DERIVING A NMR METHOD FOR USE IN ASSESSING THE STABILITY OF COLD-PRESSED RICE BRAN OIL

BY

MISS CHUTIMA PONCHURDCHAI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN CHEMISTRY
FACULTY OF SCIENCE AND TECHNOLOGY
THAMMASAT UNIVERSITY
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was approved as partial fulfillment of the requirements for
the master degree of science in chemistry

on December, 21 2015

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ABSTRACT

Oil obtained from the rice bran (*Oryza sativa* L.) has attracted much interest due to its rich source of γ-oryzanol, tocopherols and tocotrienols. Those compounds are considered to have an important role in preservation of the quality of oils, due to their antioxidant activity. Oil quality is evaluated by classical methods such as acid value, peroxide value, and iodine value and by chromatographic methods such as gas chromatography-mass spectrometry (GC-MS). In addition, a recently developed rapid and simple alternative method using nuclear magnetic resonance (NMR) spectroscopy allows for the determination of the proportions of the different acyl groups and minor components in oil.

In this research, the stability of cold-pressed RBO samples from 2 different regions (Lopburi province, L-RBO and Yasothorn province, Y-RBO) were evaluated over a year through monitoring their stability. ^{1}H NMR assignments of oils are applied to study the degradation of RBOs, normally caused by hydrolysis and oxidation over period of times. The integration of signals in the ^{1}H NMR spectra allows for the monitoring of the fatty acid compositions throughout the RBO aging process. GC-MS was also used to determine the fatty acid composition, and compared with results from ^{1}H NMR. Both ^{1}H NMR and ^{13}C NMR spectra were used to identify the formation of monoglyceride, diglyceride, and free fatty acids and to observe

oxidative changes for quality assessment. This study is the first example where RBOs are assessed using the rapid and convenient NMR method to monitor stability, and these results could be applied to the assessment of the freshness and quality of organic or non-organic, refined or cold-pressed RBOs. The aim of this work is to achieve a practical methodology for rapid quality assessment of cold-pressed RBOs used in food, cosmetic and pharmaceutical applications. The results confirm that cold-pressed can maintain their quality over one year storage under no air and light exposure. One year shelf-life of cold-pressed RBO with quality assessment has been reported in molecular level and revealed low degree of hydrolytic degradation with no primary or secondary oxidative intermediates.

Keywords: Rice bran oils, quality, stability, ^{1}H NMR, ^{13}C NMR

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Miss Chutima Ponchurdchai

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
AV	Acid value
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
CODEX	Codex Alimentarius; standard for named vegetable oils
DHA	Docosahexaenoic acid
EPA	Eicosopentaenoic acid
FAMEs	Fatty acid methyl esters
FT-IR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-Mass spectroscopy
HPLC	High performance liquid chromatography
IV	Iodine value
L	Linoleic acid
Ln	Linolenic acid
L-RBO	Rice bran oil represents from Lopburi province
***n*-3**	Polyunsaturated fatty acid or Linolenic acid
***n*-6**	Polyunsaturated fatty acid or Linoleic acid
***n*-7**	Monounsaturated fatty acid
***n*-9**	Monounsaturated fatty acid or Oleic acid
MUFA	Monounsaturated fatty acid

LIST OF ABBREVIATIONS (CONT.)

Symbols/Abbreviations	Terms
RBO	Rice bran oil
O	Oleic acid
PV	Peroxide value
S	Saturated acyl groups
SFA	Saturated fatty acids
Y-RBO	Rice bran oil represents from Yasothorn province

CHAPTER 1
INTRODUCTION

1.1 Rice

The main staple food for the world's population and the most important crops in the world is rice (*Oryza sativa* L.). At present, rice is grown more than 100 countries and more than 1 billion people's livelihood are dependent on it [1] [Thailand production of rice was estimated to 36 million tons in 2013 (FAOSTAT, 2015)]. Paddy rice storage and local rice productions are distributed over Thailand (Fig. 1). Milled rice is globally produced about 740 million tons which is used for human intake up to 85% and the remain is used for animal feed or waste over 15% [2].

