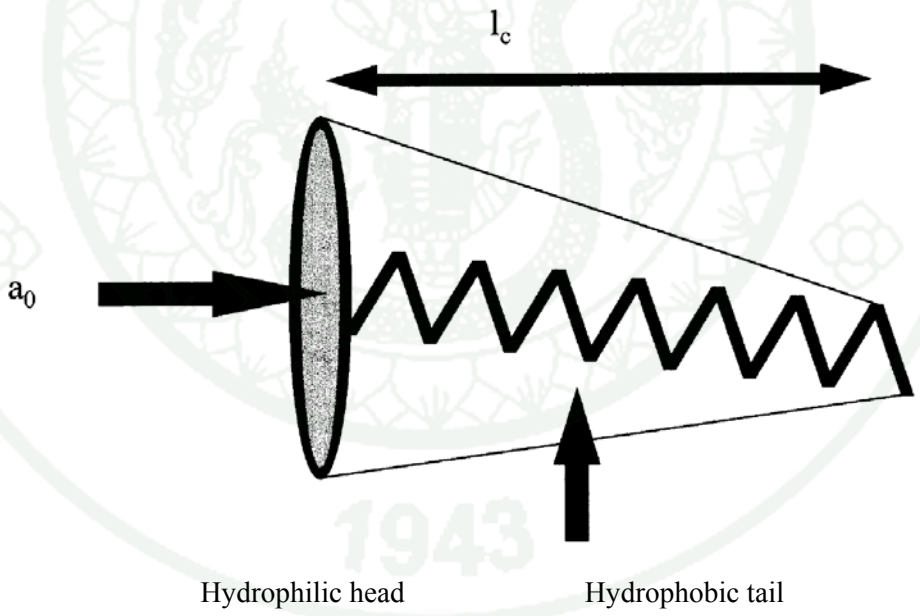


Figure 8 Schematic of surfactant stucture to calculate critical packing parameter, V is hydrophobic group volumn, l_c is the critical hydrophobic group length and a_0 is the area of hydrophilic head group

Source: Uchegbu and Vyas (1998)

Surfactant could form vesicle when the CPP value is between 0.5-1.0; while surfactants having CPP below 0.5 with large contribution of hydrophilic head group area would gave spherical micelles. In the present study Tween80 or Polyoxyethylene (20) sorbitan monooleate, which have HLB around 15 was used to structure the shape of vesicles. When CPP value exceeds 1, large contribution from hydrophobic group volume would produce inverted micelles instead of vesicles (Biswal *et al.*, 2008).

Hydration temperature is another important factor influencing the shape and size of niosome. The ideal temperature for niosome formation should be above the gel to liquid phase transition temperature of surfactant in water at high concentration. The changes in temperature during fabrication could affect assembling process of surfactants into vesicle, and induce vesicle shape transformation (Biswal *et al.*, 2008). For example, mixture of hexadecyl diglycerol ether ($C_{16}G_2$), a poly(24)oxyethylene cholesteryl ether (Solulan C24) (91:9), formed polyhedral vesicle at 25 °C but it could convert to spherical vesicle at 45 °C. It could produce small spherical niosome during cooling from 55 °C to 49 °C (Arunothayanun *et al.*, 1999; Rajera *et al.*, 2011).

Moreover, the physicochemical properties of the core materials such as drugs could affect the charge and rigidity of niosome bilayer. Vesicle size could increase due to the interactions of the encapsulated drug and surfactant head groups, that caused mutual repulsion between surfactant bilayer (Biswal *et al.*, 2008) and disrupt the vesicle structure. Niosome stability can be increased by adding membrane additive during niosome formulation along with the surfactant and encapsulated drug. The good example for membrane additive is cholesterol, which could increase rigidity and decrease drug permeability of niosome (Rajera *et al.*, 2011).

5.1.3 Stabilizing vesicle structure

Cholesterol plays an important role on vesicle formation generally known as vesicle stabilizer. Cholesterol and its derivatives have been included in liposome preparation. Cholesterol stabilizes phospholipids membrane into a more ordered structure. It can improve the fluidity of liposome bilayer membrane, reduce permeability of water soluble molecule and reduce acid sensitivity of liposome (Liu and Huang, 1989; Vemuri and Rhodes, 1995). Moreover, cholesterol is able to stabilize liposome by reducing interactions between liposome and blood proteins such as albumin, *m*-transferrin, and macroglobulin. These compounds could destabilize liposome and reduce the utilization of liposome as a delivery system (Kirby *et al.*, 1980; Liu and Huang, 1989; Vemuri and Rhodes, 1995). Cholesterol molecule inserts itself into bilayer along with phospholipid molecules by facing its hydroxyl group toward the water phase; while its tricyclic ring oriented among acyl chain of phospholipid molecule in the hydrophobic compartment of the bilayer (Vemuri and Rhodes, 1995; Chan *et al.*, 2004).

Amount of cholesterol required to stabilize vesicle system also depends on the HLB of surfactants. As HLB increases above 10, additional cholesterol should be put in to compensate the influences of large hydrophilic head groups (Kumar and Rajeshwarrao, 2011). Although cholesterol shows many beneficial effects in vesicle preparation, hypercholesterolaemia is one of major risk factor of cardiovascular disease in some people. Phytosterols could be used as cholesterol alternative due to their chemical and physicochemical properties (Folmer, 2003).

Phytosterols demonstrate their ability to regulate membrane fluidity of soybean phosphatidylcholine (PC) vesicles (Schuler *et al.*, 1990; 1991). Sitosterol and campesterol showed ability to order the acyl chain and reduce water permeability of soybean PC with equivalent or more efficient than did cholesterol. Nonetheless, the presence of double bond in stigmasterol side chain reduced its ability to execute both functions (Schuler *et al.*, 1990; 1991). Many researchers

demonstrated the feasibility of phytosterol to substitute cholesterol liposome to encapsulate many core materials. Chan and colleague (2004) demonstrated that β-sitosterol and stigmasterol could replace cholesterol during liposome-forming process to encapsulate bovine serum albumin prepared by dehydration-rehydration method. Hwang *et al.* (2010) also reported that substitution of cholesterol by phytosterols increased encapsulation efficiency of antihypertensive oligopeptides from tuna cooking juice against storage, pH, oxidation and pepsin to the similar degree compared with cholesterol prepared vesicles. However, the investigation on the use of phytosterols to stabilize oil-soluble core materials in vesicles has been limited.

5.2 Encapsulation of oil-soluble phytochemicals in pharmaceutics and cosmetics

Oil-soluble phytochemicals of rice bran oil possess many benefits in perspectives of health (antioxidant and cholesterol-lowering properties) and food additives (antioxidant activity). However, they have limited applications in aqueous systems due to their low water dispersibility. Vorarat *et al*. (2010) developed microemulsion system for skin cosmetic with high antioxidant activity and no skin irritation. They encapsulated rice bran oil and γ-oryzanol using Cremophor and Span 80 as surfactants and absolute ethanol as co-surfactant. Stable rice bran oil nanoemulsion for skin disease treatment was also formulated by Bernardi and colleague (2011). The nanoemulsion was composed of 10% rice bran oil, 10% surfactants sorbitan oleate/PEG-30 from castor oil, 0.05% antioxidant and 0.50% preservatives in distilled water. *In vitro* and *in vivo* assessment showed the potential use of rice bran oil nanoemulsion as a useful tool for skin disease treatment.

Engel and Schubert (2005) developed phytosterol-enriched o/w emulsion that could increase dose response and free phytosterol solubility in oil phase up to 30% by using crystallization inhibiter, lecithin, and Tween20 or BPS-30 (polyoxyethylene-phytosterol) as an emulsifier. Leong *et al*. 2011 prepared water-soluble phytosterol nanodispersions for food formulation using an emulsification–evaporation technique. Their results showed that the use of hexane as organic phase

was able to produce particle with monomodal distribution at a mean diameter size of approximately 50 nm. Moreover, phytosterol nanodispersions prepared with a higher homogenisation pressure and a higher organic to aqueous phase ratio resulted in significantly larger phytosterol nanoparticles in the aqueous phase.

Adel *et al.* (2010) used a mixture of β-sitosterol and γ-oryzanol to form self-assembled nano-tubules in triacylglycerol with diameter of 7.2 ± 0.1 nm and a wall thickness 0.8 ± 0.2 nm, at 16% total sterol concentration. However, Adel *et al.* (2010) found that β-sitosterol and γ-oryzanol formed crystals instead of tubules. At 32% total sterol, the tubules were formed next to the crystals of the individual compounds when assessed by small-angle X-ray scattering (SAXS). However, the tubule structure in these emulsions could change during storage and form a larger structures in the emulsion over time. Bot *et al*. (2011) prepared special type of nano-fibril structure fabricated by a mixture of β-sitosterol and γ-oryzanol. The w/o emulsion contained 16 or 32 % total sterol (esters)s in lipid phase and 10, 30 or 60% water. Izadi *et al*. (2012) improved method for the enrichment of phytosterol by dispersing phytosterol in o/w emulsion that composed of phytosterol, emulsifier, soybean oil and water. They found that the particle volume mean diameter ($D_{4,3}$) of their o/w emulsion decreased about 30% when the emulsifier concentration in the system increased, resulting in the increase in apparent viscosity of emulsion.

Neves *et al*. (2008) produced stable uniform γ-oryzanol-loaded o/w emulsion by using a microfluidic device called the microchannel (MC). In this system, refined soybean oil containing γ-oryzanol was used as the dispersant. Sucrose monolaurate or gelatin solution (1 wt. %) was used as emulsifier. The formulation result in highly monodispersed γ-oryzanol-loaded o/w emulsion with average droplets with size of 28.8 μm formulated using the grooved MC emulsification.

Due to the composition of the unevaporated fraction (UMD) after molecular distillation, which contained γ-oryzanol and mono-, di-acylglycerol (Nukit *et al*., 2014), the present study thus explored the composition of UMD further for its

oil- soluble phytochemicals and potential use of UMD as the source of core materials and noisome fabricator stabilizer with enhanced water dispersibility and stability.

6. Rice bran oil and immune response in cell model and animal model

Rice bran oil is well known as healthy oil since it is composed of many nutracueticals that possess many health benefits (Orthoefer, 2005). There are many factors that influence the immune system development including diets and nutritional status of individual. Among nutrients, lipids have a crucial role in the immune system (Sierra *et al*., 2005). The unsaponifiable components of RBO is, in part, responsible for health effect of RBO. Sierra and colleague (2005) showed that RBO modulated the immune system by enhancing B-lymphocyte proliferation and T-helper1-type (T_H1-type) cytokine such as Interleukin-2 (IL-2) or Tumor Necrosis Factors-α (TNF-α) in mice. They also suggested that RBO may have antiallergenic properties due to the reduction of the T-helper 2 (TH2) cytokine Interleukin-4 (IL-4) and immunoglobulin E (IgE) levels in mice. Moreover, they also pointed out that γ-oryzanol may partly modulate the immune system.

Vitamin E is another RBO component that plays crucial role in normal function of immune cells. Yamada *et al*. (2002) suggested that tocotrienol and α-tocopherol modulated lipid metabolism and immune functions in aged sprague-dawley rats. Supplementation of vitamin E above currently recommended levels has been showed to improve immune functions in the aged rats; which included delayed-type hypersensitivity skin response and antibody production in response to vaccination (Meydani *et al*., 2005). Vitamin E improved immune functions by mediating through the increased production of IL-2, leading to the enhanced proliferation of T cells, and through reduced production of prostaglandin E_2, a T-cell suppressive factor, as a result of a decreased peroxynitrite formation. The vitamin E-induced enhancement of immune functions in the aged animals was associated with significant improvement in resistance to influenza infection in aged mice and a

reduced risk of acquiring upper respiratory infections in nursing home residents (Meydani *et al*., 2005). Moreover, tocotrienol has been showed to contribute the immunomodulation, antibody production and resistance to implanted tumor (Nesaretnam *et al*., 2006).

Phytosterols component in RBO is well known as natural hypocholesterolimic agents. However, not only cholesterol lowering effect, phytosterol also showed other benefits such as protective effect in cancer and cardiovascular disease and immunological effects (Desai *et al*., 2009). According to Calpe-Berdiel *et al*. (2007), phytosterols modulated the T-helper immune response *in vivo*, in part independently of their hypocholesterolemic effect in a setting of acute, aseptic inflammation in a mouse model. Morover, β-sitosterol, a major member of phytosterol, is effectively modulating the secretion of pro/anti-inflammatory cytokines and showed beneficial effect in multiple sclerosis management without side effect related to statin therapy (Desai *et al*., 2009).

MATERIALS AND METHODS

Materials

1. Phytochemicals in rice bran oil

1.1 Phytochemicals in commercial vegetable oils in Thai market

1.1.1 Raw materials

Vegetable oils, including palm, soybean, corn, sunflower and rice bran oil were produced and purchased locally in Thailand. One of refined rice bran oil brand of export market was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand).

1.1.2 Reagents for phytosterols and cycloartenol determination

1.1.2.1 5α-cholestane ($C_{27}H_{48}$, Sigma, St. Louis, MO, USA)

1.1.2.2 Potassium hydroxide (KOH, Merck KGaA, Darmstadt, Germany)

1.1.2.3 Ethanol (C_2H_6O, Merck KGa, Darmstadt, Germany)

1.1.2.4 Heptane (C_7H_{16}, analytical grade, Fisher Scientific, Leicestershire, UK)

1.1.2.5 Diethyl ether ($C_4H_{10}O$, ACI Labscan, RCI Labscan limited, Bangkok, Thailand)

1.1.2.6 Sylon BFT (N,O-*bis*(trimethylsilyl) trifluoroacetamide (BSTFA) : trimethylchlorosilane (TMCS), 99:1) (Sigma, St. Louis, MO, USA)

1.1.2.7 Pyridine (C_5H_5N, Sigma, St. Louis, MO, USA)

1.1.2.8 β-Sitosterol ($C_{29}H_{50}O$, Sigma, St. Louis, MO, USA)

1.1.2.9 Campesterol ($C_{28}H_{48}O$, Sigma, St. Louis, MO, USA)

1.1.2.10 Stigmasterol ($C_{29}H_{48}O$, Sigma, St. Louis, MO, USA)

1.2 Phytosterol and γ-oryzanol in commercial refined rice bran oils

1.2.1 Reagents for γ-oryzanol determination

1.2.1.1 Hexane (C_6H_{14}, analytical grade, Mallinckrodt chemicals, Avantor Performance Materials, Inc., NJ, USA)

1.2.1.2 γ-Oryzanol purity *Japonica* rice minimum 98% was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.2.2 Ferulic derivatives of rice γ-oryzanol determination

1.2.2.1 γ-Oryzanol from *Japonica* rice purity minimum 99% was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.2.2.2 γ-Oryzanol from *Indica* rice purity minimum 98% was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.3 Potential use of rice bran oil deodorizer distillate as the source of phytochemicals

1.3.1 Raw materials

1.3.1.1 Crude rice bran oil (CRBO) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.3.1.2 Physically refined rice bran oil (RBO) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.3.1.3 Rice bran oil deodorizer distillate (DD) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.3.1.4 Evaporated fraction of molecular distillation product (EMD) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.3.1.5 Unevaporated fraction of molecular distillation product (UMD) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

1.3.2 Reagents for acid value analysis

1.3.2.1 Phenolphthalein ($C_{20}H_{14}O_4$, LABCHEM, Asia Pacific Specialty Chemicals Limited, NSW, Australia)

1.3.2.2 Isopropyl alcohol (C_3H_8O)

1.3.2.3 Toluene (C_7H_8)

1.3.2.4 Potassium hydroxide (KOH, Merck KGaA, Darmstadt, Germany)

1.3.3 Reagents for phytosterol determination

Reagents for phytosterol determination was described in Section 1.1.2.

1.3.4 Reagents for γ-oryzanol determination

Reagents for γ-oryzanol determination was described in Section 1.2.1.

2. Effect of distillation temperature during molecular distillation on rice phytochemicals

2.1 Chemical characteristic of rice bran oil deodorizer distillate

2.1.1 Raw materials

2.1.1.1 Rice bran oil deodorizer distillate (DD) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

2.1.1.2 Molecular distillation products were kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

2.1.2 Reagents for acid value analysis

Reagents for acid value analysis were described in Section 1.3.2

2.1.3 Reagents for γ-Oryzanol determination

Reagents for γ-oryzanol determination was described in Section 1.2.1.

2.1.4 Reagents for Phytosterol determination

Reagents for phytosterol determination was described in 1.1.2.

2.1.5 Reagents for Tocopherols and tocotrienols determination

2.1.5.1 α-Tocopherol (Sigma, St. Louis, MO, USA)

2.1.5.2 γ-Tocopherol (Sigma, St. Louis, MO, USA)

2.1.5.3 α-, β-, γ and δ-Tocotrienols (Davos Life Science, Biopolis, Singapore).

2.1.5.4 Pyridine (Sigma, St. Louis, MO, USA)

2.1.5.5 N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + TMCS (trimethylchlorosilane), 99:1 (sylon BFT) (Sigma, St. Louis, MO, USA)

2.1.5.6 Heptadecanyl stearate (Sigma, St. Louis, MO, USA)

2.1.6 Reagents for 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay (DPPH assay)

2.1.6.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)

2.1.6.2 Ethyl acetate

2.1.6.3 α-Tocopherol (Sigma, St. Louis, MO, USA)

2.2 Thermo-oxidative stability of rice bran oil deodorizer distillate

2.2.1 Raw material for thermo-oxidative stability analysis

2.2.1.1 Rice bran oil deodorizer distillate (DD) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

2.3 Influence of molecular distillation temperature on chemical characteristic of molecular distillation product.

2.3.1 Raw material for molecular distillation

2.3.1.1 Rice bran oil deodorizer distillate (DD) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

2.3.2 Reagents for Phytosterol determination

Reagents for phytosterol determination was described in 1.1.2.

2.3.3 Reagents for γ-oryzanol determination

Reagents for γ-oryzanol determination was described in Section 1.2.1.

2.3.4 Reagents for acid value analysis

Reagents for acid value analysis were described in Section 1.3.2

2.3.5 Reagents for tocopherols and tocotrienols determination

Reagents for tocopherols and tocotrienols were described in Section 2.1.5

2.3.6 Reagents for 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay (DPPH assay)

Reagents for DPPH assay were described in Section 2.1.6

3. Improving water dispersibility of oil-soluble rice phytochemicals

3.1 Fabrication of oil-soluble rice phytochemical vesicle

3.1.1 Raw material

3.1.1.1 Molecular distillation products (UMD) was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

3.1.1.2 Soy lecithin was kindly provided by Rama Production Co., Ltd. (Solae^{tm} de-oiled soy lecithin, St. Louis, MO, USA).

3.1.1.3 Tween80 (HLB 15) (Sigma, Sigma-Aldrich Chemical Co., St. Louis, MO, USA).

3.1.1.4 Sucrose palmitate (HLB 16) was provided by Caltech Corp., Ltd. (P1670, Mitsubishi-Kagaku Food Corporation, Tokyo, Japan).