(a)

(b)

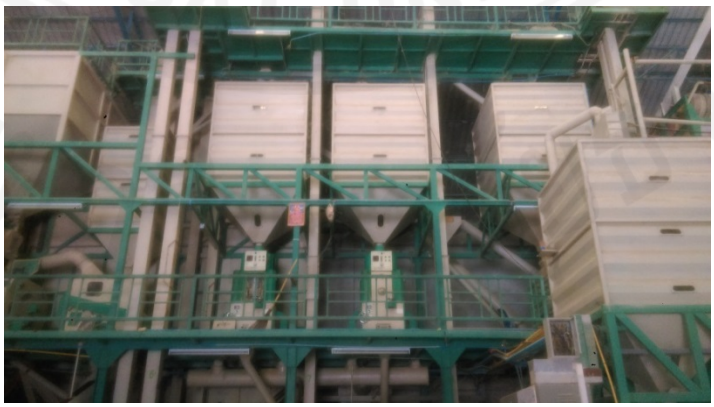


Figure 1 Rice production in Yasothorn.
(a) paddy rice storage and (b) local rice production

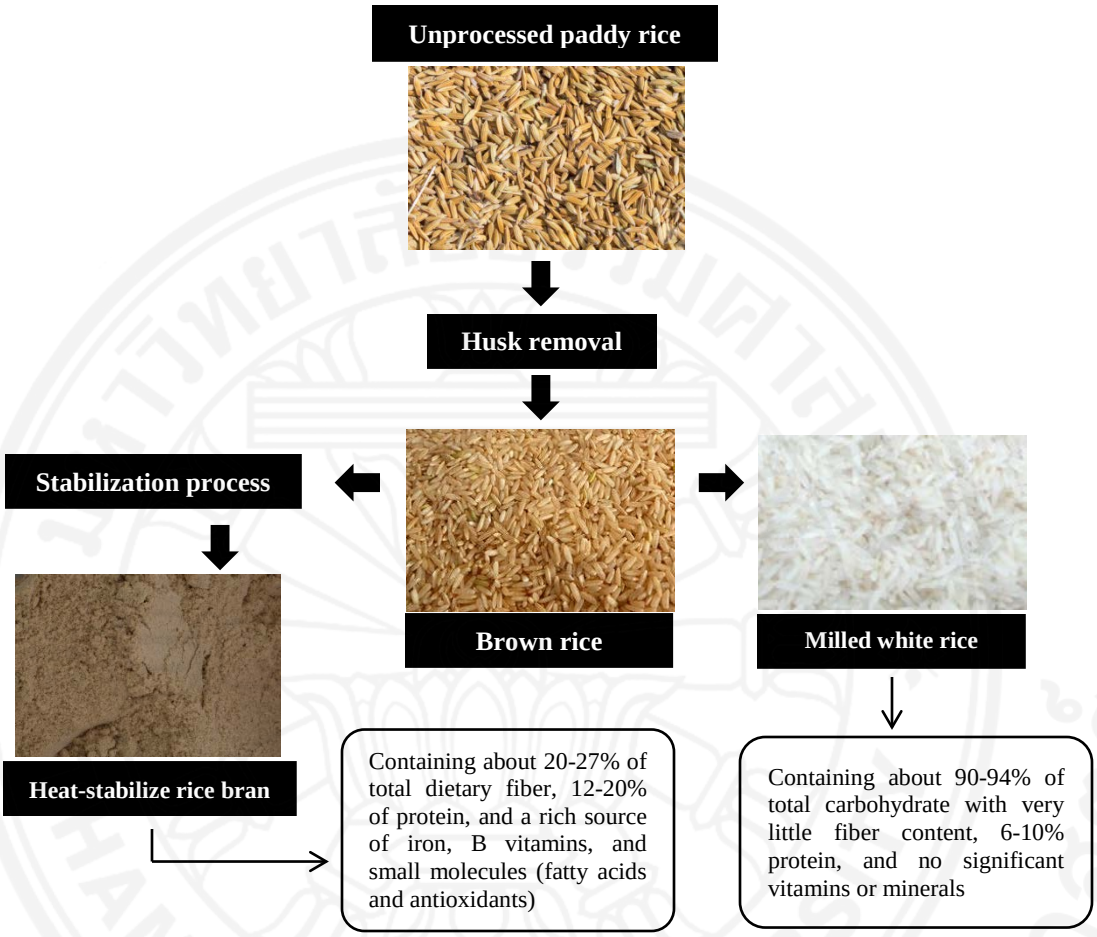


Figure 2 Whole grain rice processing and a summary of nutrient variations of white rice and end products of rice bran [2].

There are two major subspecies, one of this called *Oryza sativa* L. *japonica*, originally consumed in Southeast Asia, northern China, Japan and the United States, and other is called *Oryza sativa* L. *indica*, which is mainly consumed in India, southern China, and Southeast Asia [1]. Rice is generally employed in the polished or white grain form by rice whitening and polishing steps (Fig. 2). Therefore, many nutritious components of refined white grain has lost and remained 90-94% of carbohydrate with fiber content of 6-10% (Fig. 2). Rice bran has 10% weight ratio of bran to whole rice and contains abundant source of starch (34-62%), lipids (15-22%), protein (11-15%), soluble and insoluble dietary fibers (20-29%) [2, 3]. Moreover, rice bran contains various types

of phytochemicals such as tocopherols, tocotrienols, oryzanols, vitamin B complex, and phenolic compounds [2, 4].

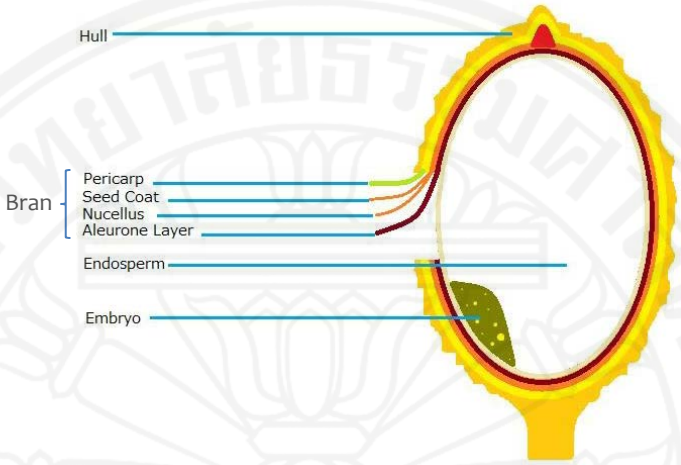


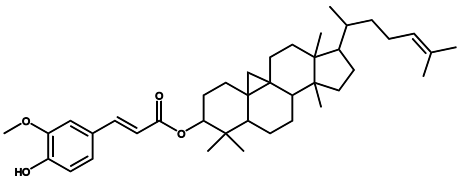
Figure 3 Illustration of the structure of the rice kernel.

1.2 Rice Bran

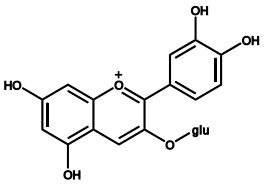
Rice bran is a part of the rice kernel that includes aleurone, pericarp and subaleurone fractions as shown in Fig. 3 [5]. It is the most abundant, but minor product (by product) which is produced in the rice milling process. Rice bran contains a unique complex of natural antioxidant compounds and pigmented bran contains more bioactive compounds than white bran [6]. Most of these bioactive compounds benefit on human health and have a great deal of potential in antioxidant activity, scavenging free radicals, improving of immune systems, and reduction of the risk for developing cancer and heart disease [7]. Bioactive compounds of rice bran are classified to five types (Table 1): (1) phenolic and cinnamic acids; (2) anthocyanins and flavonoids; (3) steroidal compounds; (4) polymeric carbohydrates; (5) others: proteins, phosphorus compounds, B vitamins and minerals. Therefore, rice bran has been globally gained high interest as a potential resource of nutrients and elements such as food supplements for diabetes [8] and supplementary source for pan breads [3]. Rice protein has been recently reported as a good functional food for food and nutritional supplements [9].

Table 1 Bioactive compounds of rice bran [5].

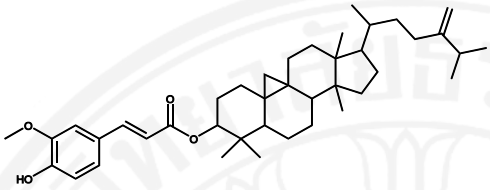
Components	**Bioactive compounds**
Phenolic and cinnamic acid	caffeic acid, coumaric acid, catechins, ferulic, gallic acid, hydroxybenzoic acid, methoxycinnamic acid, sinapic acid, syringic acid, vanillic acid
Anthocyanins, flavonoids	anthocyanin monomers, dimers, and polymers, apigenin, cyanidin glucoside **(2),** cyanidin rutinoside **(4)**, epicatechins, eriodtyol, hermnetins, hesperetin, isorhamnetins, luteolin, peonidin glucoside **(10)**, tricin **(6)**, cyanidin-gentiobioside **(8)**, Stigma glucopyranoside **(9)**, momilactone B **(11)** and phenyl glucoside **(12)**
Steroidal compounds	acylated steryl glucosides, cycloartenol ferulate **(1)**, campesteryl ferulate **(5)**, 24-methylenecycloartenyl ferulate **(3)**, γ-oryzanol, β-sitosteryl ferulate **(7)**, tocopherols and tocotrienol
Polymeric carbohydrates	arabinoxylan, glucans and hemicellulose
Others **-Proteins** **-Phosphorus compounds** **-B Vitamins [3]** **-Minerals [3]**	 gutelin, prolamin [9] phytic acid [4] thiamin (B_1), riboflavin (B_2), niacin (B_3), pyridoxamine (B_6) and pyridoxine (B_6) zinc (Zn), iron (Fe), potassium (K) and calcium (Ca)