3.1.2 Reagents oil-soluble rice phytochemical vesicle preparation

3.1.2.1 Chloroform ($CHCl_3$, analytical grade, analytical grade, ACI Labscan, RCI Labscan limited, Bangkok, Thailand)

3.1.2.2 Sodium chloride (NaCl, UNIVAR, Ajax Finechem, Auckland, New Zealand)

3.1.2.3 Potassium chloride (KCl, UNIVAR, Ajax Finechem, Auckland, New Zealand)

3.1.2.4 Disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$, UNILAB, Ajax Finechem, Auckland, New Zealand)

3.1.2.5 Potassium dihydrogen phosphate (KH_2PO_4,)

3.2 Storage stability of Tween80/UMD vesicle

3.2.1 Raw material

Raw materials were described in Section 3.1.1

3.2.2 Reagents

Reagents were described in Section 3.1.2

4. Cytotoxicity of Tween80/UMD vesicle in Caco-2 cell monolayer and THP-1 macrophages

4.1 Use of UMD to stabilize Tween80/UMD vesicle

4.1.1 Raw material

4.1.1.1 Oilve oil was purchased from a local market in The Netherlands

4.1.1.2 Molecular distillation products were kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

4.1.2 Reagents

4.1.2.1 Tween80 (Sigma, MO, USA)

4.1.2.2 Chloroform ($CHCl_3$, analytical grade, Fisher Scientific, Leicestershire, UK)

4.1.2.3 Diolein (LGC Standards GmbH, Wesel, Germany)

4.1.2.4 Triolein (Sigma, MO, USA)

4.1.2.5 β-Sitosterol (Sigma, MO, USA)

4.1.2.6 Phosphate Buffered Saline pH 7.4 (1X) [-] $CaCl_2$ [-] $MgCl_2$ (Gibco®, Life Technologies™, NY, USA)

4.2 Stability of Tween80/UMD vesicle during *in vitro* gastro-intestinal digestion

4.2.1 Raw material

4.1.1.1 Molecular distillation products were kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

4.2.2 Reagents

4.2.2.1 Tween80 (Sigma, MO, USA)

4.2.2.2 Chloroform ($CHCl_3$, analytical grade, Fisher Scientific, Leicestershire, UK)

4.2.2.3 Phosphate Buffered Saline pH 7.4 (1X) [-] $CaCl_2$ [-] $MgCl_2$ (Gibco®, Life Technologies™, NY, USA)

4.2.2.4 Sodium chloride (NaCl, SIGMA-ALDRICH, MO, USA)

4.2.2.5 Potassium chloride (KCl, Merck KGaA, Darmstadt, Germany)

4.2.2.6 Hydrochloric acid (HCl, Merck KGaA, Darmstadt, Germany)

4.2.2.7 Pepsin from porcine gastric mucosa (Sigma, MO, USA)

4.2.2.8 Sodium bicarbonate ($NaHCO_3$, Merck KGaA, Darmstadt, Germany)

4.2.2.9 Pancreatin from porcine pancrease (Sigma, MO, USA)

4.2.2.10 Lipase from porcine pancrease (Sigma, MO, USA)

4.2.2.11 Taurocholic acid sodium salt (Sigma, MO, USA)

4.2.2.12 Sodium glycodeoxycholate (Sigma, MO, USA)

4.3 Influence of Tween80/UMD vesicle on viability of Caco-2 monolayer

4.3.1 Raw material

4.3.1.1 Molecular distillation products were kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

4.3.1.2 Heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) cells passage number between 30-54 (American Type Culture Collection, Rockville, MD, USA)

4.3.2 Reagents

4.3.2.1 Tween80 (Sigma, MO, USA)

4.3.2.2 Chloroform ($CHCl_3$, analytical grade, Fisher Scientific, Leicestershire, UK)

4.3.2.3 Phosphate Buffered Saline pH 7.4 (1X) [-] $CaCl_2$ [-] $MgCl_2$ (Gibco®, Life Technologies™, NY, USA)

4.3.2.4 Dulbecco modified eagle medium, [+] 4.5g/L D-Glucose, [+] L-Glutamine, [+] 25 mM HEPES, [-] Pyruvate (DMEM (1X), Gibco®, Life Technologies™, NY, USA)

4.3.2.5 Fetal bovine serum (FBS; research grade, Hyclone®, Thermo Scientific, Hyclone UK Ltd., Northumberland, UK)

4.3.2.6 Penicillin/streptomycin (Pen/Strep) (Gibco®, Life Technologies™, NY, USA)

4.3.2.7 0.25% Trypsin-EDTA (1X) (Gibco®, Life Technologies™, NY, USA)

4.3.2.8 TritonX-100

4.3.2.9 24 well cell culture cluster flat bottom with lid (Costar 3526, Corning Incorporated, NY, USA)

4.3.2.10 Transwell insert, pore diameter 0.4 μm, translucent PET membrane (ThinCert™-24 well, Greiner bio-one®)

4.3.2.11 96-wells cell culture cluster V bottom with lid (Costar 3894, Corning Incorporated, NY, USA)

4.4 Influence of Tween80/UMD vesicle on viability of THP-1 macrophage

4.4.1 Raw material

4.4.1.1 The human monocyteic leukemia cell line (THP-1, American Type Culture Collection, Rockville, MD, USA)

4.4.1.2 Molecular distillation products were kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

4.4.2 Reagents

4.4.2.1 Tween80 (Sigma, MO, USA)

4.4.2.2 Chloroform ($CHCl_3$, analytical grade, Fisher Scientific, Leicestershire, UK)

4.4.2.3 Diolein (LGC Standards GmbH, Wesel, Germany)

4.4.2.4 Triolein (Sigma, MO, USA)

4.4.2.5 β-Sitosterol (Sigma, MO, USA)

4.4.2.6 Phosphate Buffered Saline pH 7.4 (1X) [-] $CaCl_2$ [-] $MgCl_2$ (Gibco®, Life Technologies™, NY, USA)

4.4.2.7 96 well cell culture cluster flat bottom with lid (Costar 3526, Corning Incorporated, NY, USA)

4.4.2.8 RPMI 1640 culture medium with L-Glutamine and 25 mM Hepes (Biowhittaker®, Lonza, Walkersville, MD)

4.4.2.9 Phorbol 12-myristate 13 acetate (PMA, Sigma, MO, USA)

4.4.2.10 Fetal bovine serum (FBS; research grade, Hyclone®, Thermo Scientific, Hyclone UK Ltd., Northumberland, UK)

4.4.2.11 Penicillin/Streptomycin (Pen/Strep) (Gibco®, Life TechnologiesTM, NY, USA)

4.4.2.12 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

4.5 Uptake of Tween80/UMD vesicles by Caco-2 cell monolayer

4.5.1 Raw material

4.5.1.1 Molecular distillation products were kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

4.5.1.2 Heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) cells passage number between 30-54 (American Type Culture Collection, Rockville, MD, USA)

4.5.2 Reagents

4.5.2.1 Tween80 (Sigma, MO, USA)

4.5.2.2 Chloroform ($CHCl_3$, analytical grade, Fisher Scientific, Leicestershire, UK)

4.5.2.3 Phosphate Buffer Saline pH 7.4 (1X) [-] $CaCl_2$ [-] $MgCl_2$ (Gibco®, Life Technologies™, NY, USA)

4.5.2.4 24 well cell culture cluster flat bottom with lid (Costar 3526, Corning Incorporated, NY, USA)

4.5.2.5 Transwell insert, pore diameter 0.4 μm, translucent PET membrane (ThinCert™-24 well, Greiner bio-one®)

4.5.2.6 Phenol red

4.5.2.7 Phenol red-free DMEM, [+] 4.5g/L D-Glucose, [+] L-Glutamine, [+] 25 mM HEPES, [-] Sodium Pyruvate (DMEM (1X), Gibco®, Life Technologies™, NY, USA)

4.5.2.8 Coumarin 6 (546283 ALDRICH , MO, USA)

4.5.2.9 Phosphate Buffered Saline pH 7.4 (1X) [-] $CaCl_2$ [-] $MgCl_2$ (Gibco®, Life Technologies™, NY, USA)

4.5.2.10 Fetal bovine serum (FBS; research grade, Hyclone®, Thermo Scientific, Hyclone UK Ltd., Northumberland, UK)

4.5.2.11 Penicillin/Streptomycin (Pen/Strep) (Gibco®, Life Technologies™, NY, USA)

4.5.2.12 0.25% Trypsin-EDTA (1X) (Gibco®, Life Technologies™, NY, USA)

5. Effect of oil-soluble rice bran oil phytochemical on THP-1 gene expression

5.1 Raw material

5.1.1 The human monocyteic leukemia cell line (THP-1, American Type Culture Collection, Rockville, Md.)

5.1.2 Molecular distillation products were kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

5.2 Reagents

5.2.2 Tween80 (Sigma, MO, USA)

5.2.3 Chloroform ($CHCl_3$, analytical grade, Fisher Scientific, Leicestershire, UK)

5.2.4 Diolein (LGC Standards GmbH, Wesel, Germany)

5.2.5 Triolein (Sigma, MO, USA)

5.2.6 β-Sitosterol (Sigma, MO, USA)

5.2.7 α-Tocopherol (Sigma, MO, USA)

5.2.8 γ-Oryzanol from Japonica rice purity minimum 98% was kindly supported by Surin Bran Oil Co., Ltd. (Surin, Thailand)

5.2.9 Phosphate Buffered Saline pH 7.4 (1X) [-] $CaCl_2$ [-] $MgCl_2$ (Gibco®, Life Technologies™, NY, USA)

5.2.10 96 well cell culture cluster flat bottom with lid (Costar 3526, Corning Incorporated, NY, USA)

5.2.11 RPMI 1640 culture medium with L-Glutamine and 25 mM Hepes (Biowhittaker®, Lonza, Walkersville, MD)

5.2.12 Phorbol 12-myristate 13 acetate (PMA, Sigma, MO, USA)

5.2.13 Fetal bovine serum (FBS; research grade, Hyclone®, Thermo Scientific, Hyclone UK Ltd., Northumberland, UK)

5.2.14 Penicillin/Streptomycin (Pen/Strep) (Gibco®, Life Technologies™, NY, USA)

Methods

1. Phytochemicals in rice bran oil

1.1 Phytochemicals in commercial vegetable oils in Thai market

1.1.1 Phytosterols and cycloartenol determination

Selected phytosterols, both free and esterified ones, were determined using method described by Schwartz *et al*. (2008). Briefly, 0.2-0.4 g of sample and internal standard (5α-cholestane) were saponified with 0.5 mL of saturated KOH in 8 mL ethanol at 85 °C in a water bath shaker for 30 min. Twelve milliliter distilled water was added and the saponified samples were extracted with 20 mL of heptane:diethyl ether (1:1 v/v) mixture. Five mL of the extracted organic layer was evaporated to dryness with N_2 gas. The residue was silylated with 200 μL of N,O-*bis*(trimethylsilyl) trifluoroacetamide (BSTFA) : trimethylchlorosilane (TMCS), 99:1 (Sylon BFT(and 200 μL of pyridine at room temperature overnight. The silylation reagent was evaporated and the residue was dissolved in heptane. One microliter of silylated sample was injected into a gas chromatograph equipped with flame ionization detector (HP 6890, Series GC System, Hewlett Packard, Wilmington, DE, USA) and HP-5 capillary column (30 m × 0.32 mm × 0.25 μm, Hewlett Packard, Wilmington, DE, USA). The inlet and detector temperatures were set at 300 °C and He gas was used as a carrier gas at the flow rate of 3 mL/min. The oven temperature program was set to begin at 70 °C and increase to 245 °C at the rate of 60 °C/min, hold for 1 min and increase to 275 °C at the rate of 2 °C/min and hold for 30 min. The phytosterol contents were quantified by using calibration curves of β-sitosterol, campesterol and stigmasterol. Triterpene alcohol cycloartenol was analyzed using the same method.

1.1.2 Statistical analysis

Two different batches of all vegetable oil samples were analyzed for phytosterols and cycloartenol in 3 replicates for each batch. The data were analyzed by analysis of variance (ANOVA) at a significance level of $p<0.05$. All statistical analyses were performed using SPSS software version 12 (SPSS Inc., Chicago, IL)

.

1.2 γ-Oryzanol in commercial refined rice bran oils

1.2.1 γ-Oryzanol determination

The γ-oryzanol content in oil sample was determined spectrophotometrically using method described by Khatoon and Gopala Krishna (2004). Briefly, 10 mg of sample was weighed into a 10 mL volumetric flask, dissolved in hexane and determined for γ-oryzanol content by UV spectrophotometer (Spectronic Genesys 10 UV Scanning Thermo Electron Corporation, Waltham, MA, USA) at 314 nm using 1 cm cell length. The γ-oryzanol content was determined by measuring the absorbance at 314 nm using a UV spectrophotometer (Spectronic Genesys 10 UV Scanning Thermo Electron Corp., Waltham, MA, USA). The γ-oryzanol content was then calculated as showed in Equation 9:

$$\gamma - oryzanol\,\text{content}(\text{mg}/100g) = \frac{\text{Absorbance at 314 nm in hexanesolution} * 10000}{\text{g of sample} * 358.9}$$

.....Equation (9)

1.2.2 Ferulic derivatives of rice γ-oryzanol determination

γ-Oryzanols from *Japonica* and *Indica* rice were alkaline hydrolysis to de-esterified phytosterol and triterpene. The free ferulic derivatives were determined for their phytosterol and triterpene content using gas chromatography (Schwartz *et al*., 2008) as described in Section 1.1.1

1.2.3 Statistical analysis

Two different batches of all samples were analyzed for γ-oryzanol in 3 replicates for each batch. The data were analyzed by analysis of variance (ANOVA) at a significance level of $p<0.05$. All statistical analyses were performed using SPSS software version 12 (SPSS Inc., Chicago, IL).

1.3 Potential use of rice bran oil deodorizer distillate as the source of phytochemicals

1.3.1 Acid value determination

The acid value of CRBO, RBO, DD, EMD and UMD were determined as FFA equivalent to oleic acid according to the AOCS Official Method Cd 3d-63(AOCS, 1997). Briefly, 2 mL of 1% phenolphthalein in isopropyl alcohol was added to a 125 mL of solvent mixture (isopropyl alcohol: toluene, 1:1) and neutralized to faint pink. Samples were weighed into an Erlenmeyer flask, added with 125 mL of solvent mixture containing phenolphthalein, and titrated with 0.1 M KOH. The acid value was calculated as showed in Equation 10:

$$Acid\ value\ (\text{g oleic acid equivalent in 100 g sample}) = \frac{(A-B) * Molarity\ \text{of KOH} * 56.1}{g\ \text{sample} * 1.99}$$

.....Equation (10)

where

A was mL of 0.1 M KOH titrated with sample.

B was mL of 0.1 M KOH used in titrating blank (solvent mixture).

1.3.2 Phytosterol determination

CRBO, RBO, DD, EMD and UMD were determined for phytosterol contents using gas chromatography as described in Section 1.1.1.

1.3.3 γ-Oryzanol determination

The CRBO, RBO, DD and molecular distillation products was determined for γ-oryzanol using spectrophotometer as described in Section 1.2.1.

2 Effect of distillation temperature during molecular distillation on rice phytochemicals

2.1 Chemical characteristic of rice bran oil deodorizer distillate

2.1.1 Phytosterol determination

DD were determined for phytosterol content using gas chromatography as described in Section 1.1.1.

2.1.2 γ-Oryzanol determination

DD was determined for γ-oryzanol using spectrophotometer as described in Section 1.2.1.

2.1.3 Acid value determination

The DD was determined for acid value using AOCS Official Method Cd 3d-63 as described in Section 1.3.1

2.1.4 Tocopherols and tocotrienol determination

The AOCS Recommended Practice Ce 7-87 (AOCS, 1997) was used to quantify tocopherols (α-T and γ-T form) and tocotrienols (α-T3, β-T3, γ-T3 and δ-T3 form) contents. Brifely, 20-30 mg of samples were silylated by 1 mL of pyridine and 2 mL of of N,O-*bis*(trimethylsilyl) trifluoroacetamide (BSTFA) + TMCS (trimethylchlorosilane), 99:1 (sylon BFT(. Sample was heated at 50 °C for 10 min. The internal standard (heptadecanyl stearate) was added into silylated sample and mixed well. Aliquot (1 μL) of sample was injected into the gas chromatographic apparatus and quantified for tocopherol and tocotrienol contents by using response factor (FC) as showed in Equation 11.

$$FC = \frac{A_{\text{IS}} \times C_{\text{Standard}}}{A_{\text{Standard}} \times C_{\text{IS}}} \qquad \text{.....Equation (11)}$$

where A_{IS} is the area of internal standard (heptadecanyl stearate), A_{Std} is the area of tocol standards, C_{IS} is *mg* of internal standard and C_{Std} is mg of tocol standards.

Capillary gas chromatography was performed by gas chromatograph equipped with a flame ionization detector (HP 6890 Series GC System, Hewlett Packard, USA) and HP-5 capillary column (30 m X 0.32 mm X 0.25 µm, Hewlett Packard, USA). Helium gas was used as a carrier gas at flow rate of 2 mL/min. Oven temperature was programmed to perform at 140 to 300 °C using heating rate of 10 °C/min and hold for 6 min; and the temperature was increased to 300 to 320 °C at the rate of 5 °C/min and hold for 10 min. The injector and detector were maintained at 240 and 345 °C, respectively.

2.1.5 Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay)

The total free radical-scavenging capacity of DD, determined as 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay), was evaluated using modified method described by Rossi *et al*. (2007) and Ghafoorunissa (2007). DPPH was dissolved in ethyl acetate (126.8 µM) and adjusted the dilution to obtain an absorbance at 515 nm of 0.6237 absorbance unit (AU) (UV/Vis-spectrophotometer, Infinite M200Pro, Tecan Group Ltd., Männedorf, Switzerland). Sample was diluted with HPLC-grade ethyl acetate at 5 concentrations; i.e. to obtain approximately 200-3000 µg/mL. A mixture of 100 µL of DPPH solution (final concentration 63.4 µM) and 100 µL of diluted sample was incubated in the dark at 25 ± 0.1°C for 30 min. The absorbance (*Abs*) of mixture was measured at 515 nm. The % scavenging of sample was determined as showed in Equation 12.

$$\%\ scavenging = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100 \qquad \text{.....Equation (12)}$$

where $Abs_{control}$ was the absorbance at 515 nm of DPPH solution with ethyl acetate (instead of sample) and Abs_{sample} was the absorbance at 515 nm of DPPH solution with UMD sample. The fifty percent inhibition concentration (IC_{50},

µg of sample/mL) was determined graphically by plotting the graph between % scavenging and sample concentration and calculated as µg of sample per mL of solution required to obtain 50% of a maximum scavenging capacity. *α*-Tocopherol was evaluated under same condition for comparison.

2.2 Thermo-oxidative stability of rice bran oil deodorizer distillate

2.2.1 Thermo-oxidative stability analysis

The thermo-oxidative stability of DD was characterized by Mettler Toledo DSC821e apparatus (Schwerzenbach, Switzerland). Four to five mg of DD was weighed into a 40 µL aluminum sample pan, closed with a lid with a hole of 1 mm internal diameter drilled in the center. This hole allowed sample to be in contact with oxygen or nitrogen stream. The sealed aluminum empty pan was used as a reference. The sample and reference pans were heated at the heating rates (*β*) of 2, 5, 10, 16 and 20 °C/min. Experiments were performed under nitrogen steam at 100 mL/min flow rate for thermo-stabilty evaluation. The thermo-oxidative stability of DD was determined under oxygen stream at the flow rate of 100 mL/min. When the run was completed, the onset temperature (T_o) of oxidation was determined as the intersection of extrapolated baseline and the tangent line (leading edge) of the exothermic peak (Ostrowska-Ligeza *et al*., 2010). The characterization was performed in duplicates.

The Ozawa-Flynn-Wall method (OFW) was used to determine activation energy (E_a) (Ostrowska-Ligeza *et al*., 2010). Linear regression of log β versus $1/T_o$ was plotted using the following Equation 13 to determine the slope A:

$$\log \beta = A(1/T_o) + B \qquad \text{.....Equation (13)}$$

Where β is the heating rate (°C/min) and T_o is the onset temperature of oxidation in Kelvin (K). The activation energy (E_a) was calculated from Equation 14 and A equaled to $\frac{\partial \log \beta}{\partial (1/T_0)}$ as showed in Equation 15, where R is gas constant.

$$E_a = -2.19R \frac{\partial \log \beta}{\partial (1/T_0)} \quad \text{.....Equation (14)}$$

$$E_a = -2.19R * A \quad \text{.....Equation (15)}$$

2.3 Influence of molecular distillation temperature on chemical characteristic of molecular distillation product.

2.3.1 Molecular distillation process

Distillation temperatures of 120, 140 and 160 °C were operated on a pilot-scale MD unit at *Surin Bran Oil* Co., Ltd (Surin, Thailand) using a pressure of 0.1 Pa and a flow rate of 10.14-10.66 kg/h. The unevaporated fraction after molecular distillation, designated as UMD, was analyzed for acid value, γ-oryzanol, tocotrienols, tocopherols and phytosterols, as well as DPPH antioxidant capacity, using methods described in bellowed section. All samples were kept at 4 °C in amber glass bottle before analyses.

2.3.2 Phytosterol determination

Molecular distillation products (UMD) of each molecular distillation temperature were determined for phytosterol content using gas chromatography as described in Section 1.1.1.

2.3.3 γ-Oryzanol determination

Molecular distillation products (UMD) of each molecular distillation temperature were determined for γ-oryzanol using spectrophotometer as described in Section 1.2.1.

2.3.4 Acid value determination

Molecular distillation products (UMD) of each molecular distillation temperature were determined for acid value using AOCS Official Method Cd 3d-63 as described in Section 1.3.1.

2.3.5 Tocopherols and tocotrienol determination

Molecular distillation products (UMD) of each molecular distillation temperature were determined for their tocopherol and tocotrienol content using AOCS Official Method Ce 7-87 as described in Section 2.1.4.

2.3.6 Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay)

Molecular distillation products (UMD) of each molecular distillation temperature were determined for their radical scavenging capacity by DPPH method as described in Section 2.1.5.

2.3.7 Statistical analysis

One batch of DD was distilled at different temperatures using a pilot-scale MD unit in a two separate trials. All samples were analyzed for their acid value, DPPH value, γ-oryzanol, phytosterol, tocopherol and tocotrienol content in 3 replicates for each trials. The data were analyzed by analysis of variance (ANOVA) at a significance level of $p<0.05$. All statistical analyses were performed using SPSS software version 12 (SPSS Inc., Chicago, IL).

3. Improve water dispersibility of oil-soluble rice phytochemicals

3.1 Fabrication of oil-soluble rice phytochemical vesicle

3.1.1 Preparation of rice phytochemical vesicle in PBS

Vesicle was prepared using Bangham method described by Takahashi *et al*. (2007). Commercial surfactants, namely soy lecithin, Tween80 or sucrose palmitate (0.05 to 0.16 g) and UMD sample obtained from MD operated at 140 °C (0.04-0.15 g) were dissolved in 8 mL chloroform in a screw-cap test tube and mixed thoroughly. The solvent was evaporated to dryness under N_2 stream. The residual solvent was further dried overnight in a hood. Then the thin film of surfactant-UMD was added with 8 mL of phosphate buffer saline (PBS) buffer pH 7.3 and heated at 55-60 °C for 10 min. The test tube was shaken vigorously using a vortex mixer for 5 min. The solid concentration of surfactant/UMD vesicle having different ratios of surfactant to UMD of 1:0, 1:0.25, 1:1, 1:2, 1:3, 1:4 and 1:5 in the suspension was 2.5% (w/v) in PBS.

3.1.2 Determination of particle size distribution of vesicles in PBS

The size distribution of surfactant particle and surfactant/UMD particle in PBS was analyzed by a Zetasizer Nano-ZS (Zen 3600, Malvern Instruments Ltd., Worcestershire, UK). Only treatments capable of UMD holding capacity (no observable phase separation between aqueous phase and oil phase) was used.

3.2 Storage stability of Tween80/UMD vesicle

The 0.22 μm nitrocellulose membrane (GSWP, MF-Millipore Membrane™, Millipore Corp., Ireland) was sprayed with 70% ethylalcohol and equipped with autoclaved housing and test tube before filtration. Tween80/UMD vesicles at ratio of Tween80 to UMD of 1:3 in PBS were aseptically filtered to sterilize the suspensions. The filtered sterile suspensions were kept at 4 – 5 °C or 37 °C for 0, 24, 48, 72 and 96 h before determination of size distribution using a Zeta Nano-ZS.