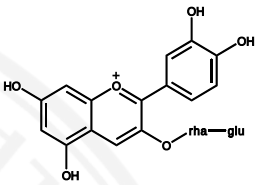
Cycloartenol ferulate **(1)**



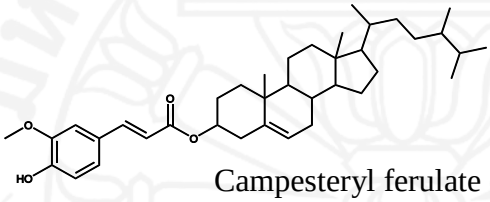
Cyanidin-3-O-glucoside **(2)**



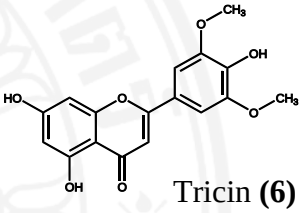
24-methylenecycloartanyl ferulate **(3)**



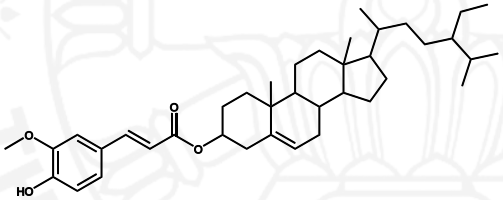
Cyanidin-3-O-rutinoside **(4)**



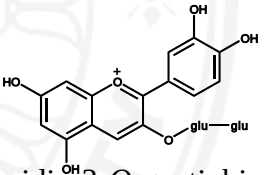
Campesteryl ferulate **(5)**



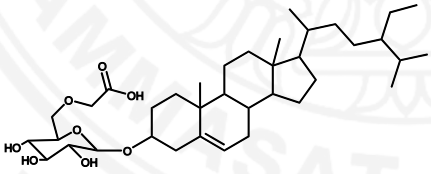
Tricin **(6)**



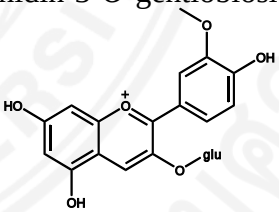
Sitosteryl ferulate **(7)**



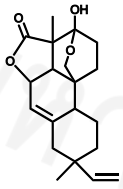
Cyanidin-3-*O*-gentiobioside **(8)**



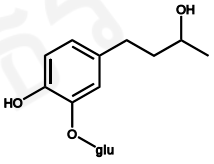
Stigma-5-en-3-*O*-β-glucopyranoside **(9)**



Peonidin-3-*O*-glucoside **(10)**



momilactone B **(11)**



2-hydroxy-5-[(3s)-3-hydroxybutyl] phenyl-β-D-glucoside **(12)**

Figure 4 Structures of selected bioactive rice bran compounds [5].

High performance liquid chromatography (HPLC) is commonly used to determine the antioxidants contents of rice bran. The contents of γ-oryzanol, phenolic

acid, anthocyanin and α-tocopherol in normal bran, red bran and black bran were compared as shown in Table 2. The high anthocyanin, phenolic acid, and γ-oryzanol contents were found in black bran while the highest contents of α-tocopherol found in normal bran [6]. Moreover, nine anthocyanins were reported to be detected by HPLC and identified by ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC/Q-TOF-MS) [10].

1.3 Rice Bran Oils (RBO)

Rice bran is extracted to produce the rice bran oil (RBO) which is many health benefits. There is generally used in many countries such as India, China, Japan, Korea, and USA not only for nutritional purposes, but also as raw materials for a wide range of industrial products which include biofuels, skin care productions and high pressure lubricants.

Table 2 The categories of bioactive rice bran compounds in different pigment [6].

Antioxidants[a]	**Contents (μg/g)**		
	Normal bran	Red bran	Black bran
Anthocyanins	-	188±8	2562±34
	0%	(5.2%)	(25.8%)
Phenolic acids	2101±175	1526±103	3289±116
	(39.5%)	(42.5%)	(33.1%)
α-Tocopherol	71±8	16±4	24±3
	(1.25%)	(0.5%)	(0.2%)
γ-Oryzanol	3681±30	1859±121	4057±464
	(62.9%)	(51.8%)	(40.9%)
Total	5853	3589	9932
	(100%)	(100%)	(100%)

[a] Numbers in parentheses are percentage of each antioxidant in rice bran extract.

The major components of RBO is triacylglycerols, minor components (consist of waxes (1.5-4%), mono- and diglycerides, free fatty acids) and other compounds

(unsaponifiable constituents, USC; consist of tocopherols and tocotrienols (0.15-0.2%), phytosterols (1.5-2%) and oryzanols (1.2-1.8%)) [11-14].

Crude rice bran oil contains a high amount of wax (1.5-4%), phosphatides (0.5-1.5%) and fatty acids (59.19%) [15, 16] including oleic acid (39-42%), linoleic acid (35-40.99%), linolenic acid (1.76%), palmitic acid (19.7-22%), stearic acid (1.56-2.3%) and arachidonic acid (0.7%). RBO has the unsaturated fatty acid in higher level than other vegetable oils; over 70% of oleic acid and linoleic acid and comprising 22% of palmitic acid [16-18]. Moreover, RBO is a rich source of natural antioxidant compound called γ-oryzanols which is reported to be essential to health for decreasing plasma cholesterol, lowering serum cholesterol concentration and decreasing platelet aggregation [18-20]. Consequently, RBO can be a good candidate of raw materials for bioactive components which has increased oxidative stability compared to other vegetable oils.

The composition of γ-oryzanol including 4-hydroxy-3-methoxycinnamic acid (ferulate) esters of cycloartenyl ferulate **(1)**, 24-methylenecycloartanyl ferulate **(3)**, β-sitosterol ferulate **(7)**, and campesterol ferulate **(5)** [5] (Fig. 4) can be separated and quantified by high performance liquid chromatography [18]. Rice bran has been reported ester levels of 3.4 mg/g of bran, and RBO has high levels of cyclobromol esters of 15.7 mg/g of oil (**1**, **5**, and **7** in Fig. 4) [21].

The antioxidant activities of vitamin E and γ-oryzanol component from rice bran were studied and compared [22] that 24-methylenecycloartanyl ferulate **(3)** revealed with highest antioxidant activities (34-44% of total) [23]. Furthermore, all the γ-oryzanol components (**1**, **3**, **5** and **7**) showed their antioxidant activities higher than vitamin E components (α-tocopherol, α-tocotrienol, γ-tocopherol and γ-tocotrienol) [22].

RBO properties are continually and widely studied on humans and animals involving in a wide range of biological activities. The important properties have shown the decrease of low density lipoprotein (LDL) cholesterol and total serum cholesterol. Also, increasing of high density lipoprotein (HDL) cholesterol to some extent either by influencing absorption of dietary cholesterol or by enhancing the conversion of cholesterol to fecal bile acid and sterols [24]. Moreover, γ-oryzanol has

been reported to increase endorphin releasing and help muscle development by intaking with exercise training [25].

To ensure a high oil quality, mechanical pressing is preferred (lower process temperature and no solvent) although lower yields are achieved. In Thailand, cold pressing process has been used in small and medium factories for producing commercial RBO. This technique has more advantage in safety and simplicity than solvent extraction technique. In addition, these oils show native properties due to no heat and chemical treatments; therefore it is suitable for small and medium scale production in Thailand (Fig. 5) for local domestic use. Recently, cold-pressed plants of similar oils have been received great attention, such as sesame oil.

Figure 5 Rice bran oil production process by using screw pressing.

Rice bran obtained from rice milling process is transferred directly for cold-pressed process of RBO within 24 hours included feeding the rice bran into chamber,

and then pressed at temperature. The oil is filtrated through cheesecloth and filtrated again with filter paper. The crude RBO is set to stand until most of the particles are participated. Then, the RBO was filtrated by high performance pressed filter several times to receive cold-pressed RBO ready for bottles.

Considering oil yield, many studies demonstrated that the factors contributing to higher pressure and temperature in the screw barrel had a major positive influence on oil yield. Nevertheless, high temperature could be responsible for oil degradation. Moreover, cold-pressed process still has a challenge in enhancing oil yield as the effect of screw pressing parameters, such as die diameter and screw rotation speed, cannot be applied to increase the yield [26].

1.4 Objectives

Rice bran oil samples were obtained from local small and medium size factories in Thailand for assessing their high quality products. There are two important regions of rice production in Thailand which are selected as samples in this study. RBOs from Yasothorn province represent from Northeast region of Thailand and RBOs from Lopburi province represent from central region of Thailand.

The aim of this research was to study the stability of cold-pressed rice bran oil from two different regions in a period of one year and monitor some parameters such as acid value, peroxide value and iodine value for determining the quality of rice bran oil. In addition, those data are related to the fatty acid compositions in rice bran oils and some compounds, formed during storage and caused by degradation process such as mono- or diglycerides and epoxide were investigated. The degradation behaviors of RBO disclosed the safety issue for further uses of RBO in formulation, required in pharmaceutical, food and cosmetic applications.

1.5 Scope and limitations of the study

The invention of NMR techniques had led to a high throughput application to monitor the degradation of triglyceride. This offers a better method alternatively to evaluate the oil by acid value, iodine value and peroxide value while GC-MS can be troublesome for inaccurately and larger amount of samples used. Therefore, all

techniques will be used in this study and compared the results. Conversely, NMR analysis is required skillful operator and experienced interpreter to evaluate the data.