4. Cytotoxicity of Tween80/UMD vesicle in Caco-2 cell monolayer and THP-1 macrophages

4.1 Effect of UMD on the fabrication of Tween80-based vesicles

Tween80 based vesicle was prepared by using Bangham method as described in Section 3.1.1 to obtain the final concentration of surfactant/UMD at 2.5% (w/v), which would contain tocols 453 μg/mL, phytosterol 814 μg/mL, γ-oryzanol 201 μg/mL and Tween80 6,250 μg/mL. Tween80 was used as surfactant fabricating vesicles to encapsulate UMD in dispersion. Extra virgin olive oil was used as lipid phase in the absence of UMD. β-Sitosterol and dioleoylglycerol surfactant were tested for their ability for vesicle stabilizer and co-surfactant, respectively. Tween80 based vesicle composition in each treatment is presented in Table 9. The suspensions

were prepared by dissolving the chemicals and UMD in 8 mL chloroform in a screw-cap test tube and mixed thoroughly. The solvent was evaporated to dryness under N_2 stream. The residual solvent was further dried overnight in a hood. Then the thin film of surfactant-UMD was added with 8 mL of phosphate buffered saline (PBS) pH 7.3 and heated at 55-60 °C for 10 min. The test tube was shaken vigorously using vortex mixer for 5 min. The suspensions were tested for size distribution by using Zetasizer Nano-ZS (Zen 3600, Malvern Instruments Ltd., Worcestershire, UK).

Table 9 Lipid vesicle composition

Treatment	Weight of components added in 8 mL PBS (mg)						
	Tween80	Added β-sitosterol	Added dioleoylglycerol	Added olive oil	UMD composition (150 mg)		
					γ-Oryzanol	Tocols	Phytosterol
a	50	-	-	113.5	-	-	-
b	50	-	-	-	1.6	3.6	6.5
c	50	6.5	-	113.5	-	-	-
d	50	-	30	113.5	-	-	-
e	50	6.5	30	113.5	-	-	-
Treatment	Concentration of components added in 8 mL PBS (mg/mL)						
	Tween80	Added β-Sitosterol	Added Dioleoylglycerol	Added Olive oil	UMD composition (18.8 mg/mL)		
					γ-Oryzanol	Tocols	Phytosterol
a	6.3	-	-	14.2	-	-	-
b	6.3	-	-	-	0.2	0.5	0.8
c	6.3	0.8	-	14.2	-	-	-
d	6.3	-	3.8	14.2	-	-	-
e	6.3	0.8	3.8	14.2	-	-	-

4.2 Stability of Tween80/UMD vesicle during *in vitro* gastro-intestinal digestion

The prepared Tween80/UMD vesicles at Tween80 to UMD ratio of 1:3 were exposed to various *in vitro* digestion steps as previously described by Vreeburg *et al.* (2012). Briefly, 10 g of Tween 80/UMD vesicle suspension (25 mg/mL) was mixed with 140 mM NaCl 5 mM KCL 10 mL in 50 mL tube and adjusted pH to 2 with 1 M HCl. A 0.667 mL of 40 g/ L porcine pepsin in 0.1 M HCl was added to suspension and incubated for 30 min at 37 °C. After incubation, 1 M $NaHCO_3$ was used to raise the pH to 5.8, then 0.95 mL of 40 g/ L pancreatin in 0.1 M $NaHCO_3$, 40 g/L lipase in 0.1 M $NaHCO_3$ and 0.5 mL of bile salt were added to the suspension and pH was adjusted to 6.5 by 1 M $NaHCO_3$. Tube headspace was flushed with N_2 gas and incubated at 37 °C for 30 min. The digestion was stopped by adjusting pH to 7.5 using 1 M $NaHCO_3$. The digested sample was further analysed for their size distribution by a Zetasizer Nano-ZS to estimate stability of vesicles against physiological conditions of the GI tract. All experiments were performed in triplicate.

4.3 Influence of Tween80/UMD vesicle on viability of Caco-2 monolayer

The colorimetric MTT metabolic activity assay was used to determine viability of Caco-2 cells monolayer after they were exposed to Tween80/UMD vesicles. Briefly, Caco-2 cells were seeded at density of 1.95 X 10^5 cells per insert in a 24 transwell plates format and grew to confluence for 21 d. Culture medium was replaced every other day. The transeptihelial electrical resistance (TEER) value was measured by using MilliCell-ERS Ω-meter to assess the integrity of Caco-2 monolayers. Caco-2 monolayers with a TEER value above 200 Ω/cm^2 were used for viability test. The 21-day old Caco-2 monolayers were exposed to Tween80/UMD vesicle at concentration 0.1, 1.0 and 5.0 mg/mL for 3 h at 37 °C in 5% CO_2 humidified incubator. After incubation, medium in basolateral and apical chambers were removed, and the cells were washed with fresh PBS, added with 50 µL of 0.25%

trypsin-EDTA into transwell apical chamber and incubated for 10 min at 37 °C to detach cells from transwell membrane. The 100 µL of DMEM medium was added into the apical chambers to inhibit trypsin activity. Detached cell suspension was transferred to 96-wells V bottom plate. The plate was centrifuged at 500 relative centrifugation force (rcf) for 5 min. The supernatant was removed by using multi-channel micropipette. The sediment cells was resuspended in 100 µL of MTT solution containing 0.5 mg/mL MTT in DMEM and 10% FBS and incubated for 2 h at 37°C in 5% CO_2 humidified incubator. The plate was centrifuged at 500 rcf for 5 min and MTT solution was discarded using multi-channel micropipette. A mixture of 50 µL of DMSO:ethanol (1:1) was added into each well and the plate was mildly shaken for 5 min. The absorbance was measured at 570 nm using microplate reader (Infinite 200 PRO, Tecan Group Ltd., Männedorf, Switzerland). All experiments were performed in duplicate, and the viability of Caco-2 in the absence of Tween80/UMD (designated as control), but contains PBS in similar amount as the treatment groups, was used to compare cytotoxicity of Tween80/UMD.

4.4 Influence of Tween80/UMD vesicle on viability of THP-1 macrophage

Differentiation of THP-1 monocyte into THP-1 macrophage was performed. The human monocyteic leukemia cell line (THP-1, American Type Culture Collection, Rockville, Md.) was grown in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (P/S). THP-1 monocytes were differentiated into macrophages by addition of phorbol 12-myristate 13 acetate (PMA, Sigma). In brief, 0.5 mL (5 x 10^5 cells) or 82 µL (8.2 x 10^4 cells) of cell suspension containing PMA (final concentration 100 ng/ mL of cell suspension) were seeded into 24 wells and 96 wells cell culture plate, respectively, and incubated in an incubator humidified with 5% CO_2 at 37 ^oC for 48 h. After incubation, undifferentiated monocyte and PMA were discarded and differentiated macrophages were washed twice with RPMI 1640 culture medium containing 10% FBS and 1% penicillin/streptomycin (P/S) and let the cells rest for 24 h before experiment.

The colorimetric MTT metabolic activity assay was used to determine viability of THP-1 macrophage after exposure to Tween80/UMD vesicles. The differentiated macrophages were treated with 100 uL of sample, i.e. PBS (control) and Tween80/UMD vesicles at concentration 0.01, 0.1 and 1.0 mg/mL into the designed well and incubated for 24 h. After incubation, the stimulating mediums were removed and 100 μL of MTT solution (0.5 mg/mL) were added to each well and incubated for 2 h. MTT solutions were discarded and 30 μL DMSO: ethanol (1:1 v/v) were added and mildly shake the plate for 5 minutes and read absorbance at 570 nm.

4.5 Uptake of Tween80/UMD vesicles by Caco-2 cell monolayer

Caco-2 cells was seeded at a density of 1.95 X 10^5 cells per insert on a 24 well-plate format and grew to confluence for 21 d. The 21-days old Caco-2 monolayer was incubated with encapsulated 6-cumarin entrapped in Tween80/UMD vesicle at final concentration of 5 mg/mL in DMEM medium for 3 h. The volume of Tween80/UMD vesicle in PBS added to media was comtrolled not to exceed 20% of original media. Treated monolayer was washed 3 times with PBS to remove excess vesicle. The Caco-2 monolayer on transwell membrane was cut and placed on glass slide and viewed under confocal laser scanning microscopy (CLSM, Zeiss LSM510) to observe the uptake of Tween80/UMD vesicle into Caco-2 cells. The excitation wavelength for 6-coumarin was 495 nm and the emission wavelength was 520 nm. The uptake of Tween80/UMD containing 6-coumarin would fluorescent green colour under CLSM.

5 Effect of oil-soluble rice bran oil phytochemical on THP-1 gene expression

Various types of Tween80-vesicles were prepared by using Bangham method as describe in Section 3.1.1. Tween80-vesicle containing UMD was designated as Tween80/UMD vesicle (UV). The 8 mL of vesicle suspensions contained Tween80 (50 mg), dioleoylglycerol (30 mg), trioleoylglycerol (113.5 mg) and β-sitosterol (6.5 mg) was designated as Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol vesicle (EV). Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol vesicle containing either α-tocopherol (0.06 mg/mL) or γ-oryzanol (0.2 mg/mL) was designated as Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol/tocopherol (TV) and Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol/γ-oryzanol (OV) vesicle, respectively (Appendix Table A1).

The differentiated THP-1 macrophages were stimulated for 24 h with Tween80-vesicles at concentration 0.01, 0.1 and 1 mg/mL. Stimulated macrophages were harvested and immunomodulatory activities were investigated by measuring gene expression. The expression of genes encoding for pro-inflammatory cytokines, i.e. IL-1β, IL-8 and TNF-α were evaluated.

The gene expression by real-time qPCR was conducted by isolation of total RNA by using RNeasy mini kit (Qiagen, USA) with RNase-free DNase (Qiagen, USA) treatment for 15 min. The 1% agarose gel was used for checking the purity of RNA sample and Nanodrop was used to calculate the concentration of RNA in sample. The complementary DNA (cDNA) was synthesized from isolated RNA sample with iScript cDNA synthesis kit (Bio-Rad, USA). cDNA 200 ng was mixed with 10 μL of IQ^{TM} SYBR Green Supermix (Bio-Rad, USA) and primer pair in 20 μL reaction volume. The mixture was preheated at 95 °C for 90 s, followed by PCR for 40 cycles, denaturing at 95 °C for 10 s, annealing at 58 °C for 10 s, elongation at 72 °C for 15 s, and finally elongation at 72 °C for 2 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen for normalization. The values expressed as

fold change relative to the value at time point zero, calculated as ΔΔCt as described in Equation 16-19.

$$\Delta Ct_{sample} = Ct_{sample} - Ct_{GAPDH} \quad \text{.....Equation (16)}$$

$$\Delta Ct_{media} = Ct_{media} - Ct_{GAPDH} \quad \text{.....Equation (17)}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{media} \quad \text{.....Equation (18)}$$

$$relative\ fold\ change = 2^{-(\Delta\Delta Ct)} \quad \text{.....Equation (19)}$$

Where Ct_{GAPDH} is glyceraldehyde-3-phosphate dehydrogenase threshold cycle, Ct_{sample} is sample threshold cycle and Ct_{media} is threshold cycle of media.

RESULTS AND DISCUSSION

1. Phytochemicals in rice bran oil

1.1 Phytochemicals in commercial vegetable oils in Thai market

Phytosterol content in commercial vegetable oils investigated ranged from 55 to 1,034 mg/100 g (Table 10). The highest content was found in all brands of RBO, which contained phytosterols around 972-1,034 mg/100 g, followed by corn oil, soybean oil, sunflower oil and palm oil, respectively. β-Sitosterol was the most abundant phytosterol, followed by campesterol and stigmasterol. The phytosterol contents in palm, soybean, corn and sunflower oil were found in the same range of those oils sold in the US market (Phillips *et al*., 2002). Phytosterol contents in sunflower oil reported in this study agreed with the contents previous reported in Spain, Finland and Egypt (Jiménez-Escrig *et al*., 2006; Schwartz *et al*., 2008; Hassanien, 2012) but much lower than those found in sunflower oil reported by Normén *et al*. (2007). Phytosterol composition in the same type of oil was also varied, which likely reflected the differences in processing, growing season, or variety of a particular plant source and analytical method (Phillips *et al*., 2002; Van Hoed *et al*., 2006). Phytosterol instability during refining step may cause the variation in phytosterol contents in the refined oils. Verleyen and colleague (2002) demonstrated that phytosterol dehydrated and formed steradienes during bleaching and deodorizing process. Beside dehydration product, the formation of monomeric and dimeric thermo-oxidative products could explain the loss in phytosterol contents during vegetable oil refining process (Struijs *et al.*, 2010).

The contents of three major phytosterols namely; β-sitosterol, campesterol and stigmasterol, in RBO from different commercial brands were summarized in Table 10. All commercial RBO contained more than 800 mg/100g of those phytosterols. The results are in good agreement with those reported by Van Hoed *et al.* (2010). The most abundant phytosterol found was β-sitosterol, followed by campesterol and stigmasterol.

Table 10 Selected phytosterol and cycloartenol contents in refined vegetable oils.

Types of oil	Brands	Phytosterol content (mg/100g)				Cycloartenol (mg/100g)
		Campesterol	Stigmasterol	β-Sitosterol	Sum	
Palm	1	$17.6^{gh} \pm 2.0$	$13.9^{f} \pm 0.7$	$48.3^{e} \pm 5.8$	$79.7^{h} \pm 7.9$	Not detected
	2	$12.1^{h} \pm 1.9$	$10.3^{f} \pm 0.3$	$33.3^{e} \pm 5.4$	$55.7^{h} \pm 7.5$	Not detected
	3	$22.0^{gh} \pm 6.1$	$17.7^{f} \pm 4.5$	$57.5^{e} \pm 16.5$	$97.1^{h} \pm 26.7$	Not detected
	4	$16.2^{gh} \pm 2.6$	$14.1^{f} \pm 0.5$	$42.7^{e} \pm 8.2$	$73.1^{h} \pm 10.9$	Not detected
Soybean	1	$44.3^{ef} \pm 4.2$	$62.6^{c} \pm 1.0$	$130.1^{d} \pm 11.9$	$237.1^{f} \pm 16.0$	$11.1^{gh} \pm 0.9$
	2	$44.7^{ef} \pm 5.1$	$61.0^{c} \pm 4.0$	$138.9^{d} \pm 15.0$	$249.5^{f} \pm 19.5$	$11.7^{gh} \pm 5.7$
	3	$53.7^{e} \pm 4.9$	$57.7^{c} \pm 0.9$	$137.6^{d} \pm 12.8$	$249.0^{f} \pm 17.3$	$9.1^{h} \pm 0.4$
Corn	1	$92.1^{d} \pm 9.6$	$77.3^{b} \pm 19.1$	$438.2^{c} \pm 32.7$	$607.6^{e} \pm 50.0$	$21.3^{fgh} \pm 1.7$
	2	$182.3^{c} \pm 26.8$	$61.2^{c} \pm 5.3$	$528.7^{b} \pm 73.0$	$772.2^{d} \pm 102.7$	$23.1^{efg} \pm 2.1$

Means in the same column followed by different superscripts are significantly different ($p<0.05$).

Table 10 Selected phytosterol and cycloartenol contents in refined vegetable oils (continue).

Types of oil	Brands	Phytosterol content (mg/100g)				Cycloartenol (mg/100g)
		Campesterol	Stigmasterol	β-Sitosterol	Sum	
Rice bran	1	$226.8^{a} \pm 30.0$	$172.1^{a} \pm 11.3$	$573.6^{ab} \pm 78.2$	$972.5^{ab} \pm 114.5$	$92.2^{ab} \pm 14.7$
	2	$211.8^{b} \pm 14.7$	$162.7^{a} \pm 5.2$	$535.8^{b} \pm 22.4$	$910.2^{bc} \pm 33.6$	$101.2^{ab} \pm 16.1$
	3	$208.0^{b} \pm 13.0$	$169.2^{a} \pm 6.0$	$568.0^{ab} \pm 44.1$	$945.2^{abc} \pm 52.6$	$87.4^{b} \pm 5.9$
	4	$240.3^{a} \pm 13.9$	$169.1^{a} \pm 7.0$	$625.0^{a} \pm 66.5$	$1{,}034.4^{a} \pm 72.4$	$110.4^{a} \pm 26.4$
	5	$178.5^{c} \pm 22.0$	$131.3^{b} \pm 8.7$	$547.8^{b} \pm 56.4$	$857.6^{c} \pm 78.0$	$89.3^{ab} \pm 12.6$
Sunflower	1	$26.2^{gh} \pm 2.1$	$27.2^{e} \pm 0.3$	$153.4^{d} \pm 11.1$	$206.7^{fg} \pm 13.4$	$35.6^{de} \pm 3.3$
	2	$20.7^{gh} \pm 1.0$	$18.4^{f} \pm 0.5$	$134.2^{d} \pm 7.3$	$174.4^{g} \pm 8.8$	$31.2^{def} \pm 2.5$
	3	$29.7^{fg} \pm 6.0$	$37.1^{d} \pm 14.5$	$165.7^{d} \pm 10.6$	$232.5^{fg} \pm 28.2$	$38.1^{d} \pm 4.4$

Means in the same column followed by different superscripts are significantly different ($p<0.05$).

Table 10 shows cycloartenol content in refined rice bran oil, sunflower oil, corn oil and soybean oil, respectively. However, it was not detected in palm oil. RBO contained significant amount of unique group of ferulic acid esters of triterpene alcohols and sterols called γ-oryzanol in its unsaponifiable fraction. Sitosteryl ferulate, campesteryl ferulate, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate are the major components of γ-oryzanol (Norton, 1995; Azrina *et al.*, 2008). In phytosterol analysis used in this study, alkaline hydrolysis was included to cleave phytosterol from their esterified fatty acid and esterified ferulic acid. The triterpene alcohol from γ-oryzanol such as cycloartenol and 24-methylenecycloartanol originally esterified with ferulic acid could therefore be liberated and were apparently showed in RBO chromatogram. Figure 9 shows phytosterol profiles of refined vegetable oils investigated. RBO chromatograms showed other peaks after the retention time of 25 min, which were not found in palm oil. These peaks were likely to be cycloartenol and 24-methylenecycloartanol, which is the major triterpene alcohols in γ-oryznol (Norton, 1995; Azrina *et al.*, 2008). γ-Oryznol in rice bran oil occurred as phytosterol and triterpene alcohol esterified with ferulic acid, which could liberate by alkaline hydrolysis step in phytosterol analysis result in free phytosterol such as β-sitosterol and campesterol, and free triterpene alcohol such as cycloartenol and 24-methylenecycloartanol (Moreau *et al*., 2002, Miller and Engel, 2006). For that reason, these free triterpene alcohols are found in RBO chromatogram.

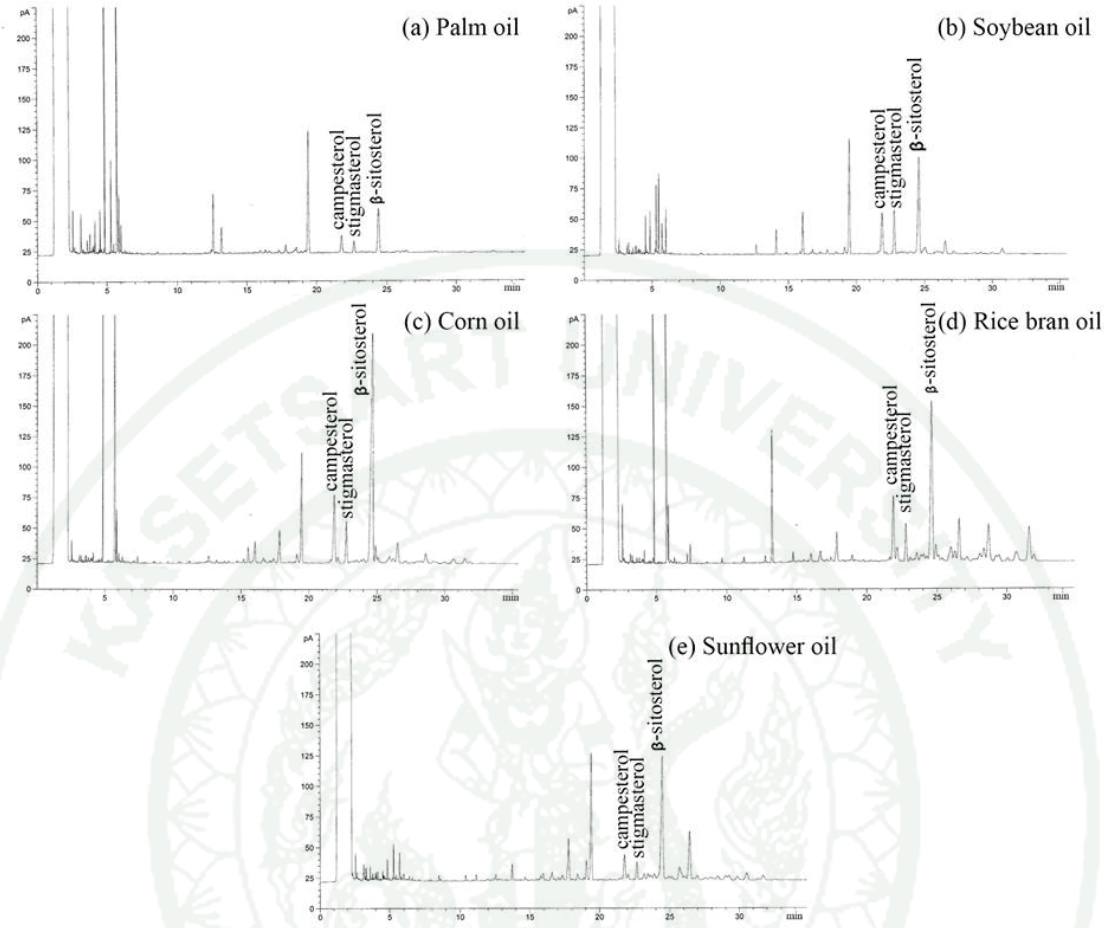


Figure 9 Phytosterol profiles of commercial vegetable oil, (a) palm oil, (b) soybean oil, (c) corn oil, (d) rice bran oil and (e) sunflower oil.

1.2 γ-Oryzanol in commercial refined rice bran oils

The commercial RBO also contained various contents of γ-oryzanol, ranging from 248 to 887 mg/100 g (Table 11). The γ-oryzanol contents were in the same range as those reported in RBO from *Indica* rice (Gopala Krishna *et al.,* 2001; Van Hoed *et al.,* 2006).

Table 11 γ-Oryzanol contents in commercial refined rice bran oil of different brands.