- All chemical analysis of quality parameter was carried out according to the AOAC method [75].
- Fatty acid composition of RBOs was determined by using ^{1}H NMR technique and compared results with GC-MS technique.
- ^{1}H and ^{13}C NMR techniques were used in this research for observing degradation of oil, involved mono- or diglyceride formation.
- The factors such as RBO stability in vary cooking temperature, emulsion formula, different in rice variety are not included in this current study.

1.6 Expected outcomes

Stability of RBOs can be monitoring by using NMR technique which is rapid and reliable methodology for both fatty acid composition and degradation. This fundamental study can be applied to identify or approve a specific character of organic cold-pressed rice bran oil, especially any claimed cold-pressed RBOs products in the market. In future work, the NMR analysis of cold-pressed RBOs will be approached to study RBO-in-water emulsions in order to certify the products, formulated by organic cold-pressed rice bran oil.

CHAPTER 2
LITERATURE REVIEW

2.1 Degradation of oil

Triglycerides are the main components of vegetable oil. Their structures consist of glycerol part and different acyl group parts such as saturated, monounsaturated and polyunsaturated acyl groups (Fig. 6). When the oil is submitted to air exposure or even high temperature, degradation of oil will be occurred through oxidation or hydrolysis reaction which is mostly affected to fatty acid transformation and triglyceride. This effect has been attributed to oil properties and quality and represented in physical and chemical characters.

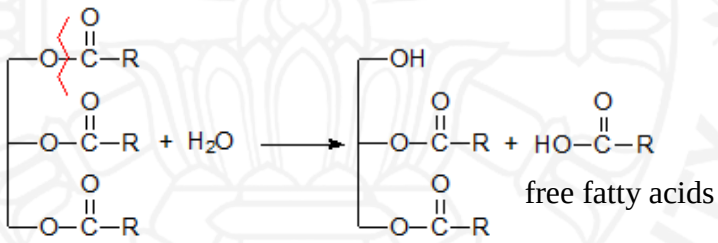


Figure 6 Hydrolysis reaction scheme.

Hydrolysis is a main reaction that breaks down the triglyceride molecule and causes formation of mono-, diglycerides and free fatty acids.

The oxidation of polyunsaturated acyl group is reported to be given unstable intermediate primary oxidation products containing both hydroperoxide groups and dienic conjugated systems, which consequently evolved to give secondary oxidation products. Aldehydes, ketones, free acids and hydroxilic compounds are reported to be mostly produced from thermal oxidation [27, 28].

The oxidation process can be degraded oil, which has been reported not only to reduce both shelf life and the nutritional value of oil but also produce toxic compounds.

Moreover, formation of some compounds can effect on human health, especially, toxic oxygenated *α,β*-unsaturated aldehydes from oxidation of polyunsaturated fatty acid [29].

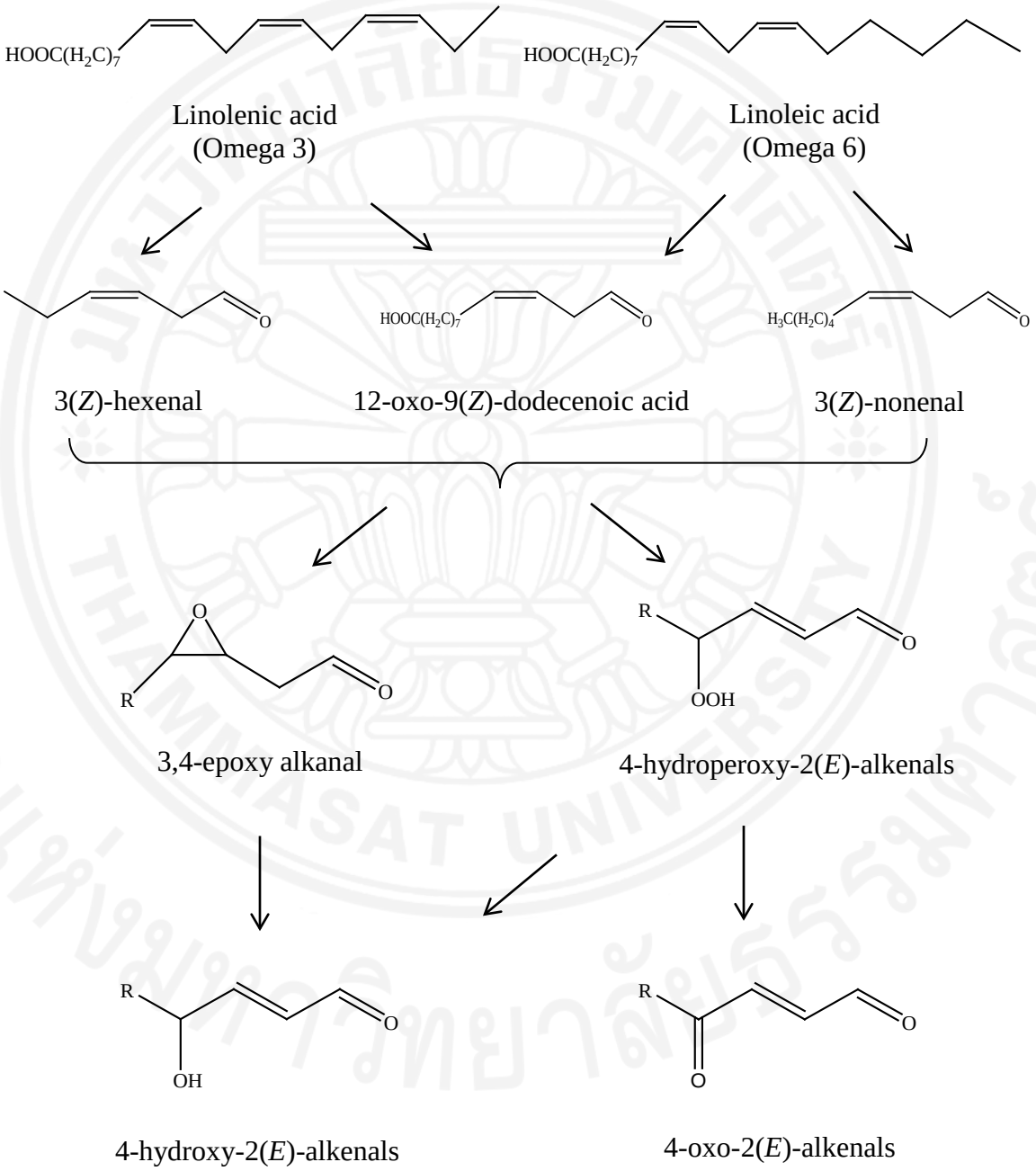


Figure 7 Oxidation of polyunsaturated acyl group [29].

Fig. 7 showed pathways of formation of different oxygenated α,β-unsaturated aldehydes (OαβUAs) from polyunsaturated acyl group. The oxidation of omega-3 and omega-6 polyunsaturated acids produced hydroperoxide derivatives which evolved to give 12-oxo-*cis*-9-dodecenoic acid, *cis*-3-hexenal or *cis*-3-nonenal. The reduction of these three *cis*-unsaturated aldehydes given 4-hydroxy-2(*E*)-alkenals or dehydration of these *cis*-unsaturated aldehydes produced 4-oxo-2(*E*)-alkenals through hydroperoxy or epoxy intermediate derivatives [29].

In fact previous studies have proved the formation of genotoxic and cytotoxic 4-hydroperoxy-(*E*)-2-alkenals and 4-hydroxy-(*E*)-2-alkenals from different edible oils submitted to a broad range of degradative conditions as shown in Table 3.

Table 3 Degradative conditions to formation the genotoxic and cytotoxic compounds from different edible oils.

Authors	**Oil types**	**Degradative conditions**
Guillén & Ruiz (2004, 2005, 2008) [30-34]	Sesame oil, Edible oil (rapeseed, walnut, linseed oils)	Submitted to 70°C and 190°C both with aeration
Guillén & Ruiz (2006) [35]	Edible oil (virgin olive, corn, linseed oils)	Submitted to microwave action below 190°C
Guillén & Goicoechea (2007) [36]	Sunflower oil	Storage at room temperature in closed receptacles
Guillén & Uriarte (2012) [37]	Sunflower oil	Heated at 190°C

There is evidence that these toxic compounds are also produced in the thermoxidative degradation of edible oils provoked under different conditions; furthermore, it has been proved that the amount and formation of these toxic aldehydes

produced depends on both the oxidative conditions and oil nature. It is suggested that these toxic compound are mostly started from the oils with high content of linolenic acid.

Oil properties and quality depend on its fatty acid composition which is related to health benefit in term of shelf-life and could be monitoring over period of time. For these reasons the quantification of the proportions of the fatty acid in oils is a very important task in order to determine the quality of oil and investigate the degradation in a term of time and temperature.

2.2 Characterization of oil

The most common method to evaluate oil quality is by classical methods such as acid value, peroxide value, iodine value, etc. There are also chromatographic methods for determining the proportion of fatty acid, minor components, etc. [38] to characterize the oil in molecular level.

2.1.1 Gas chromatography technique

Gas chromatography [39] is one of among the most common methods for determining the fatty acid of vegetable oils, animal fats and their derivatives. For this purpose, the oils or fats are usually converted to the corresponding methyl esters, followed by standard method and detailed by the International Union of Pure and Applied Chemistry (IUPAC). It involves the alkaline saponification of extracted fat to break down glycerides, and the liberated FAs, being esterified in the presence of methanol and boron trifluoride. Fatty acid methyl esters (FAMEs) are then extracted with organic solvent and analyzed using GC with flame ionization detector (FID) [40]. There are many methods to prepare the oils or fats samples as FAMEs as described in Table 4.