Brands	γ-Oryzanol (mg/100g)
1	$287.0^{d} \pm 1.9$
2	$422.3^{c} \pm 74.2$
3	$248.0^{e} \pm 3.4$
4	$517.2^{b} \pm 19.6$
5	$887.0^{a} \pm 19.3$

Means in the same column followed by different superscripts are significantly different ($p<0.05$).

The variation of γ-oryzanol contents in commercial RBO was likely due to the different refining process and processing parameters used for each commercial brand. The chemical refining process employed during the production of RBO brands 1-4 could result in RBO with low content of γ-oryzanol (Gopala Krishna *et al.,* 2001; Van Hoed *et al.,* 2006). This was due to the loss of γ-oryzanol during neutralization step. In the chemical refining process, γ-oryzanol is concentrated in the soapstock (Gopala Krishna *et al.,* 2001; Van Hoed *et al.,* 2006; Pestana *et al.,* 2008). A major loss of γ-oryzanol content of over 80% of their original content in crude oil during neutralization of RBO was reported; while the steps of degumming, dewaxing and deodorizing did not affect the γ-oryzanol content in RBO (Gopala Krishna *et al.,* 2001; Van Hoed *et al.,* 2006).

The physical refining process, however, resulted in the lower loss of γ-oryzanol compared to the chemical refining process as showed in Brand 5. This was

because the neutralization step was not included in the physical refining process. Consequently, the physical refining process was able to retain most of the original amount of γ-oryzanol in RBO (Gopala Krishna *et al.,* 2001; 2006).

The γ-oryzanol content in RBO was dependent not only on the refining factors as mentioned above, but also on the genetic and environmental factors during rice growing (Lerma-García *et al.,* 2009). Different phytochemical contents in commercial RBO investigated in the present study may result from different rice cultivars as well. Some commercial brands investigated used rice cultivars grown in the central part of Thailand (Brands 1-4) while Brand 5 from the cultivar grown in the north-eastern part, i.e. Khao Dawk Mali 105. Therefore, the effect of physical refining process on the distribution of rice phytochemicals investigated in the following section study was emphasized on Brand 5 that used fresh rice bran from Khao Dawk Mali 105 as raw material for RBO production.

Alkaline hydrolysis was used to de-esterify phytosterols and triterpene alcohols in γ-oryzanols from *Japonica* rice and *Indica* rice into their free forms. Figure 10 shows that γ-oryzanol from *Japonica* rice was composed of 4 major components. They were identified as campesterol, β-sitosterol and cycloartenol, and most likely 24-methylenecycloartanol, respectively at the retention time of 28 min. γ-Oryzanol from *Japonica* rice contained mixture of both phytosterols and triterpene alcohols. The γ-oryzanol from *Indica* rice, however, showed much lower peaks of campesterol, β-sitosterol and cycloartenol. Nonetheless, *Indica* rice γ-oryzanol showed additional peak in the chromatogram at retention time of 31 min, which need further identification. The significant γ-oryzanol variation among rice cultivars observed could reflect their applications. The antioxidant activity of each γ-oryzanol components were varied among experimental system and test method. DPPH radical scavenging activity of all four major γ-oryzanol constituents were similar due to hydroxyl group their esterified ferulic acid was the active unit in γ-oryzanol constituents for DPPH radical scavenging activity (Akiyama *et al*., 2005; Nyström *et*

al., 2005). While 24-methylenecycloartanyl ferulate was more effective in inhibiting oxidation of the cholesterol than cycloartenyl ferulate or campesteryl ferulate (Xu *et al*., 2001). Moreover, sitostanyl ferulate was a capable antioxidant and antipolymerization for high temperature applications (Wang *et al*., 2002; Nyström *et al*., 2007). Moreover, γ-oryzanol that contained high content of phytosterol ester could provide cholesterol-lowering properties (Berger *et al*., 2005; Wilson *et al*., 2007), which may be influenced by their phytosterol composition, β-sitosterol and campesterol, that was hydrolysed into their free form in digestive tract by intestinal enzymes (Mandak and Nyström. 2012). Ferulated triterpene fraction of γ-oryzanol showed anti-inflammatory and immune related effect (Akihisa *et al*., 2000; Nagasaka *et al*., 2007; Oka *et al*., 2010). Cycloartenyl ferulate and 24-methylenecycloartanol showed strong anti-inflammatory activity against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice (Akihisa *et al*., 2000). Moreover, cycloartenyl ferulate captured IgE and prevented it from binding to high-affinity IgE receptor (FcεRI), resulting in the attenuation of allergic reaction. In addition, cycloartenyl ferulate also inhibited nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity, indicating that these cycloartenyl ferulate may also prevent the late phase of allergic inflammation (Oka *et al*., 2010). Cycloartenol ferulate showed inhibitory effect on tumor promotion in carcinogenesis in mouse skin compare to sitosterol ferulate and 24-methylenecycloartanol ferulate (Yasukawa *et al*., 1998).

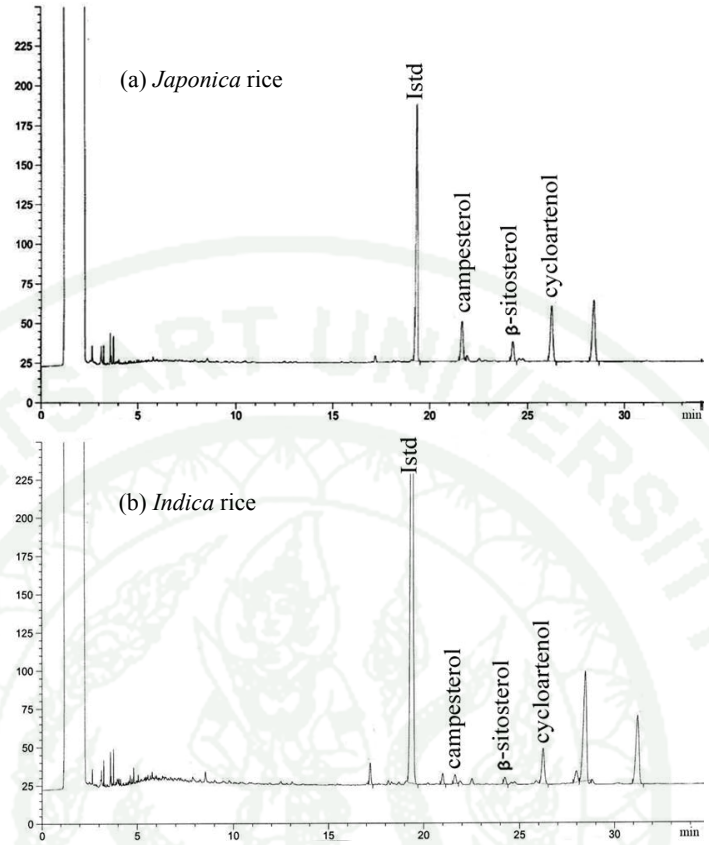


Figure 10 Ferulic derivatives of rice γ-oryzanols from (a) *Japonica* rice and (b) *Indica* rice

1.3 Potential use of rice bran oil deodorizer distillate as the source of phytochemicals

Crude rice bran oil (CRBO), refined rice bran oil (RBO) and deodorizer distillate (DD) obtained from two production batches using physical refining process were investigated. The CRBO contained 1,362 – 1,376 mg phytosterol/100 g (Table 12, Figure 11). Physical refining process resulted in RBO containing 820 – 895 mg phytosterol/100g. Not only the reduction in phytosterol content, but also the content of γ-oryzanol was reduced from 1,599 – 1,666 mg/100 g in CRBO to 933 – 960

mg/100g in RBO, indicating the loss of rice phytochemicals in refined RBO during refining process.

However, both phytosterols and γ-oryzanol were found in significant amounts in DD (Table 12), which contained 457 – 564 mg phytosterol/100 g and 840 – 1,148 mg γ-oryzanol/100 g. Results also indicated that the FFAs in CRBO were effectively reduced by deodorization, resulting in the low acid value in RBO, that is, 0.08 mg KOH/g RBO since the requirement of residual FFAs in the refined oil should not exceed 0.6 mgKOH/g oil (Codex, 1999; Thai Ministry of Public Health, 2000). The acid value of reported RBO was slightly higher than standard of refined oil. In this study, acid value was determined by titration with KOH according to AOCS Cd 3d-63. However, RBO contained significant amount of γ-oryzanol, which phenolic group in ferulic moiety could react with alkali and contribute significantly increase to measured acid value (Gopala Krishna *et al.,* 2006).

Table 12 Ranges of phytochemical contents and acid values of crude rice bran oil (CRBO), refined rice bran oil (RBO) and deodorizer distillate (DD) from 2 production batches of physical refining process.

Chemical constituents	Types of sample		
	CRBO	RBO	DD
Phytosterol (mg/100g)			
Compesterol	281 ± 5 – 292 ± 40	164 ± 24 – 193 ± 3	89 ± 7 – 100 ± 5
Stigmasterol	235 ± 3 – 246 ± 11	126 ± 7 – 136 ± 8	65 ± 4 – 106 ± 2
β-Sitosterol	839 ± 117 – 846 ± 17	520 ± 73 – 576 ± 15	303 ± 15 – 358 ± 18
Sum	1,362 ± 22 – 1,376 ± 168	820 ± 104 – 895 ± 15	457 ± 25 – 564 ± 25
γ-Oryzanol (mg/100g)	1,599 ± 26 – 1,666 ± 10	933 ± 18 – 960 ± 15	840 ± 15 – 1,148 ± 21
Acid value (mg KOH/g)	21.1 ± 1.2 – 21.7 ± 0.8	0.08 ± 0.0 – 0.13 ± 0.0	138.7 ± 2.6 – 154.4 ± 0.6

Mean ± standard deviation of each production batch (n=3/batch) presented in concentration range.

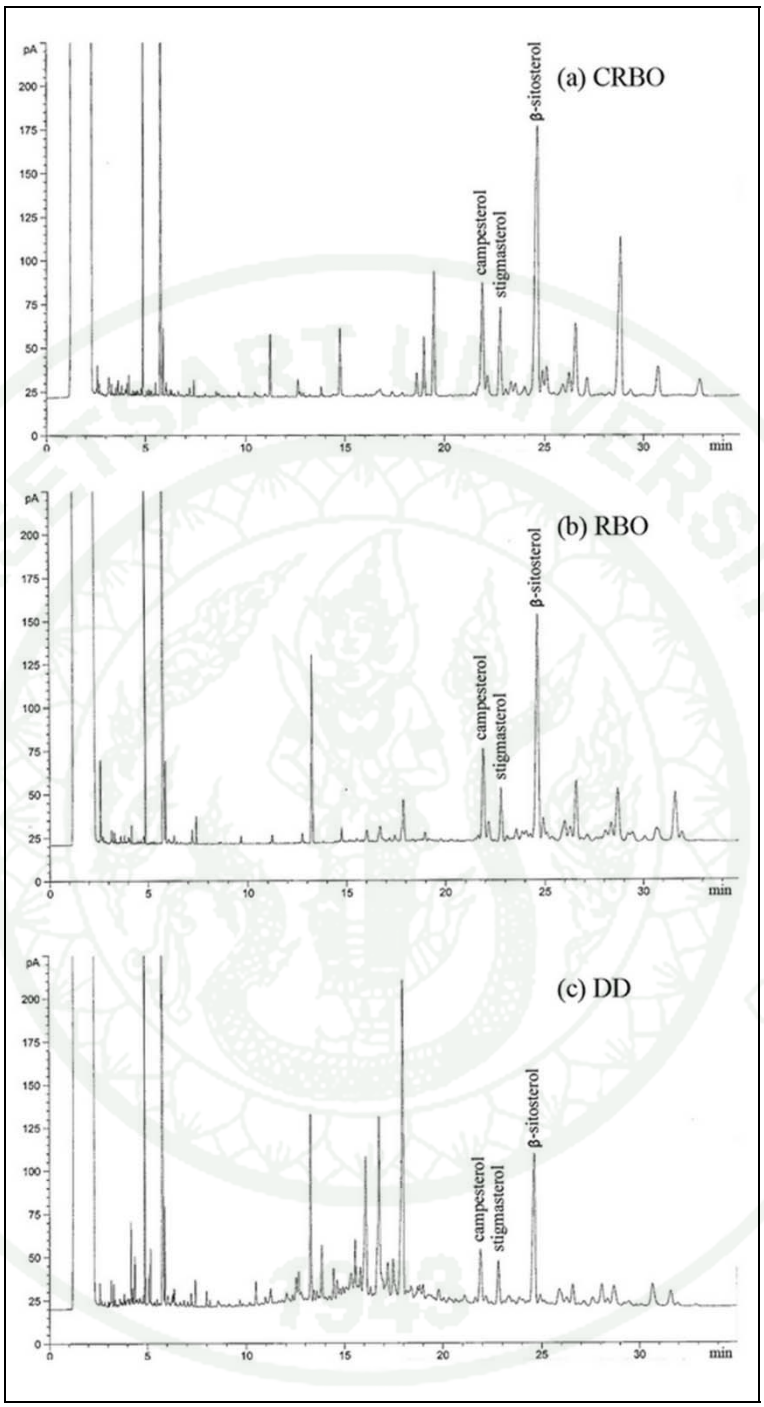


Figure 11 Effect of physical refining process on phytosterol profiles, (a) crude rice bran oil (CRBO), (b) refined rice bran oil (RBO) and (c) deodorizer distillate (DD)

In the present study, the molecular distillation (MD) was used to reduce FFAs in DD and concentrate phytosterols and γ-oryzanol, which had higher boiling point than did FFAs. It was found that the phytosterols were concentrated in the unevaporated fraction (UMD, Table 13). However, DD from both production batches investigated in this study had wide variation of γ-oryzanol (Table 13), resulting in the inconsistency of γ-oryzanol distribution in EMD and UMD. This was probably due to the wide temperature range used during deodorizing step at 240 – 260 °C under vacuum (533- 800 Pa) of both production batches to keep low acid value and the consistency of γ-oryzanol content in refined RBO, which is the final product of the oil refining process. Nonetheless, the present study showed that the MD could effectively remove FFAs from the DD, resulting in the UMD having low acid value of 5.6 – 9.3 mg KOH/g.

Generally, β-sitosterol was the most abundant phytosterol (50-70% of sum phytosterols) found in plants, followed by campesterol and stigmasterol in equal amounts (Phillips *et al.,* 2002; Normén *et al.,* 2007; Derakhshan-Honarparvar *et al.,* 2010). Phytosterol composition resulted from the differences in growing season, the variety of a particular plant source, as well as the process parameters used during oil refining process (Phillips *et al.,* 2002; Van Hoed *et al.,*, 2006). In the present study, phytosterols originally found in CRBO were retained in RBO at a higher concentration than in DD after refining process. The γ-oryzanol, however, was accumulated in DD at fairly high concentration.

Table 13 Range of phytochemical contents and acid values of deodorizer distillate (DD), evaporated fraction after molecular distillation (EMD) and unevaporated fraction after molecular distillation (UMD) from two production batches.

Chemical constituents	Types of sample		
	DD	EMD	UMD
Phytosterol (mg/100g)			
Campesterol	89 ± 7 – 100 ± 5	33 ± 2 – 65 ± 6	309 ± 21 – 566 ± 26
Stigmasterol	65 ± 4 – 106 ± 2	30 ± 0 – 86 ± 4	256 ± 2 – 702 ± 18
β-Sitosterol	303 ± 15 – 358 ± 18	108 ± 1 – 240 ± 21	1,020 ± 54 – 2,123 ± 100
Sum	457 ± 25 – 564 ± 25	171 ± 2 – 392 ± 24	1,585 ± 75 – 3,391 ± 143
γ-Oryzanol (mg/100g)	840 ± 15 – 1,148 ± 21	781 ± 9 – 848 ± 51	592 ± 12 – 1095 ± 11
Acid value (mg KOH/g)	138.7 ± 2.6 – 154.4 ± 0.6	196.3 ± 0.5 – 197.0 ± 4.9	5.6 ± 0.3 – 9.3 ± 0.2

Mean ± standard deviation of each production batch (n=3/batch) presented in concentration range.

During the steam deodorization step, phytosterols were stripped from the CRBO due to their relatively high volatility compared to γ-oryzanol (Gopala Krishna *et al.,* 2001; 2006; Van Hoed *et al.,* 2006). At high temperature during deodorization step of 220-250 °C under 400-667 mmHg vacuum (Orthoefer 2005), phytosterols and tocols could be lost. The loss of phytosterols in CRBO during refining process could also be due to the formation of steradiene and steratrienes, sterol and hydroxyl sterol dehydration products formed during bleaching and deodorizing process (Verleyen *et al.,* 2002; Bortolomeazzi *et al.,* 2003). Thermo-oxidation is one of the major deterioration of phytosterols, which resulted in the formation of hydroxyl, keto and epoxy compounds (Lampi *et al.,* 2002). Nonetheless, the present study indicated reasonable recovery yield of the selected phytosterols from DD when MD was used to further evaporated out the FFAs.

The γ-oryzanol was reported to retain in physically refined RBO for almost 100 % of the original oil by Gopala Krishna *et al.* (2001). However, this is not the case for γ-oryzanol retention in RBO reported in the present study. This may be due to the differences in process parameters used during steam deodorizing step of Thai RBO refining process investigated compared to those reported by Gopala Krishna *et al.* (2001).

From practical standpoints, it is possible that the DD, which showed potential use as the raw material for the production of rice phytochemicals in the present study, could have wide variations since it is the co-product from oil refining process. The influences of processing parameters used during MD operation (e.g. feed flow rate, evaporating temperature, vacuum, etc.) on the partition of rice phytochemicals in the UMD thus need further investigation. Although MD could be used to concentrate phytosterols in the UMD fraction, further investigation in oxidative-thermo stability of rice phytochemicals is reported in Section 2.

2. Effect of distillation temperature during molecular distillation on rice phytochemicals

2.1 Chemical characterization of rice bran oil deodorizer distillate

DD has been showed as potential source of rice phytochemicals that can be recovered by pilot-scale MD unit (Sawadikiat and Hongsprabhas, 2014). The γ-oryzanol content in DD was 598.9 mg/100 g (Table 4). The DD also contained high contents of tocotrienols, tocopherols and phytosterols. The antioxidant capacity, expressed as the IC_{50}, was 2,301.6 μg/mL. However, the acid value was very high, i.e. 125.4 mg KOH/g which is unsuitable for food use.

2.2 Thermo-oxidative stability of rice bran oil deodorizer distillate

DSC thermogram illustrates that in the absence of O_2 (under N_2 stream), DD obtained from physical refining process was thermo-stable between 25-300 °C (Figure 12). However, when O_2 was present, the oxidation of DD started at 121.2 °C at the heating rate of 2 °C/min. Table 15 indicated that raising heating rate increased the onset temperature (T_o) of oxidation. The calculated activation energy (E_a) of DD was 111.3 kJ/mol, which was higher than those of sunflower oil, soybean oil and corn oil (Adhvaryua *et al*., 2000). The *Ea* of rice DD reported in the present study was within the same range of olive oil (Ostrowska-Ligeza *et al*., 2010). The reason was probably that RBO contained relatively higher saturated fatty acid (22 %) and lower polyunsaturated fatty acid (33%) (Van Hoed *et al*., 2006) compared to sunflower oil, soybean oil and corn oil in Adhvaryua *et al*. (2000) studied. While, olive oils in Ostrowska-Ligeza *et al*. (2010) studies contains lower polyunsaturated fatty acid (2%) and high monounsaturated fatty acid around 73%; while sunflower oil, soybean oil and corn oil contain around 46-61% of polyunsaturated fatty acid (Adhvaryua *et al*., 2000; Ostrowska-Ligeza *et al*., 2010). It is generally recognize that Ea value was influenced by fatty acid composition of analyzed oil by increasing in polyunsaturated fatty acid composition could decreasing Ea while increasing monounsaturated and

saturated fatty acid composition could increasing Ea value. Increasing saturated-CH_2 carbon in fatty acid composition would improve the resistance to initial thermal breakdown. Consequently, the activation energy required for such system was also high (Adhvaryua *et al*., 2000).

Table 14 Chemical characteristics of rice bran oil deodorizer distillate (DD) obtained from physical refining process

Chemical constituents	Mean ± s.d.
Acid value (mg KOH/g)	125.4 ± 1.0
γ-Oryzanol (mg/100 g)	598.9 ± 0.5
Tocotrienol contents (mg/100 g)	
α-Tocotrienol	77.7 ± 1.4
β-Tocotrienol	1,132.6 ± 16.1
γ-Tocotrienol	139.2 ± 3.2
δ-Tocotrienol	612.9 ± 15.5
Tocopherol contents (mg/100 g)	
α-Tocopherol	166.1 ± 1.2
γ -Tocopherol	57.2 ± 0.4
Phytosterol contents (mg/100 g)	
β-Sitosterol	970.8 ± 77.9
Campesterol	259.2 ± 24.3
Stigmasterol	310.8 ± 17.4
DPPH radical scavenging capacity (IC_{50}; μg/mL)	2,301.6 ± 45.3

Mean ± standard deviation of one production batch (n=3/batch)

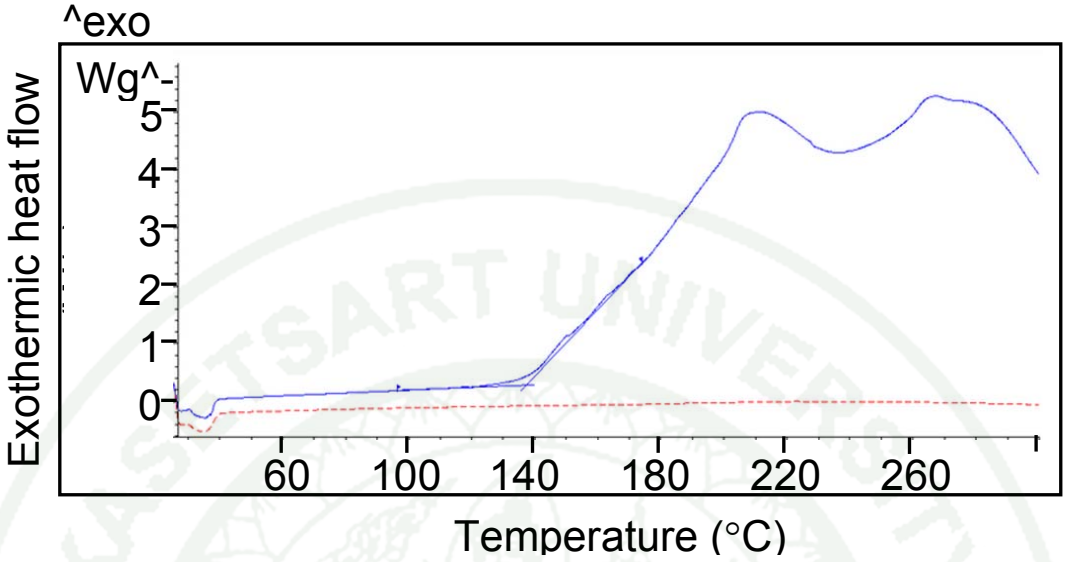


Figure 12 DSC thermograms of DD at heating rate 10°C/min under O_2 (solid line) and N_2 (dotted line) stream at flow rate 100 mL/min.

Table 15 Effect of heating rate on onset temperature of oxidation (T_o) and activation energy (E_a) of deodorizer distillate.

Heating rate (°C/min)	T_o (°C)
2	$121.2^{e} \pm 0.2$
5	$128.8^{d} \pm 0.4$
10	$137.1^{c} \pm 0.4$
16	$142.2^{b} \pm 0.8$
20	$149.0^{a} \pm 0.5$
E_a (kJ/mol), calculated from Ozawa Flynn Wall method	111.2 ± 2.1

2.3 Influence of molecular distillation temperature on chemical characteristic of molecular distillation product.

Nonetheless, most FFAs were evaporated after the MD process, resulting in the UMD having low acid values (Table 16). The acid values of UMDs were reduced dramatically when distillation temperature at and above 140 °C was used and reached the minimum values of less than 2.2 mg KOH/g. After FFAs were removed, some oil-soluble rice phytochemicals were concentrated in the UMD fractions. Table 16 indicated that γ-oryzanol content was the highest in UMD obtained at distillation temperature of 140 °C ($p<0.05$).

Tocotrienols were the main tocols in DD and all UMD samples. Table 14 and 16 indicated that β-tocotrienol was the most abundant isomer, followed by δ-tocotrienol, γ-tocotrienol and α-tocotrienol, respectively. Both tocopherols and tocotrienols were effectively concentrated at low distillation temperature of 120 °C. As distillation temperature increased to 160 °C, all tocols concentrations in UMDs were dramatically decreased ($p<0.05$). The chromatogram (Figure 13) indicated that high distillation temperature of 160 °C reduced some unsaponifiable matters, i.e. tocols having the retention time between 12 – 20 min, showed as less numbers of peak in UMD obtained after distillation at 160 °C.

Table 16 Effect of distillation temperature during molecular distillation on chemical contents of unevaporated fraction after molecular distillation (UMD)

Chemical constituents	Distillation Temperature		
	120 °C	140 °C	160 °C
Acid value (mg KOH/g)	$42.9^{a} \pm 3.4$	$2.2^{b} \pm 0.7$	$1.5^{b} \pm 0.1$
γ-Oryzanol (mg/100 g)	$870.0^{b} \pm 25.1$	$1070.3^{a} \pm 4.1$	$1018.1^{a} \pm 28.1$
Tocotrienol contents (mg/100 g)			
α-Tocotrienol	$150.5^{a} \pm 5.2$	$151.6^{a} \pm 6.7$	$81.8^{b} \pm 6.4$
β-Tocotrienol	$1804.0^{a} \pm 42.2$	$1206.4^{b} \pm 179.0$	$471.6^{c} \pm 6.3$
γ-Tocotrienol	$205.7^{b} \pm 14.9$	$257.7^{a} \pm 18.3$	$83.4^{c} \pm 7.4$
δ-Tocotrienol	$955.1^{a} \pm 20.3$	$429.8^{b} \pm 72.8$	$152.7^{c} \pm 21.2$
Tocopherol contents (mg/100 g)			
α-Tocopherol	$265.5^{b} \pm 11.9$	$295.8^{a} \pm 19.4$	$61.0^{c} \pm 3.9$
γ -Tocopherol	$108.9^{a} \pm 0.4$	$76.0^{b} \pm 5.8$	not detected
Total tocols (mg/100g)	$3484.0^{a} \pm 67.49$	$2416.7^{b} \pm 388.38$	$850.4^{c} \pm 6.6$
Phytosterol contents (mg/100g)			
β-Sitosterol	$2635.0^{b} \pm 134.6$	$2778.7^{b} \pm 190.4$	$3160.7^{a} \pm 37.5$
Campesterol	$763.4^{b} \pm 48.5$	$748.0^{b} \pm 54.5$	$860.8^{a} \pm 17.6$
Stigmasterol	$591.8^{b} \pm 44.1$	$816.2^{a} \pm 60.1$	$883.3^{a} \pm 23.5$
Total Phytosterol (mg/100g)	$3990.2^{c} \pm 211.0$	$4342.9^{b} \pm 287.9$	$4904.8^{a} \pm 76.3$
DPPH radical scavenging capacity (IC_{50}; µg/mL)	$1053.9^{b} \pm 61.7$	$899.0^{c} \pm 30.7$	$1385.8^{a} \pm 1.1$

Means in the same row followed by different superscripts are significantly different ($p<0.05$).

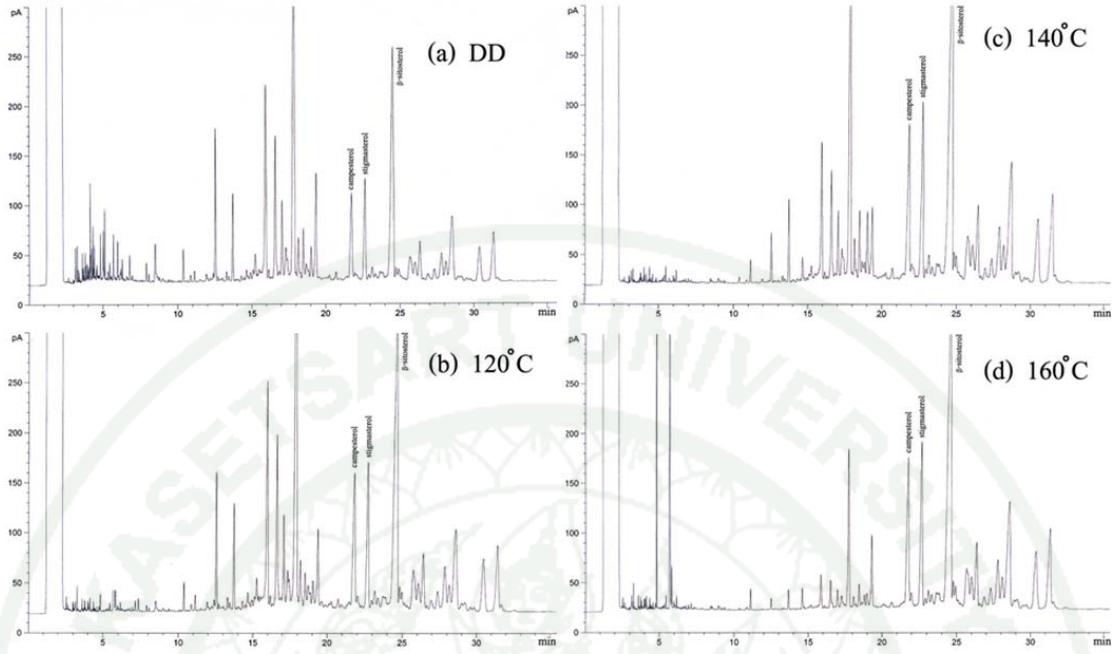


Figure 13 Effect of distillation temperature on phytosterol profiles of deodorizer distillate (a) and unevaporated fractions obtained after molecular distillation at 120 °C (b), 140 °C (c) and 160 °C (d).

Among all 4 isomers of tocotrienol, the δ isomer lost the most during MD process when the distillation temperature in MD was increased from 120 °C to 160 °C. More α-isomer, however, was retained at high distillation temperature than the others. The variation in the reduction of different isomers may be due to the difference in thermal stability and boiling point of each isomer. The boiling point of each isomer at 760 mmHg (or 101,324.7 Pa) was predicted using ACD/PhysChem Suite software from ACD/Labs (Royal Society of Chemistry, 2013). The α-tocotrienol isomer had boiling point at 542 °C while δ-tocotrienol had a lower boiling point of 517 °C. The difference in the boiling point could be due to the different structure of the chromanol head group, of which α-tocotrienol has 3 methyl-substituted groups; whereas δ-tocotrienol has only one methyl-substituted group. The increase in methyl-substituted group on chromanol head group likely results in an increase in intermolecular force. Consequently, the α-tocotrienol isomer has a higher boiling point compared with the δ-tocotrienol isomer, and retained to the high extent when MD was operated at 140 °C and 160 °C.

The MD process in the present study was performed under low pressure (i.e. 0.1 Pa). As a result, oxidation of tocols was likely minimized due to low O_2 content. Verleyen *et al*. (2001b; 2001c) reported that loss of α-tocopherol strongly related to heating temperature and time while the headspace pressure and O_2 concentration above the α-tocopherol in triolein hardly influenced the degradation of α-tocopherol at the high temperature around 180-260 °C and reduced pressure of 400-4000 Pa. Therefore, it is most likely that the loss of tocols was mainly due to the evaporation rather than the thermo-oxidative degradation. This was due to the thermal stability of DD against high temperature (Figure 11) and very low pressure at 0.1 Pa used during MD process investigated in the current study. The γ-oryzanol and phytosterols, however, had high boiling points than did the tocols, and thus were concentrated more at higher distillation temperature.

Although the γ-oryzanol was concentrated by MD, the reduction in tocol contents influenced the DPPH radical scavenging capacity showed as an increase in the IC_{50} of UMD obtained after distillation at 160 °C (Table 16). In this study, DPPH radical scavenging capacity presented by IC_{50}, the high IC_{50} mean low DPPH radical scavenging capacity. Tocols and γ-oryzanol, not phytosterol, are antioxidant responsible for DPPH radical scavenging in rice bran which tocols showed much higher activity than did γ-oryzanol. The γ-oryzanol contents of UMD obtained after distillation at 140 and 160 °C were not significantly difference, however, reduction in tocols resulted in lower DPPH radical scavenging capacity. This suggested that tocols were responsible for differences in DPPH radical scavenging capacity among UMD. However, the high content of tocols were concentrated by MD at low distillation temperature of 120 °C, DPPH radical scavenging capacity of UMD obtained after distillation at 120 °C showed higher IC_{50} compared to UMD obtained after distillation at 140 °C. This suggested that high concentration of tocols in UMD, tocols could act as both antioxidant and pro-oxidant (Naumov and Vasil'ev, 2003; Ouchi *et al*., 2009). Tocols radical could react with non-radical molecule which induce free radical formation and compete for antioxidant molecule result in lower DPPH radical scavenging activity (Ouchi *et al*., 2009). Tocols were the most influential antioxidant

in all UMDs while γ-oryzanol slightly influenced on DPPH radical scavenging activity. Distillation temperature of 140 °C which resulted in UMD containing low acid value, high antioxidant capacity and suitable amount of phytochemicals was then used in further investigation as the source of phytosterols, tocols and γ-oryzanols to be encapsulated in the vesicles; as reported in Section 3.

3. Improving water dispersibility of oil-soluble rice phytochemicals

3.1 Fabrication of oil-soluble rice phytochemical vesicle

Vesicles containing UMD in the presence of commercial surfactant, i.e. soy lecithin, Tween80 and sucrose palmitate were fabricated. In the absence of UMD, soy lecithin vesicles showed a polymodal size distribution with high % intensity of the particles having average sizes around 140 nm and 700 nm (Figure 14a). Incorporation of UMD into soy lecithin/UMD vesicle using soy lecithin to UMD ratio of 1:0.25 (w/w) resulted in a bimodal size distribution with particle size around 60 nm and 400 nm. Further increasing the UMD ratios in lecithin/UMD vesicle to 1:1, 1:2 and 1:3 resulted in polydispersed colloidal suspensions. This indicated instability of soy lecithin/UMD vesicles in PBS.

The size distribution of vesicles fabricated using Tween80 in PBS showed polymodal distribution (Figure 14b). Considering from % intensity, most Tween80 vesicles had the size around 20 nm; whereas a much lower population had a diameter around micrometer range. The incorporation of UMD into Tween80/UMD vesicles at a Tween80 to UMD ratio of 1:0.25 (w/w) resulted in 2 major groups of vesicles. The first group with high % intensity had a size about 20 nm. The second group of vesicles, with lower % intensity, had a size range between 200 to 300 nm. When the UMD ratio was increased to 1:1, 1:2 and 1:3 (w/w), the Tween80/UMD vesicles showed monomodal size distribution, with the majority having the size around 200 to 300 nm, and no lipid separation was observed. This suggested that the

Tween80/UMD vesicles fabricated could hold high content of UMD, indicating the potential for encapsulating rice phytochemicals concentrated by MD process. Increasing the ratio of UMD to 1:4 and 1:5, however, resulted in the aggregation of Tween80/UMD vesicles at a size of around 3000-4000 nm (results not showed), which separated from the aqueous phase after 3 h of preparation. In this study Tween80/UMD vesicles had an average diameter greater than that of micellar Tween80 of 35 Å (Amani *et al*., 2011).

Sucrose palmitate (SP), however, gelled in the PBS at the concentration used in current study. Therefore, the size distribution of vesicle in PBS could not be determined. This result was in agreement with those reported by Szüts *et al*. (2010) that the sucrose palmitate (HLB 16) gel could be formed in water. At concentration above 2% in water, sucrose palmitate could form gel at above 40 °C. With the temperature rising, the H-bond between sucrose ester and water is breakdown, which enhances micelles growing. This phenomenon could increase micellar size or the transition from spherical to wormlike micelles that changed the viscosity of the systems and possibly formed gel (Szüts *et al*., 2010). Nonetheless, incorporation of UMD into SP/UMD vesicle resulted in suspensions instead of gel. However, the SP/UMD vesicles showed polydispersed distribution, ranging from submicron to micrometer size (Figure. 14c). This occurrence could be due to tri-acylglycerol and small molecular weight surfactant, i.e. mono- and di-acylglycerol in UMD (Nukit *et al.,* 2014) that acted as oil and co-surfactant; which disrupted wormlike micellar structure of sucrose palmitate. Rodriguez-Abreu et al. (2005) showed that addition small amount of lipophilic co-surfactant to sucrose palmitate induced micellar growth and led to the formation of wormlike micelles. However, these worm micelles were disrupted when the co-surfactant fraction was increased over some critical value. They also found that worm like micelles of sucrose palmitate could solubilize in oil.

The ability of Tween80, compared to soy lecithin and sucrose palmitate, in aiding the formation of surfactant/UMD vesicles may be due to the structure of surfactant and the composition of UMD itself. The UMD was composed of triacylglycerol, diacylglycerol and monoacylglycerol with an oleoyl chain as the

major esterified fatty acid (Nukit *et al.*, 2014). Therefore, the hydrophobic tail of oleyl chain in Tween80 molecules and the oleyl chain in the UMD reported in the current study, which acted as co-surfactant, may self-assembled into stable vesicles.

Despite the highly negative charged group of phosphatidyl choline and phosphatidyl ethanolamine that generally favor liposome or vesicle formation by soy lecithin, the major acyl groups of commercial soy lecithin are usually linoleyl (65.9 %) and oleyl (10.6%) chains (Magil *et al*., 1981). The double bond of linoleyl chain and oleyl chain may not favor the formation of thermodynamically stable bilayer of vesicle. Similar results were observed in SP/UMD vesicles, of which the esterified palmitic was the major acyl chain.

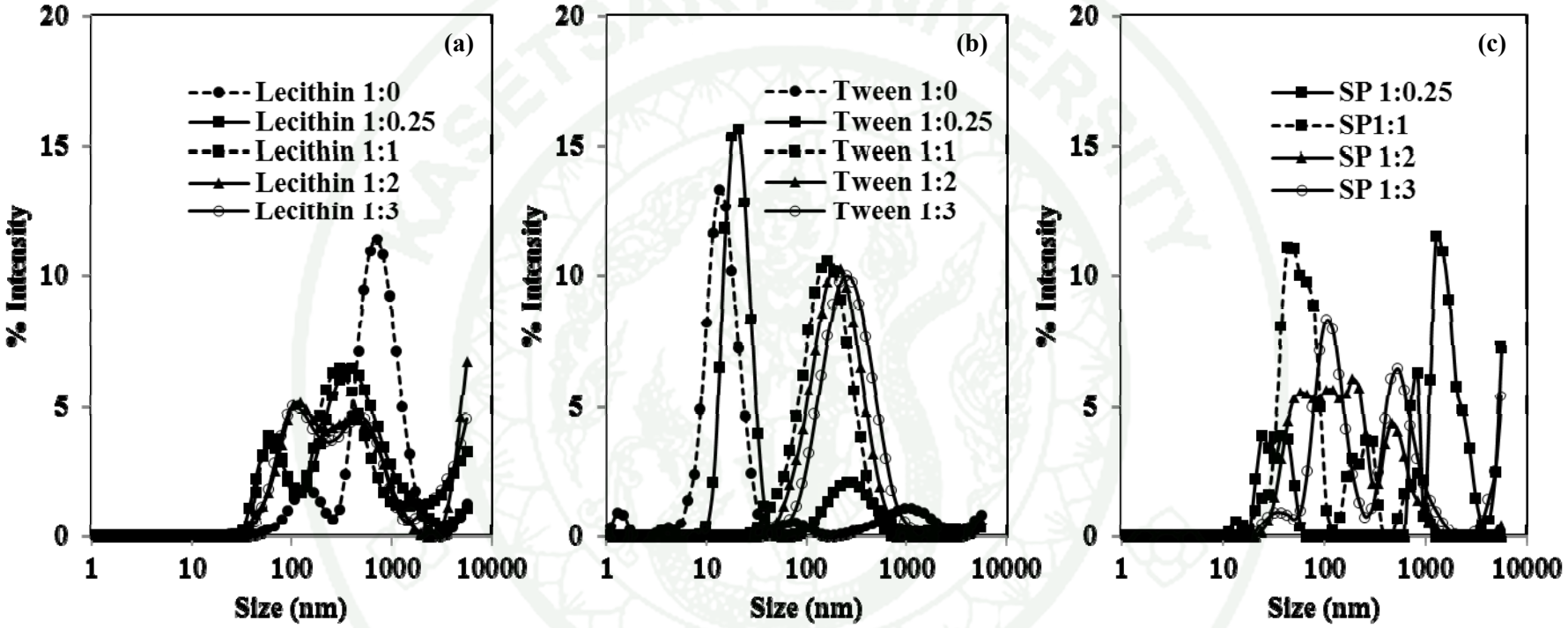


Figure 14 Particle size distributions of surfactant-UMD vesicles prepared using different ratios of surfactant to UMD. (a) soy lecithin/UMD vesicles, (b) Tween80/UMD vesicles, and (c) sucrose palmitate (SP)/UMD vesicles.

3.2 Storage stability of Tween80/UMD vesicle

Tween80/UMD vesicles formed by using Tween80/UMD at the ratio 1:3 were aseptically filtered through 0.22 μm nitrocellulose membrane. This procedure was performed to eliminate microorganism and estimate the storage stability of vesicles at refrigerated and cell culture incubation temperature which could be used in further investigation.

After filtered sterilization, the size distribution of Tween80/UMD vesicles in PBS showed monomodal distribution of around 200 nm with narrow size range. The sterile suspensions were quite stable at low temperature of 4-5 °C and at 37 °C for 0, 24, 48, 72 and 96 h (Figure 15). After storage, the average diameter size was maintained around 200 nm (Figure 15). Nonetheless, the Tween80/UMD vesicles had a wider range of size compared to that of 0 h. This was possibly due to the flocculation of the vesicle. Nevertheless, the stability of Tween80/UMD vesicles in PBS showed potential use in the formulation of vesicle suspensions that were stable over a temperature range from chilled storage to body temperature.

Results indicated that Tween80 could be used as a major surfactant to improve water dispersibility of oil-soluble rice phytochemicals in UMD by fabricating Tween80/UMD vesicles. Such vesicular system could control vesicle size of 200 nm with high stability. Therefore, the Tween80/UMD vesicles formed by using the ratio of Tween80 to UMD as 1:3 was used in further investigation to evaluate cytotoxicity of UMD in Caco-2 cell model and reported in Section 4.

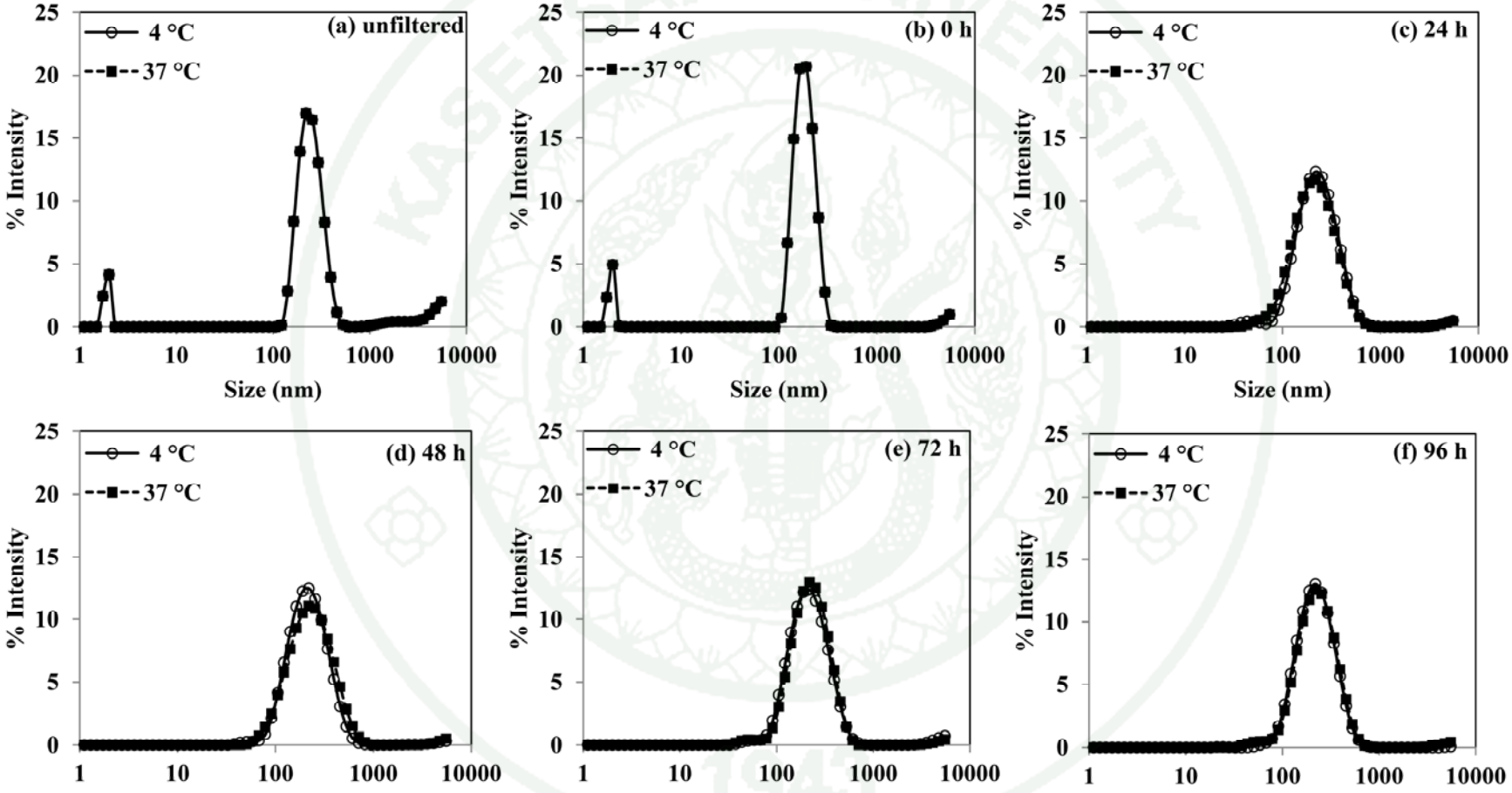


Figure 15 Effect of storage time and temperature on particle size distribution of Tween80/UMD vesicles stored at different temperatures.

It is apparent that the UMD obtained from MD process could be used as the source of rice phytochemicals. In the presence of UMD, the Tween80/UMD vesicle could be fabricated. It is possible that Tween80, which has HLB around 15, as well as the co-surfactant mono, diacylglycerol concentrated by MD process were structurally compatible for vesicle formation through oleyl chain. Tween80 composed of polyethoxylated sorbitan hydrophilic head group and oleic acid tail. From its structural point of view, Tween80 has very large head group compared to its tail, which could result in high HLB value that not favor the formation of vesicle without cholesterol (Uchegbu and Florence, 1995; Manosroi *et al*., 2003; Rajera *et al*., 2011). Manosroi *et al*. (2003) increased hydrophobicity of Tween 61 polyethoxylated sorbitan hydrophilic head group and stearic acid tail by adding cholesterol with molar ratio of Tween61 and cholesterol as 1:1. They reported that suitable CPP value of 0.5-1 was found to form vesicle. In this study, Tween80/UMD vesicle is Tween80 mixing with co-surfactant, mono-, di-acylglycerol and vesicle stabilizer that highly hydrophobic, phytosterol, which could increase hydrophobic of system and leading to vesicle formation. Phytosterol, which is cholesterol structured identical compound, was reported for their ability to act as vesicle stabilizer in place of cholesterol (Schuler *et al*., 1990; 1991; Chan *et al*., 2004; Hwang *et al*., 2010). In this study, rice phytosterols existed in the UMD could further stabilize vesicular structure by facing their hydroxyl group at C-23 position toward aqueous phase, while their tricyclic ring oriented among acyl chain of Tween80. However, γ-oryzanol, OH-group at position C-3 of phytosterol was esterified with ferulic acid. The esterified ferulic acid also contains OH-group at their phenol ring. As a result, γ-oryzanol structure could also had ability to be vesicle stabilizer and oriented their OH-group facing the aqueous phase along with the polar head of Tween80. It is also possible that tocols, which also contained OH-group in their chromanol head, could also turn their polar OH-group toward aqueous phase and arranged their phytal tail along with the hydrophobic compartment.

4. Cytotoxicity of Tween80/UMD vesicle in Caco-2 cell monolayer and THP-1 macrophages

4.1 Effect of UMD on the fabrication of Tween80-based vesicles

The effectiveness of UMD, which contained rice phytosterols, tocols and γ-oryzanol, on the fabrication of lipid vesicles was tested in an oil model not containing tocols and γ-oryzanol: a Tween80/olive oil/dioleoylglycerol/β-sitosterol vesicle. Figure 16 illustrates that Tween80 alone could not stabilize Tween80/olive oil vesicle. The vesicles in PBS were polydispersed and had range of 20 nm to above 6 μm. Therefore, Tween80 alone could not emulsify olive oil and PBS when the thin film method was used (Figure 16a). On the other hand, UMD, which contained residual triacylglycerol, monoacyl glycerol, diacyl glycerol (Nukit *et al*., 2014), as well as β-sitosterol, campesterol, stigmasterol, tocols and γ-oryzanol, was able to maintain the average size of vesicle 30 to 1,000 nm, with the average size of 200-300 nm (Figure 16b).

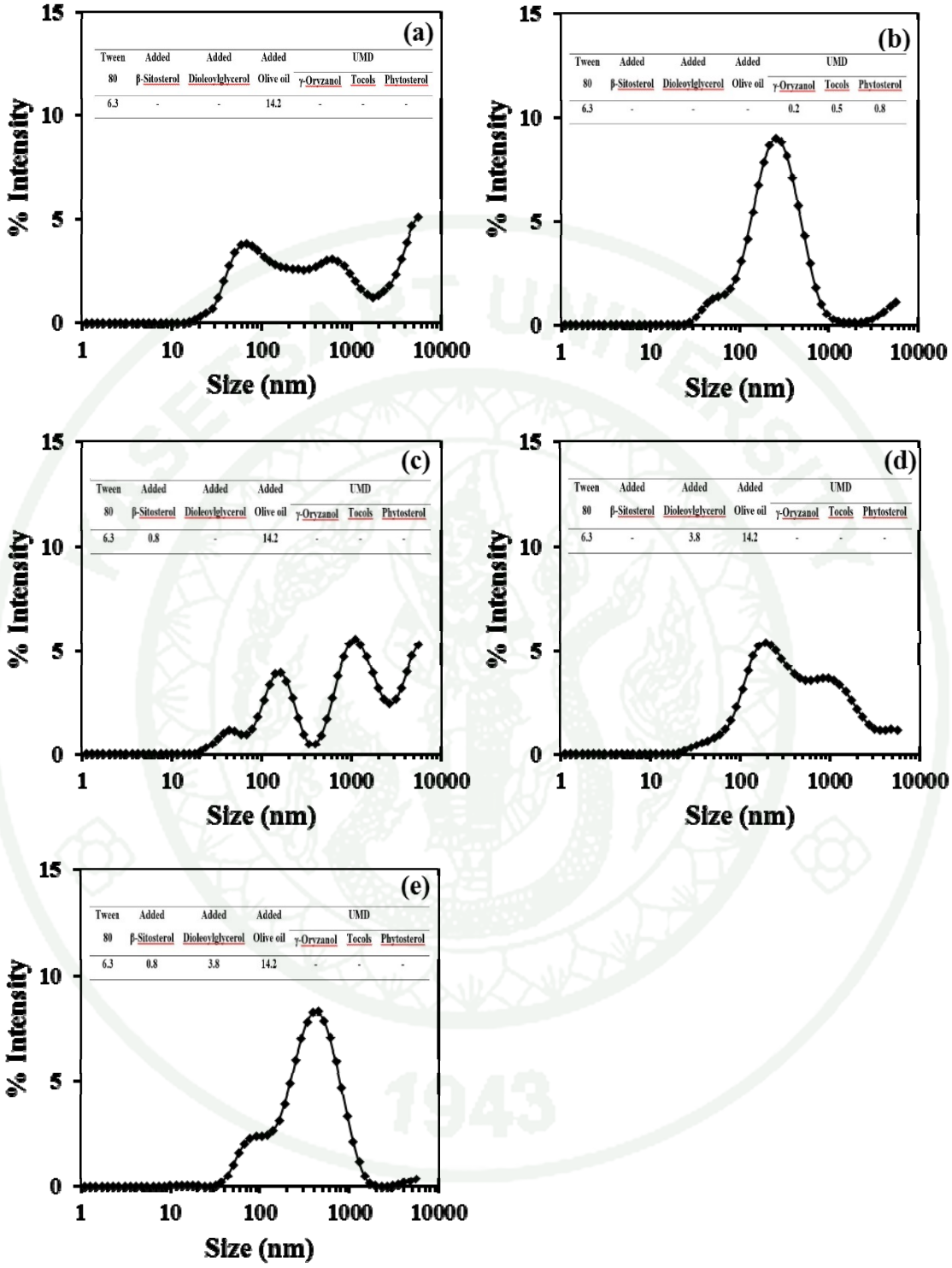


Figure 16 Effect of vesicle composition on particle size distribution of vesicle in PBS.

The fabrication of vesicles containing Tween80, olive oil and β-sitosterol (Figure 16c) and those containing Tween80, olive oil and dioleoylglycerol (Figure 16d) resulted in polydispersed suspensions. Only vesicles containing Tween80, olive oil, dioleoylglycerol and β-sitosterol (Figure 16e) could maintain the size range of 50-1500 nm with the highest % intensity at 400 nm. The result confirmed that both dioleoylglycerol and β-sitosterol were essential in the formation of Tween80-based vesicles. However, the vesicles size of Figure 16b was smaller than vesicles showed in Figure 16e. Both vesicle types contained Tween80, olive oil, dioleoylglycerol and β-sitosterol while Tween80/UMD also (Figure 16b) contained small MW surfactant like monoacylglycerol (Nukit *et al*., 2014). Although the result implicated that UMD could played significant roles in stabilizing the vesicle compare to β-sitosterol alone it should be noted that the composition of the vesicles were different. More work should be done to optimize the concentration range of vesicle fabricator and stabilizer suitable for the formation of small vesicles of the size range between 100-300 nm.

4.2 Stability of Tween80-UMD vesicle during *in vitro* gastro-intestinal digestion

The change in particle size of lipid vesicles is one of the important parameter with an effect on cellular uptake of Tween80/UMD vesicles. The passage of lipid vesicles through intestinal mucosa might be inversely related to lipid vesicle particle size (Desai et al., 1997). The *in vitro* digestion model was used to investigate the effect of the human digestion process on the stability of Tween80/UMD vesicles. Table 17 showed that the average particle size of Tween80/UMD vesicles were significantly increased after adjusted to pH 5.8 ($p<0.05$). However, Tween80/UMD vesicle remained unchanged after intestinal enzyme and bile salt were present. This result suggested that the elevation of pH 5.8 influenced Tween80/vesicle size by inducing vesicle flocculation. Increasing of pH further to pH 6.5 and 7.5 showed no significant change in vesicle size. This result indicated that particle size of Tween80/UMD vesicles was slightly changed throughout the *in vitro* digestion

process. The passage of lipid vesicles through intestinal mucosa might be inversely related to lipid vesicle particle size. Desai *et al*. (1997) demonstrated that the uptake of microparticle in Caco-2 cell depended on microparticle diameter, concentration, incubation time and temperature. The 100 nm-diameter particles could be uptaken at 2.5 fold greater than the 1000 nm-diameter microparticles. From current study, Tween80/UMD vesicle could retain size at 200-300 nm after *in vitro* digestion process. This indicated that Tween80/UMD vesicle could be used as carrier to transport lipophilic active compound through intestinal tract. However, the vesicle uptake rate and active compound released from Tween80/UMD vesicle still need further investigation.

Table 17 Effects of *in vitro* digestion on lipid vesicle particle size distribution

In vitro digestion steps	Particle size (nm)
Undigested vesicle in PBS pH 7.4	$237^{bc} \pm 6$
Added salt solution	$228^{c} \pm 5$
Adjusted to pH 2	$272^{abc} \pm 38$
Digested with pepsin	$270^{abc} \pm 14$
Adjusted to pH 5.8	$309^{a} \pm 50$
Digested with intestinal enzymes and bile salt	$303^{a} \pm 29$
Adjusted to pH 6.5	$261^{abc} \pm 21$
Adjusted to pH 7.5	$294^{ab} \pm 48$

Mean ± standard deviation of 3 replications

4.3 Influence of Tween80/UMD vesicle on viability of Caco-2 monolayer

Figure 17 showed the viability of 21 day–old Caco-2 cell monolayers after 3 h incubation with Tween80/UMD vesicles at different concentrations. The viability of Caco-2 cell was calculated in relation to PBS treated cells (control). The presence of Tween80/UMD vesicle up to 2 mg/mL slightly increased cell viability.

However, increasing Tween80/UMD vesicle concentration up to 4 mg/mL lowered viability of Caco-2 cells to 90%. Increasing Tween80/UMD vesicle to high concentration at 5 mg/mL decreased cell viability down to 80%. In this study, Tween80 concentration in tested sample was up to 0.125 % (w/v). However, the cytotoxicity of Tween80 was not tested. O'Sullivan *et al*. (2004) reported no cytotoxic effect was in the differentiated Caco-2 cell tested by MTT assay after Tween80 treatment at 1mL/L (0.1 % w/v) for 24 h. Later on Lu and colleague (2014) showed that Tween80 was not toxic to Caco-2 cell up to concentration 0.125 % but cell viability was decreased at Tween80 concentration of 0.25%.

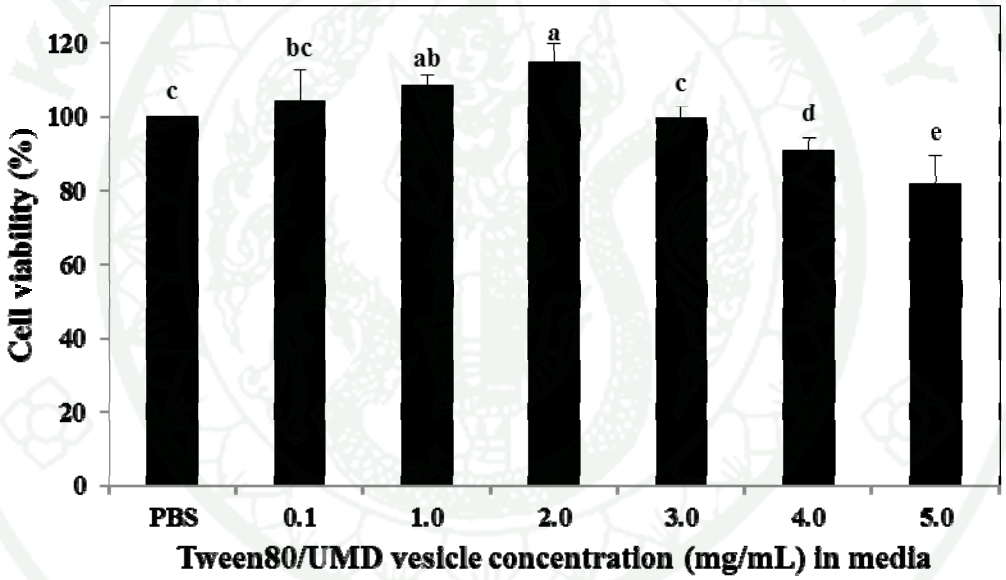


Figure 17 Effect of Tween80/UMD vesicles at different concentration on Caco-2 cell viability after 3h incubation. Bars represent standard deviation from two independent biological and two technical replications.

4.4 Influence of Tween80/UMD vesicle on viability of THP-1 macrophage

Tween80/UMD vesicle was investigated for potential cytotoxicity to THP-1 macrophages after incubation for 24 h. The viability of THP-1 macrophages was calculated as relative to PBS. Figure 18 indicated that Tween80/UMD vesicle was not toxic to THP-1 macrophages up to 1.0 mg/mL. At this concentration of the

vesicles, Tween80/UMD contained 32.6 μg/mL phytosterol, 18.1 μg/mL tocols and 8.0 μg/mL γ-oryzanol. The influence of Tween80 on viability of THP-1 macrophages in this study was not tested. For better interpretation, cytotoxicity of Tween80 should be included. Tween80 highest concentration in this study was 0.025 % w/v, which was too low to affect the viability of THP-1 macrophages. According to Geys *et al*. (2010) Tween80 did not affect viability of THP-1 macrophages up to concentration 0.1 %.

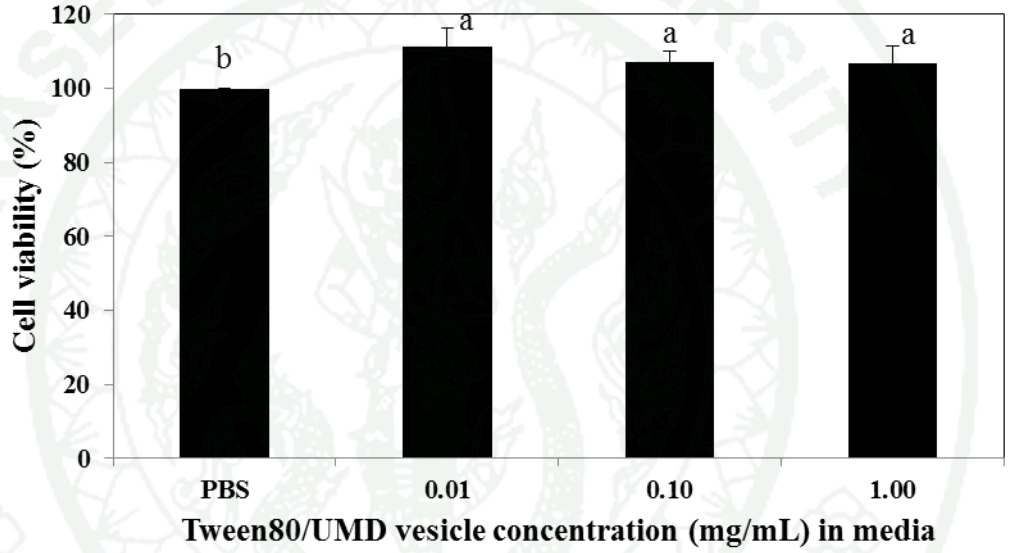


Figure 18 Effect of Tween80/UMD vesicles at different concentration on THP-1 macrophages viability after 24 h incubation. Bars represent standard deviation from six independent biological and two technical replications

4.5 Uptake of lipid vesicle by Caco-2 cell monolayer

The 21 day-old Caco-2 monolayer in transwells was exposed to Tween80/UMD lipid vesicle at final concentration in medium 0.1, 1 and 5 mg/mL for 3 h. The integrity of Caco-2 cell monolayer tight junction was monitored using TEER measurement. Figure 19 showed the TEER values of all the treatment including PBS. It was found that the TEER values gradually decreased over 3 h of incubation. It should be noted that Caco-2 cell monolayer could be sensitive to many factors

including the fluctuation of CO_2 and temperature during TEER measurement, which could lead to the reduction of TEER value. Moreover, the incubation time was too short to observe TEER value change trend. For further investigation, the incubation time should be prolonged in order to investigate the influence of vesicle on the change in TEER value.

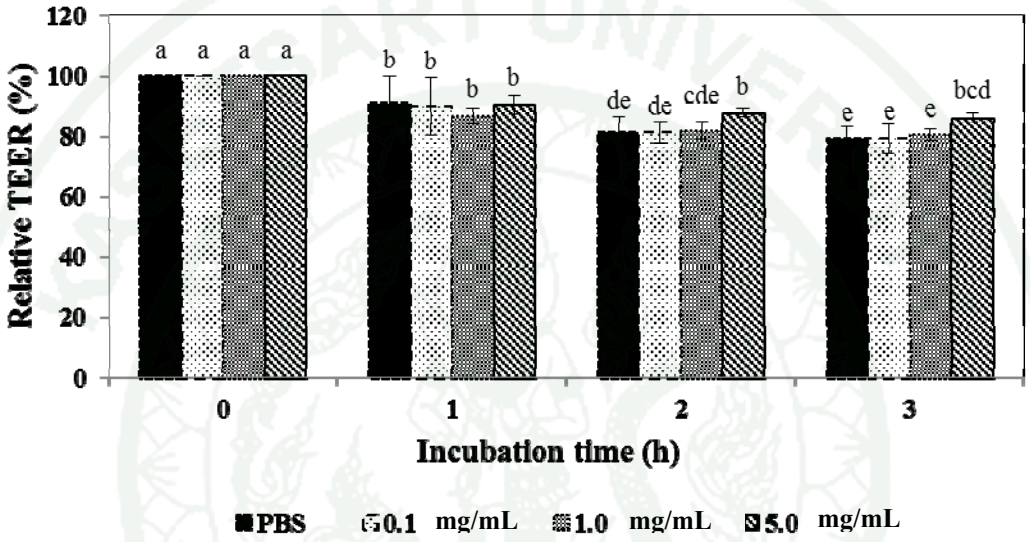


Figure 19 Effect of Tween80/UMD vesicles at different concentration of TEER-values of Caco-2 cell monolayer over incubation time 3 h.

Tween80/UMD vesicles loaded with 6-coumarin were used to track the intracellular uptake by Caco-2 cell monolayer. Figure 20, Tween80/UMD vesicles were present on the cell surface. However, this picture could not indicate uptake of Tween80/UMD vesicle since Caco-2 cell structure are not clearly determined. For better result, the nuclei and tight junction should be stained in order to indicate cell structure and vesicle localization.

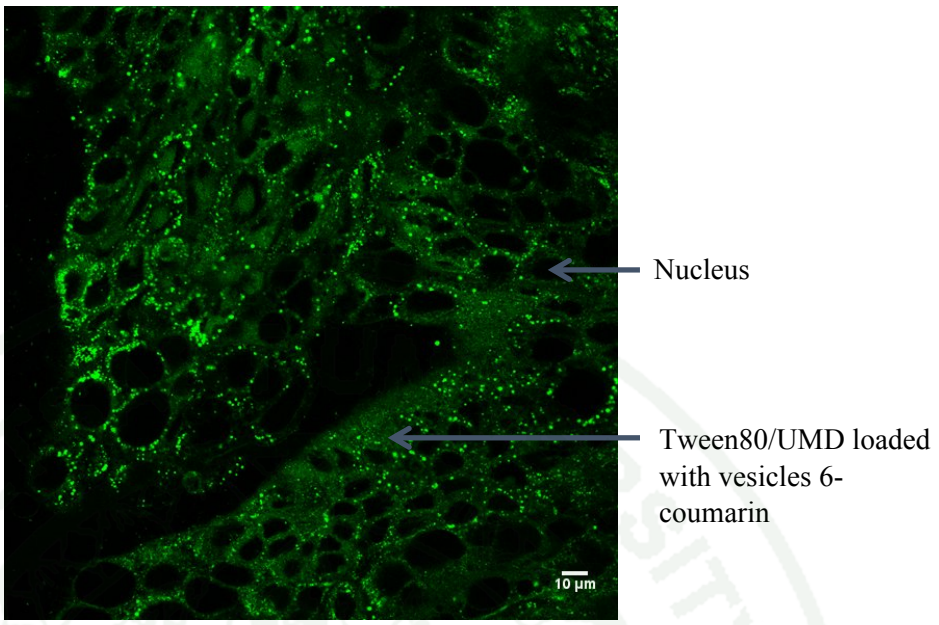


Figure 20 CLSM micrograph of Caco-2 cell monolayer after Tween80/UMD vesicles were uptaken. Tween80/UMD vesicles were loaded with 6-coumarin fluorescence in green.

5. Effect of oil-soluble rice bran oil phytochemical on THP-1 gene expression

The immunomodulatory properties of oil-soluble rice phytochemical encapsulated in Tween80/UMD vesicle were investigated by using THP-1 macrophages model. In preliminary study, THP-1 macrophages were stimulated with Tween80/UMD vesicles at various concentrations up to 5 mg/mL for 3 and 6 h. Gene expression of inflammatory-related cytokine, i.e. IL-1β, IL-8 and TNF-α remained unchange compared to cell grown in PBS (data not show). In this investigation, THP-1 macrophages were exposed with Tween80-vesicles, i.e. Tween80/UMD (UV), Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol vesicle (EV), Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol/α-tocopherol vesicle (TV) and Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol/γ-oryzanol vesicle (OV) for 24 h. The gene expression level of pro-inflammatory cytokine such as IL-1β, IL-8 and TNF-α were investigated compared to PBS. In all vesicle types, the elevation of

observed THP-1 macrophages cytokine gene expressions were only found at high vesicle concentration at 1 mg/mL (UV contained γ-oryzanol 8.0 μg/mL, tocols 18.1 μg/mL and phytosterol 32.6 μg/mL; EV contained phytosterol 32.5 μg/mL; TV contained phytosterol 32.5 μg/mL and α-tocopherol 0.2 μg/mL; OV contained phytosterol 32.5 μg/mL γ-oryzanol 8.0 μg/mL) (Figure 21). The increasing gene expression of IL-1β after being exposed to Tween80-vesicle was the most stand out compared to gene expression of other cytokines. When consider IL-1β gene expression of THP-1 macrophages after they were exposed to Tween80-vesicle at high concentration, THP-1 macrophages exposed to UV showed increased IL-1β gene expression in the same extent to THP-1 macrophages exposed to EV. This result could indicate that the mixture of oil-soluble rice phytochemical in UMD such as tocols and γ-oryzanol did not influence IL-1β gene expression. However, IL-1β gene expression after being exposed to TV and OV were increased in even higher extent than both UV and EV. IL-1β gene expression after being exposed to TV was very high even though α-tocopherol concentration (equal to α-tocopherol content in UV) in TV was much lower than total tocols concentration in UMD. It is possible that the effect of α-tocopherol or γ-oryzanol in IL-1β stimulation were more pronounced when used as single compound than a mixture. The influence of RBO and their oil-soluble phytochemicals such as tocols, γ-orzanol and phytosterol, as well as its mixture on their insight immunomodulation properties still needs further investigation.

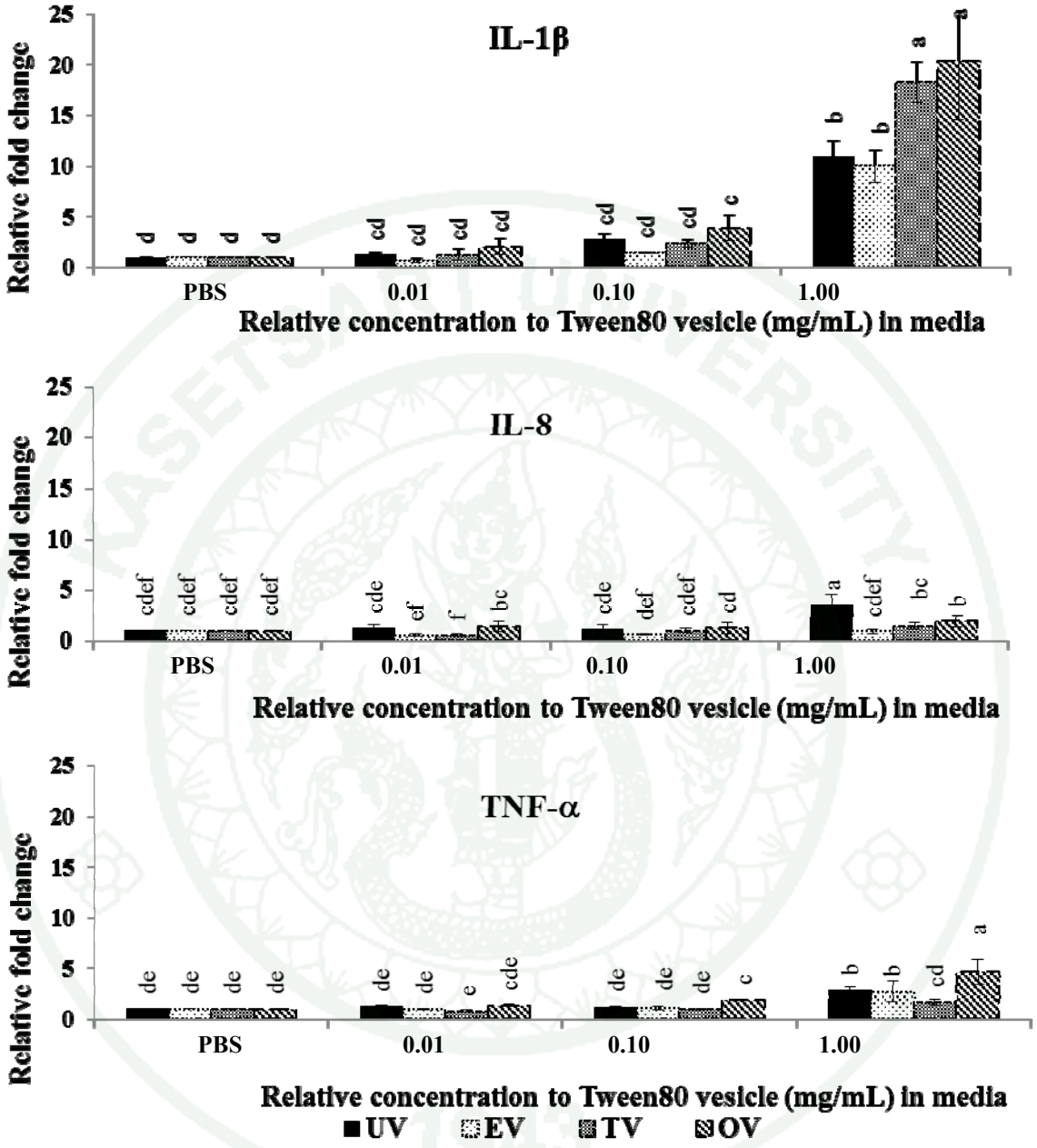


Figure 21 Expression of pro-inflammatory cytokine genes expression of THP-1 macrophages after stimulation with UV, EV, TV and OV for 24 h. Gene expression was normalize to GAPDH and non-stimulate macrophages at individual concentration ($2^{-\Delta\Delta CT}$). Data showed are means and standard deviations from two biological and two technical replications.

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrated the use of rice bran oil deodorizer distillate (DD) as a potential source of oil-soluble rice phytochemicals, i.e. γ-oryzanol, phytosterols and tocols. This study also showed potential use of molecular distillation (MD) in concentrating rice phytochemicals DD obtained from the physical refining process of RBO. However, the temperatures applied during MD significantly affected phytochemicals retention and antioxidant capacity of the unevaporated fraction (UMD). Among tocotrienol isomers found in rice bran oil, this thesis first reported the existence of β-tocotrienol, which was the most abundant isoform found in rice. The loss in tocol contents, both tocopherols and tocotrienols affect the loss in DPPH antioxidant capacity although γ-oryzanol was present at high concentration. The UMD compositions were dependent on distillation temperature and the effects of volatility of individual components. These results could permit the manufactures to tailor the UMD products to a preferred composition for maximum health benefits by varying distillation temperature during MD operations.

Unsaponifiable matters and small molecular weight surfactants in crude rice bran oil phytochemical derived from MD process, i.e. phytosterols and mono-, di-acylglycerol could enhance water dispersibility of Tween80/UMD vesicles fabricated by thin film hydration method. The comprehension of the present study could be applied to optimize the production of water-dispersible rice phytochemicals; i.e. tocols, γ-oryzanol and phytosterols from DD, the by-products in aqueous liquid foods or drinks. Encapsulate oil-soluble rice phytochemical in Tween80-vesicle could facilitate cellular uptake of oil-soluble rice bran oil phytochemical, although Tween80/UMD vesicle slightly decreased cell viability of Caco-2 monolayer at concentration above 3 mg/mL. Moreover, oil-soluble rice phytochemicals such as tocols and γ-oryzanol stimulated pro-inflammatory cytokine gene expression suggested that oil-soluble rice phytochemicals could have immunomodulation

property. The comprehension of this work could be expanded the use of oil-soluble rice phytochemical in immumodulatory application.

This study suggested the water dispersibility improvement of oil-soluble phytochemical by Tween80/UMD vesicle formation, which enhanced by phytosterol and small molecular weight surfactant. The insight mechanisms of Tween80-vesicle formation are still needed. Moreover, current study also showed potential use of Tween80-vesicle as carrier to encapsulate oil-soluble rice phytochemical and deliver to cell as well as their immunomodulation properties. However, the influences of individual rice phytochemical, as well as their mixture, on immunomodulation still need further investigation.

LITERATURE CITED

Adel, R.D., P. C M Heussen and A. Bot. 2010. Effect of water on self-assembled tubules in β-sitosterol + γ-oryzanol-based organogels. **Journal of Physics Conference Series**. 247(1): 1-12.

Adhvaryua, A., S.Z. Erhana, Z.S. Liua and J.M. Perez. 2000. Oxidation kinetic studies of oils derived from unmodified and genetically modified vegetables using pressurized differential scanning calorimetry and nuclear magnetic resonance spectroscopy. **Thermochimica Acta.** 364: 87-97.

Akihisa , T., K. Yasukawa , M. Yamaura , M. Ukiya ,Y. Kimura , N. Shimizu and K. Arai. 2000. Triterpene Alcohol and Sterol Ferulates from Rice Bran and Their Anti-inflammatory Effects. **Journal of Agricultural and Food Chemistry**. 48 (6): 2313–2319.

Akiyama, Y., K. Hori, T. Takahashi and Y. Yoshiki. 2005. Free radical scavenging activities of γ-oryzanol constituents. **Food Science and Technology Research**. 11(3): 295-297.

AOCS. 1997. **Official Methods and Recommended Practices of American Oil Chemist′ s Society**. AOCS Press, Illinois.

Amani, A., P. York, H. De Waard and J. Anwar. 2011. Molecular dynamic simulation of a polysorbate 80 micelle in water. **Soft Matter**. 7: 2900-2908.

Arunothayanun, P. IF Uchegbu and AT Florence. 1999. Osmotic behavior of polyhedral non-ionic surfactant vesicles (niosomes). **The Journal of Pharmacy and Pharmacology**. 51(6):651-657.

Azrina, A., I. Maznah and A.H. Azizah. 2008. Extraction and determination of oryzanol in rice bran of mixed Herbarium UKMB; AZ 6807: MR 185, AZ 6808: MR 211, AZ6809: MR 29. **ASEAN Food Journal**. 15 (1): 89-96.

Batistella, C.B., E.B. Moraes, R. Maciel Filho and M.R. Wolf Maciel. 2002. Molecular distillation: Rigorous modeling and simulation for recovering vitamin E from vegetal oils. **Applied Biochemistry and Biotechnology**. 98–100: 1187-1206.

Bagatolli, L.A. 2009. Lipid Membrane Technology, pp. 713-740. In H. G. Bohr, eds. **Handbook of molecular biophysics: methods and applications**. Wiley-Vch Verlag GmbH and Co. KGaA, Weinheim.

Berger, A., D. Rein, A. Schäfer, I. Monnard, G. Gremaud, P. Lambelet and C. Bertoli. 2005. Similar cholesterol–lowering properties of rice bran oil, with varied γ–oryzanol, in mildly hypercholesterolemic men. **European Journal of Nutrition.** 44(3): 163-173.

Bernardi,D.S., T. A Pereira**,** N. R Maciel**,** J. Bortoloto**,** G. S Viera**,** G. C. Oliveira and P. A Rocha-Filho. 2011. Formation and stability of oil-in-water nanoemulsions containing rice bran oil: ***in vitro*** and ***in vivo*** assessments. **Journal of Nanobiotechnology**. 9:44

Biswal, S., P.N. Murthy, J. Sahu , P. Sahoo and F.Amir. 2008. Vesicles of non-ionic surfactants (niosomes) and drug delivery potential. **International Journal of Pharmaceutical Sciences and Nanotechnology**. 1(1): 1-8.

Bockisch, M. 1998. **Fats and Oils Handbook**. AOCS Press, Champaign, Illinois

Bonsdorff-Nikander, A., M. Karjalainen, J. Rantanen, L. Christiansen and J. Yliruusi. 2003. Physical stability of a microcrystalline β-sitosterol suspension in oil. **European Journal of Pharmaceutical Sciences.** 19:173–179.

Bortolomeazzi, R., F. Cordaro, L. Pizzale and L.S. Conte. 2003. Presence of phytosterol oxides in crude vegetable oils and their fate during refining. **Journal of Agricultural and Food Chemistry**. 51(8): 2394-2401.

Bot, A., R. den Adel, C. Regkos, H. Sawalha, P. Venema and E. FlÖter. 2011. Structuring in β-sitosterol + γ-oryzanol-based emulsion gels during various stages of a temperature cycle. **Food Hydrocolloids**. 25(4): 639–646.

Burton, G. W. and M. G. Traber. 1990. Vitamin E: antioxidant activity, biokinetics, and bioavailability. **Annual Review of Nutrition**. 10:357-382.

Calpe-Berdiel, L., J.C. Escolà-Gil, S. Benítez, C. Bancells, F. González-Sastre, X. Palomer, F. Blanco-Vaca. 2007. Dietary phytosterols modulate T-helper immune response but do not induce apparent anti-inflammatory effects in a mouse model of acute, aseptic inflammation. **Life Science.** 80: 1951–1956.

Chan, Y-H, B-H Chen, C. P. Chiu and Y-F Lu. 2004. The influence of phytosterols on the encapsulation efficiency of cholesterol liposomes. **International Journal of Food Science and Technology**. 39, 985–995.

Christiansen, LI., J.T. Rantanen, A.K. Bonsdorff, M.A. Karjalainen and J.K. Yliruusi. 2002. A novel method of producing a microcrystalline β-sitosterol suspension in oil. **European Journal of Pharmaceutical Sciences**. 15(3):261-269.

Cicero, A.F.G. and A. Gaddi. 2001. Rice bran oil and γ-oryzanol in the treatment of hyperlipoproteinaemias and other conditions. **Phytotherapy Research**. 15(4): 277-289.

Čmolík, J and J. Pokorný. 2000. Physical refining of edible oils. **European Journal of Lipid Science and Technology**. 102. 472–486.

Copeland, D. and M.W. Belcher. 2003. **Methods for treating deodorizer distillate**. W.O. Patent 03080778.

Codex, 1999. **Codex Standard for Named Vegetable Oil.** Codex Stan 210-1999**.**

Cvengroš, J. 1995. Physical Refining of Edible Oils. **Journal of the American Chemists' Society.** 72(10): 1193-1196.

Derakhshan-Honarparvar1, M., M. M. Hamedi, M. Kh. Pirouzifard. 2010. Rice bran phytosterols of three widespread Iranian cultivars. **Journal of Agricultural Science and Technology**. 12: 167-172.

Desai, M.P., V. Labhasetwar, E. Walter, R.J. Levy and G.L. Amidon. 1997. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. **Pharmaceutical Research**. 14(11): 1568-1573.

Desai, F., M. Ramanathan, C.S. Fink, G. E. Wilding, B. Weinstock-Guttman, A. B. Awad. 2009. Comparison of the immunomodulatory effects of the plant sterol β-sitosterol to simvastatin in peripheral blood cells from multiple sclerosis patients. **International Immunopharmacology**. 9: 153–157.

Desai, M.P., V. Labhasetwar, E. Walter, R.J. Levy and G.L. Amidon. 1997. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. **Pharmaceutical Research**. 14(11): 1568-1573.

Dobbins, T.A., D. B. Wiley and D.C. Dobbins. 2008**. Process for isolating phytosterols and tocopherols from deodorizer distillate**.**USPTO** 20080015367.

Eitenmiller, R. and J. Lee. 2004. **Vitamin E: Food Chemistry, Composition, and Analysis**. Marcel Dekker, Inc., New York.

Engel, R. and H. Schubert. 2005. Formulation of phytosterols in emulsions for increased dose response in functional foods. **Innovative Food Science Emerging Technologies**. 6: 233– 237.

Fernandes, P. and Cabral, J.M.S. 2007. Phytosterols: Applications and recovery methods. **Bioresource Technology**. 98: 2335-2350.

Folmer, B.M. 2003. Sterol surfactants: from synthesis to applications. **Advances in Colloid and Interface Science.** 103:99-119

Frank, N., F.M. Andrews, S.B. Elliott, Lewt, J. and R.C Boston. 2005. Effects of rice bran oil on plasma lipid concentrations, lipoprotein composition, and glucose dynamics in mares. **Journal of Animal Science**. 83(11): 2509-2518.

Geys, J., B. Nemery and P.H.M. Hoet. 2010. Assay conditions can influence the outcome of cytotoxicity tests of nanomaterials: better assay characterization is needed to compare studies. **Toxicology in Vitro**. 24(2):620-629.

Ghafoorunissa, S.H. 2007. Sesame lignans enhance the thermal stability of edible vegetable oils. **Food Chemistry**. 105: 1076–1085.

Gopala Krishna, A.G., S. Khatoon, P.M. Shiela, C.V. Sarmandal, T.N. Indira and A. Mishra. 2001. Effect of refining of crude rice bran oil on the retention of oryzanol in the refined oil. **Journal of the American Oil Chemists' Society**. 78(2): 127-131.

Gopala Krishna, A.G., P.A. Prashanth, A. Pragasam, K.V. Raghavendra and S. Matoon. 2003. Unsaponifiable matter and oxidative stability of commercially produced Indian rice bran oil. **Journal of Food Lipids**. 10(4):329-340.

Gopala Krishna, A.G., K.H. Hemakumar and S. Khatoon. 2006. Acidity of oryzanol and its contribution to free fatty acid value in vegetable oils. **Journal of the American Oil Chemists' Society**. 83(12): 999-1005.

Hassanien, M. F. R. 2012. Tocol and phytosterol composition of edible oils in the Egyptian market. **Journal of Food Processing and Preservation**. 36: 531–538.

Huang, C-C. 2003. **Potential Functionality and Digestibility of Oryzanol as Determined Using in *Vitro* Cell Culture Models.** PhD Thesis, Louisiana State University and Agricultural and Mechanical College.

Huang, Z-R., Y-K Lin and J-Y Fang. 2009. Review: Biological and pharmacological activities of squalene and related compounds: potential uses in cosmetic dermatology. **Molecules**. 14(1): 540-554.

Hwang, J-S., Y-L Tsai and K-C Hsu. 2010. The feasibility of antihypertensive oligopeptides encapsulated in liposomes prepared with phytosterols-b-sitosterol or stigmasterol. **Food Research International**. 43: 133–139.

Izadi, Z., A. Nasirpour and G. Garousi. 2012. Optimization of phytosterols dispersion in an Oil/Water emulsion using mixture design approach. **Journal of Dispersion Science and Technology.** 33(12): 1715-1722.

Jiménez-Escrig, A., A.B. Santos-Hidalgo and F. Saura-Calixto. 2006. Common sources and estimated intake of plant sterols in the Spanish diet. **Journal of Agricultural and Food Chemistry**. 54(9): 3462-71.

Juliano, C., M. Cossu, M.C. Alamanni and L. Piu. 2005. Antioxidant activity of gamma oryzanol: Mechanism of action and its effect on oxidative stability of pharmaceutical oils. **International Journal of Pharmaceutics**. 299: 146–154.

Kamal-Eldin, A. and L-Å Appelqvist. 1996. The chemistry and antioxidant properties of tocopherols and tocotrienols. **Lipids**, Vol. 31, no. 7: 671-701.

Kazi, K.M., A.S. Mandal, N. Biswas, A. Guha, S. Chatterjee, M. Behera and K. Kuotsu. 2010. Niosome: A future of targeted drug delivery systems. **Journal of Advanced Pharmaceutical Technology and Research**. 1(4): 374–380.

Khatoon, S and A.G. Gopala Krishna. 2004. Fat-soluble nutraceuticals and fatty acid composition of selected Indian rice varieties. **Journal of the American Oil Chemists' Society**. 81(10): 939-943.

Kirby,C., J. Clarke and G. Gregoriadis. 1980. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. **Biochemical Journal**.186: 591-598.

Kumar, G.P., P. Rajeshwarrao. 2011. Nonionic surfactant vesicular systems for effective drug delivery—an overview. **Acta Pharmaceutica Sinica B.** 1(4): 208-219.

Lagarda, M.J., G. García-Llatas and R. Farré. 2006. Analysis of phytosterols in foods. **Journal of Pharmaceutical and Biomedical**. 41(5): 1486-1496.

Lampi, A.-M., L. Juntunen, J. Toivo and V. Piironen. 2002. Determination of thermo-oxidation products of plant sterols. **Journal of Chromatography B**. 777: 83-92.

Liu, A.L. 2008. **Advances in Planar Lipid Bilayers and Liposomes**. Volume 7. Elsevier Inc., Amsterdam.

Lei, Z., B. Chen and Z. Ding. 2005. **Special distillation process**. Elsevier B.V., Amsterdam.

Leong, W-F., O-M. Lai, K. Long, Y. B. CheMan, M. Misran, C-P Tan. 2011. Preparation and characterisation of water-soluble phytosterol nanodispersions. **Food Chemistry**. 129(1): 77-83.

Lerma-García, M.J., J.M. Herrero-Martínez, E.F. Simó-Alfonso, C.R.B. Mendonça, G. Ramis-Ramos. 2009. Composition, industrial processing and applications of rice bran γ-oryzanol. **Food Chemistry**. 115(2): 389-404.

Lin, K-M and S.S. Koseoglu. 2003. Separation of sterols from deodorizer distillate by crystallization. **Journal of Food Lipids**. 10:107-127.

Liu D. and L. Huang. 1989. Role of cholesterol in the stability of pH-sensitive, large unilamellar liposomes prepared by the detergent-dialysis method. **Biochimica et Biophysica Acta**. 981(2):254-60.

Liu, D., J. Shi, L. R. Posada, Y. Kakuda and S. J. Xue. 2008. Separating tocotrienols from palm oil by molecular distillation. **Food Reviews International**. 24:376–391.

Lu, Y., Y-Y Wang, N. Yang, D. Zhang, F-Y Zhang, H-T Gao, W-T Rong, S-Q Yu and Q. Xu. 2014. Food emulsifier polysorbate 80 increases intestinal absorption of di-(2-ethylhexyl) phthalate in rats. **Toxicological Sciences**. 139(2):317-327.

Magil, S.G., S.H. Zeisel and R.J. Wurtman. 1981. Effects of ingesting soy or egg lecithins on serum choline, brain choline and brain acetylcholine. **The Journal of Nutrition**. 111(1):166-70.

Mahale, N.B., P.D. Thakkar, R.G. Mali, D.R. Walunj and S.R. Chauhari. 2012. Niosomes: novel sustained release nonionic stable vesicular systems- an overview. **Advances in Colloid and Interface Science.** 1833-184: 46-54.

Manosroi, A., P. Wongtrakul, J. Manosroi, H. Sakai, F. Sugawara, M. Yuasa, M. Abe. 2003. Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. **Colloids and Surfaces B.** 30: 129-138.

Mandak, E. and L. Nyström. 2012. The effect of in vitro digestion on steryl ferulates from rice (*Oryza sativa* L.) and Other Grains. **Journal of Agricultural and Food Chemistry**. 60: 6123−6130.

Martins, P.F., V.M. Ito, C.B. Batistella and M.R.W. Maciel. 2006. Free fatty acid separation from vegetable oil deodorizer distillate using molecular distillation process. **Separation and Purification Technology**. 48: 78–84.

Meydani, S.N., S.N. Han and D. Wu. 2005. Vitamin E and immune response in the aged: molecular mechanisms and clinical implications. **Immunological Reveiws**. 205(1): 269 – 284.

Miller, A. and K-H. Engel. 2006. Content of γ-oryzanol and composition of steryl ferulates in brown rice (*Oryza sativa* L.) of European origin. **Journal of Agricultural and Food Chemistry**. 54: 8127-8133.

Mustacich, D.J., R.S. Bruno and M.G. Traber. 2007. Vitamin E. *In* Litwack, G., eds. **Vitamins and Hormones: Volume 76.** Academic Press, California.

Mollet, H. and A. Grubenmann. 2001. **Formulation Technology: Emulsions, Suspensions, Solid Forms.** 1st edition. WILEY-VCH Verlag GmbH. Weinheim.

Moreau, RA., B.D. Whitaker and K.B. Hicks. 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. **Progress in Lipid Research**. 41(6): 457-500.

Most M.M., R. Tulley, S. Morales and M. Lefevre. 2005. Rice bran oil, not fiber, lowers cholesterol in humans. **The American Journal of Clinical Nutrition**. 81:64–8.

Nagasaka, R., C. Chotimarkorn, I. Md. Shafiqul, M. Hori, H. Ozaki and H. Ushio. 2007. Anti-inflammatory effect of hydroxycinnamic acid derivatives. **Biochemical and Biophysical Research Communication.** 358: 615-619.

Nagesha, G.K., B. Manohar and K.U. Sankar. 2003. Enrichment of tocopherols in modified soy deodorizer distillate using supercritical carbon dioxide extraction. **European Food Research and Technology**. 217(5): 427-433.

Naumov, V.V. and R.F. Vasil'ev. 2003. Antioxidant and prooxidant effect of tocopherol. **Kinetics and Catalysis**. 44(1): 101-105.

Nesaretnam, K., R. Ambra, K.R. Selvaduray, A. Radhakrishnan, R. Canail and F. Virgili. 2006. Tocotrienol-rich fraction from palm oil and gene expression in human breast cancer cells. **Annals of the New York Academy of Sciences. 1031 (Vitamin E and Health)**: 143-157.

Neves, M.A., H.S. Ribeiro, I. Kobayashi and M. Nakajima. 2008. Encapsulation of lipophilic bioactive molecules by microchannel emulsification. **Food Biophysics**. 3(2): 126-131.

Normén, L., L. Ellegård, H. Brants, P. Dutta, H. Andersson. 2007. A phytosterol database: Fatty foods consumed in Sweden and the Netherlands. **Journal of Food Composition and Analysis**. 20: 193–201.

Norton, R.A. 1995. Quantitation of steryl ferulate and ρ-Coumarate esters from corn and rice. **Lipids**. 30(3): 269-274.

Nukit, N., P. Setwipattanachai, S.Chaiseri. and P. Hongsprabhas. 2014. Effects of surfactants and aging time on solidification of rice bran oil at room temperature. **Journal of Oleo Science**. In press.

Nyström, L., T. Achrenius, A-M. Lampi, R.A. Moreau and V. Piironen. 2007. A comparison of the antioxidant properties of steryl ferulates with tocopherol at high temperatures. **Food Chemistry.** 101: 947–954.

O'Brien, R.D., W.E. Farr and P.J. Wan. 2000. **Introduction to Fats and Oils Technology**. 2nd ed. AOCS Press. Champaign, Illinois.

O'Brien, R.D. 2009**. Fats and Oils: Formulating and Processing for Applications.** 3rd ed. CRC Press. Boca Raton.

O'Sullivan, SM., J.A. Woods and N.M. O'Brien. 2004. Use of Tween 40 and Tween 80 to deliver a mixture of phytochemicals to human colonic adenocarcinoma cell (CaCo-2) monolayers. **British Journal of Nutrition**. 91(5):757-764.

Office of Agriculture Economic, 2014. **Thailand's agricultural statistic 2014: Rice**. Available Source. http://www.oae.go.th/ewt_news.php?nid=13577 April 16, 2014.

Oka,T., M. Fujimoto, R. Nagasaka, H. Ushio, M. Hori and H. Ozaki. 2010. Cycloartenyl ferulate, a component of rice bran oil-derived γ-oryzanol, attenuates mast cell degranulation. **Phytomedicine**. 17(2): 152–156.

Orthoefer, F.T. 2005. Rice bran oil, pp. 464-489. *In* F. Shahidi, ed. **Bailey's Industrial Oil & Fat: Volume 5: Edible Oil and Fat Products: Processing Technologies.** 6th ed. John Wiley & Sons, Inc. New Jersey.

Ostrowska-Ligeza, E., W. Bekas, D. Kowalska, M. Lobacz, M. Wroniak and B. Kowalski. 2010. Kinetics of commercial olive oil oxidation: Dynamic differential scanning calorimetry and Rancimat studies. **European Journal of Lipid Science and Technology.** 112(2): 268-274.

Ouchi, A., M. Ishikura, K. Konishi, S. Nagaoka, K. Mukai. 2009. Kinetic study of the prooxidant effect of α-tocopherol. Hydrogen abstraction from lipids by α-tocopheroxyl radical. **Lipids**. 44: 935-943.

Pegg, R.B. and F. Shahidi. 2007. Encapsulation,stabilization, and controlled release of food ingredients and bioactives. pp. 510-568 In M.S. Rahman. 2007. **Handbook of Food Preservation.** 2nd ed. CRC Press Taylor and Francis Group, Boca Raton.

Pestana, V.R., R.C. Zambiazi, C.R.B. Mendonça, M.H. Bruscatto, M.J. Lerma-Garcı´a, G. Ramis-Ramos. 2008. Quality changes and tocopherols and γ-orizanol concentrations in rice bran oil during the refining process. **Journal of the American Oil Chemists' Society.** 85:1013–1019.

Phillips, K.M., D.M. Ruggio, J.I. Toivo, M.A. Swank and A.H. Simpkins. 2002. Free and esterified sterol composition of edible oils and fats. **Journal of Food Composition and Analysis.** 15: 123–142.

Piironen, V., J. Toivo, and A.-M. Lampi. 2000. Natural sources of dietary plant sterols. **Journal of Food Composition and Analysis**. 13: 619-624.

Posada, L.R., J. Shi, Y. Kakuda and S.J. Xue. 2007. Extraction of tocotrienols from palm fatty acid distillates using molecular distillation. **Separation and Purification Technology**. 57(2): 220-229.

Rajera, R., K. Nagpal, S. K. Singh and D.N. Mishra. 2011. Review: Niosome: a controlled and novel drug delivery system. **Biological and Pharmaceutical Bulletin**. 34(7): 945-953.

Rajnarayana, K., M.C. Prabhakar and D.R.Krishna. 2001. Influence of rice bran oil on serum lipid peroxides and lipids in human subjects. **Indian Journal of Physiology and Pharmacology**. 45(4): 442-444.

Rodriguez-Abreu, C., K. Aramaki, Y. Tanaka, M.A. Lopez-Quintela, M. Ishitobi and H. Kunieda. 2005. Wormlike micelles and microemulsions in aqueous mixtures of sucrose esters and nonionic cosurfactants. **Journal of Colloid and Interface Science**. 291: 560–569.

Rossi, M., C. Alamprese, S. Ratti. 2007. Tocopherols and tocotrienols as free radical-scavengers in refined vegetable oils and their stability during deep-fat frying**. Food Chemistry**. 102: 812–817.

Rozner, S. and N. Garti. 2006. The activity and absorption relationship of cholesterol and phytosterols. **Colloid Surface A.** 282–283: 435–456.

Royal Society of Chemistry. 2013. **Properties: Predicted-ACD/Labs**. Available Source: http://www.chemspider.com/, September 12, 2013.

Rukmini, C. and T.C. Raghuram. 1991. Nutritional and biochemical aspects of the hypolipidemic action of rice bran oil: A review**. Journal of American College Nutrition**. 10 (6): 593-601.

Sawadikiat, P. and P. Hongsprabhas. 2014. Phytosterols and γ-oryzanol in rice bran oils and distillates from physical refining process. International Journal of Food Science & Technology. In press.

Sen, C. K., S. Khanna and S. Roy. 2007. Tocotrienols in health and disease: the other half of the natural vitamin E family: Review. **Molecular Aspects of Medicine**. 28: 692–728.

Schuler, I., G. Duportail, N. Glasser, P. Benveniste and M.-A. Hartmann. 1990. Soybean phosphatidylcholine vesicles containing plant sterols: a fluorescence anisotropy study. **Biochimica et Biophysica Acta**. 1028: 82-88.

Schuler, I, A. Milon, Y. Nakatani, G. Ourisson, A.-M. Albrecht, P. Benveniste and M.-A. Hartmann.1991. Differential effects of plant sterols on water permeability and on acyl chain ordering of soybean phosphatidylcholine bilayers. **Proceeding of the National Academy of Science of the United State of America.** 88: 6926-6930

Schwartz, H., V. Ollilainen, V. Piironen and A-M. Lampi. 2008. Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. **Journal of Food Composition and Analysis.** 21: 152–161.

Shahiwala, A. and A. Misra. 2002. Studies in topical application of niosomally entrapped Nimesulide. **Journal of Pharmacy & Pharmaceutical Sciences**. 5(3):220-5.

Sharma, R.D. and C. Rukmini. 1986. Rice bran oil and hypocholesterolemia in rats. **Lipids**. 21(11): 715-717.

Shimada, Y., S. Nakai, M. Suenaga, A. Sugihara, M. Kitano and Y. Tominaga. 2000. Facile purification of tocopherols from soybean oil deodorizer distillate in high yield using lipase. **Journal of the American Oil Chemists' Society**. 77(10): 1009-1013.

Sierra, S., F. Lara-Villoslada, M. Olivares, J. Jiménez, J. Boza, J. Xaus. 2005. Increased immune response in mice consuming rice bran oil. **European Journal of Nutrition**. 44 : 509–516.

Struijs, K., A.-M. Lampi, V. Ollilainen, V. Piironen. 2010. Dimer formation during the thermo-oxidation of stigmasterol. **European Food Research and Technology**. 231(6): 853-863.

Szüts, A., M. Budai-Szücs, I. Erös , N. Otomo and P. Szabó-Révész. 2010. Study of gel-forming properties of sucrose esters for thermosensitive drug delivery systems. **International Journal of Pharmaceutics**. 383(1-2):132-137.

Takahashi, M., K. Inafuku, T. Miyagi, H. Oku, K. Wada, T. Imura and D. Kitamoto. 2007. Efficient preparation of liposomes encapsulating food materials using lecithin by a mechanochemical method. **Journal of Oleo Science**. 56(1); 35-42.

Thai Ministry of Public Health. 2000. **Notification of the Ministry of Public Health (No 205): Fat and Oil.**

Uchegbu, I.F. and A.T. Florence. 1995. Non-ionic surfactant vesicles (niosomes): physical and pharmaceutical chemistry. **Advance in Colloid and Interface Science**. 58:1-55

Uchegbu, I.F. and S. P. Vyas. 1998. Non-ionic surfactant based vesicles (niosomes) in drug delivery. **International Journal of Pharmaceutics**. 172: 33–70.

U.S. Department of Agriculture, Agricultural Research Service. 2013. **USDA National Nutrient Database for Standard Reference, Release 26**. Nutrient Data Laboratory Home Page. Available Source: http://www.ars.usda.gov/ba/bhnrc/ndl, April 16, 2014.

Van Hoed, V.G. Depaemelaere, J. Vila Ayala, P. Santiwattana, R. Verhé, and W. De Greyt. 2006. Influence of chemical refining on the major and minor components of rice bran oil. **Journal of the American Oil Chemists' Society** . 83(4): 315-321.

Van Hoed, V., J.V. Ayala, M. Czarnowska, W. De Greyt, R. Verhe. 2010. Optimization of physical refining to produce rice bran oil with light color and high oryzanol content. **Journal of the American Oil Chemists' Society**. 87:1227–1234.

Vemuri, s., C.T. Rhodes. 1995. Preparation and characterization of liposomes as therapeutic delivery systems: a review. **Pharmaceutica Acta Helvetiae**. 70: 95-111.

Verleyen, T., R. Verhe, L. Garcia, K. Dewettinck, A. Huyghebaert and W. De Greyt. 2001a. Gas chromatographic characterization of vegetable oil deodorization distillate. **Journal of Chromatography A.** 921: 277–285.

Verleyen, T., R. Verhe, A. Huyghebaert, K. Dewettinck and W.D. Greyt. 2001b. Identification of α-tocopherol oxidation products in triolein at elevated temperatures. **Journal of Agricultural and Food Chemistry** 49: 1508-1511.

Verleyen,T., A. Kamal-Eldin, C. Dobarganes, R. Verhe, K. Dewettinck and A. Huyghebaert. 2001c. Modeling of α-tocopherol loss and oxidation products formed during thermoxidation in triolein and tripalmitin mixtures. **Lipids**. 36 (7): 719-726.

Verleyen, T., A. Kamal-Eldin, R. Mozuraityte, R. Verhé, K. Dewettinck, A. Huyghebaert, W. D. Greyt. 2002a. Oxidation at elevated temperatures: competition between α-tocopherol and unsaturated triacylglycerols. **European Journal of Lipid Science and Technology**. 104: 228–233.

Verleyen, T., E. Cortes, R. Verhe, K. Dewettinck, A. Huyghebaert and W.D. Greyt. 2002b. Factors determining the steradiene formation in bleaching and deodorization. **European Journal of Lipid Science and Technology** 104: 331–339.

Vissers, M.N., P.L., Zock, G.W. Meijer, M.B. Katan. 2000. Effect of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum lipoprotein concentrations in humans. **The American Journal of Clinical Nutrition**. 72(6): 1510-1515.

Vorarat, S., C. Managit, L. Ianthanakul, W. Soparat and N. Kamkaen. 2010. Examination of antioxidant activity and development of rice bran oil and gamma-oryzanol microemulsion. **Journal of Health Research**. 24(2): 67-72.

Vreeburg, R.A.M., E.E. van Wezel, F. Ocaňa-Calahorro and J.J. Mes. 2012. Apple extract induces increased epithelial resistance and claudin 4 expression in Caco-2 cells. **Journal of the Science of Food and Agricultural.** 92(2): 439-444.

Wang, T., K. B. Hick, and R. Moreau. 2002. Antioxidant activity of phytosterols, oryzanol, and other phytosterol conjugates. **Journal of the American Oil Chemists' Society**. 799 (12): 1201-1206.

Wilson, T.A., L.M. Ausman, C.W. Lawton, D.M. Hegsted and R.J. Nicolosi. 2000. Comparative cholesterol lowering properties of vegetable oils: beyond fatty acids. **The Journal of the American College of Nutrition.** 19(5): 601-607.

Wilson, T.A., R.J. Nicolosi, B. Woolfrey and D. Kritchevsky. 2007. Rice bran oil and oryzanol reduce plasma lipid and lipoprotein cholesterol concentrations and aortic cholesterol ester accumulation to a greater extent than ferulic acid in hypercholesterolemic hamsters. **The Journal of Nutritional Biochemistry**. 18(2): 105-112.

Xu, Z., N. Hua and J. S. Godber. 2001. Antioxidative activity of tocopherols, tocotrienols, and γ-oryzanol components from rice bran against cholesterol oxidation accelerated by 2, 2'-azobis(2-methylpropionamidine) dihydrochloride. **Journal of Agricultural and Food Chemistry**. 49: 2077-2081.

Yamada, K., M. Okabe, K.-I Ohkura, M. Oji, M. Nonaka, I. Ikeda and H. Tachibana. 2002. Dietary effect of α-tocopherol and tocotrienols on lipid metabolism and immune function of aged sprague-dawley rats. **Food Science and Technology Research**. 8 (1): 59–63.

Yan, F., H. Yang, J. Li and H. Wang. 2012. Optimization of phytosterols recovery from soybean oil deodorizer distillate. **Journal of the American Oil Chemists' Society**. 89(7): 1363-1370.

Yang, H., F. Yan, D. Wu, M. Huo, J. Li, Y. Cao and Y. Jiang. 2010. Recovery of phytosterols from waste residue of soybean oil deodorizer distillate. **Bioresource Technology**. 101(5): 1471-1476.

Yasukawa K., T.Akihisa, Y. Kimura, T. Tamura and M. Takido. 1998. Inhibitory effect of cycloartenol ferulate, a component of rice bran, on tumor promotion in two-stage carcinogenesis in mouse skin. **Biological & Pharmaceutical Bulletin**. 21(10):1072-1076.

Yean, J.N., H.N. Seok and Y.K. Mi. 2008. Cholesterol-lowering efficacy of unrefined bran oil from the pigmented black rice (*Oryza sativa* L. cv. Suwon 415) in hypercholesterolemic rats. **Food Science and Biotechnology**. 17(3): 457-463.

APPENDIX

Appendix Table 1 Tween80-vesicle composition

Vesicle types	Code	Tween 80	Concentration of added components in 8 mL PBS (mg/mL)					
			β-Sitosterol	Dioleoyl glycerol	Trioleoyl glycerol	γ-Oryzanol	α-Tocopherol	UMD
Tween80/UMD	UV	6.3	-	-	-	-	-	18.8
Tween80/dioleoylglycerol/ trioleoylglycerol/β-sitosterol	EV	6.3	0.8	3.8	14.2	-	-	-
Tween80/dioleoylglycerol/ trioleoylglycerol/β-sitosterol/ α-tocopherol	TV	6.3	0.8	3.8	14.2	0.2	-	-
Tween80/dioleoylglycerol/ trioleoylglycerol/β-sitosterol/ γ-oryzanol	OV	6.3	0.8	3.8	14.2	-	0.06	-

CURRICULUM VITAE

NAME : Ms Pattong Sawadikiat

BIRTH DATE : May 26, 1980

BIRTH PLACE : Khon Kaen, Thailand

EDUCATION

YEAR	INSTITUTE	DEGREE/DIPLOMA
2002	Khon Kaen Univ.	B.Sc. (Food Technology)
2006	Kasetsart Univ.	M.S. (Food Science)

POSITION/TITLE : Assistant Product manager (2006-2007)

WORK PLACE : Topflight Interfoods Co., Ltd, Bangkok, Thailand (2006-2007)

SCHOLARSHIP : The Royal Golden Jubilee Ph.D. Program (RGJ) under The Thai Research Found 2007-2010

AWARDS : Outstanding oral presentation, RGJ-Ph.D. Congress X April 3-5, 2009

: Outstanding poster presentation, RGJ-Ph.D. Congress XI April 1-3, 2010