Although gas chromatography (GC) methods have previously been widely employed for determining fatty acid in oils, these methods are complicate, required a lot of time and cost, and in some cases the results are not very accurate. Some problems related with oxidation of the sample, with the formation of some compounds during transesterification, or with the separation and identification of the methyl esters in the chromatographic run have been described [41, 42]. Taking this into account, the

development of new methods for oil characterization is great interest as greener analytical technique

Table 4 Methods for converting the oil to determine fatty acid composition.

Authors	**Method**
Fernando D. Goffman et al. (2003) [43]	Transmethylation with solution of sodium methylate in methanol
Y. C. Mitei et al. (2008) [44]	Transesterified oil samples by refluxing in dry methanol
E. L. Bakota et al. (2013) [45]	Transmethylation with methanolic KOH
I.A. Nehdi et al. (2013) [46]	Transesterified with sodium methoxide

2.2.2 Alternative methods by Spectroscopy technique

The chemical analysis by other techniques, including FT-IR and NMR can be applied to determine the different acyl groups in oils and fats. These techniques are fast and usually require little time to prepare the sample and therefore can be cost-effective, and provide a great deal of information with only one test. There is no excessive sample treatment: consequently, the use of high amount of hazardous solvents and reagents can be avoided.

2.2.2.1 Fourier Transform Infrared (FT-IR) Spectroscopy

The intensity of concrete infrared spectroscopic bands for determining the degree of unsaturation of oils and fats has been known for a long time [47] and this technique provides on the fast acquisition of a great number of spectral data [48].

The C-H stretching, and sometimes from the C=C stretching region of the spectra were chosen for FT-IR determination. In addition, infrared spectroscopy (IR) has been widely used for determination of *trans* unsaturated fatty acids in oils and fats industry, and the method has been standardized by AOCS and IUPAC [47]. This method was applied to Fourier transform infrared spectroscopy (FT-IR) by F. Ulberth et

al. [49] who reported the rapid methodology for determination of *trans* unsaturation in edible fats.

The absorbance of some bands of the FT-IR spectrum related to unsaturation degree of edible oils and fats [38]. It has also been proved that frequency values of some bands are also closely related to the proportions of saturated, monounsaturated and polyunsaturated acyl groups. FT-IR spectroscopy was improved for determination of iodine value and saponification number [50], free fatty acid [51], peroxide value [52] and *cis-trans* content [53] of edible oils. The band at 1711 cm^{-1} and 3750-3150 cm^{-1} were used for determine the free fatty acid and peroxide value, respectively.

M.D. Guillén and N. Cabo (1997) [47] reported the assignment of functional group vibrations of different edible oils on FT-IR spectra and predicted the proportions of saturated, monounsaturated and polyunsaturated acyl groups by using equation obtained from frequency of some band.

Moya Moreno et al. (1999) [27, 28] mixed oil sample with *n*-valeronitrile as internal standard. A peak height at 3008 cm^{-1} was used to determine unsaturation by using absorbance band at 2246 cm^{-1} (C≡N stretching) as reference band. In addition, this technique could be used for monitoring oxidation processes of edible oil.

M.D. Guillén and N. Cabo (1999) [33] studied oxidation process of edible oils by FT-IR spectroscopy. They heated the oil samples at 70°C and recorded spectra every day. The most of the bands of the spectra showed the change of frequency value. The different stages of the oxidation process could be explained by the change of frequency value of specific bands and the oxidation degree of each oil sample was obtained from this observed here.

2.2.2.2 Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has been used in edible oil analysis since 1976. Several applications on oil have been investigated, for example, characterization of fatty acid composition, assessment of oil

quality, the study of lipid oxidation, and the assessment of varietal differences and geographical origin of oils [54] and showed in Table 5.

All these studies are based on the assignment of ^{1}H NMR spectral signals to different kind of protons and on the subsequent treatment of the values obtained from the integration of these signals.

Y. Miyake et al. (1998) [55] compared unsaturated fatty acid composition of vegetable oil between ^{1}H NMR and ^{13}C NMR. The results from ^{13}C NMR obtained the difference between GC methods at most ±5% in case of unsaturated fatty acid content less than 5%. In addition, Y. Miyake et al. (1998) [56] determined iodine value by using ^{1}H NMR method compared with traditional Wijs–cyclohexane methods and ^{1}H NMR method showed the error within ±1.

Table 5 The literature of the use of ^{1}H NMR technique in several oils.

Authors	**Oil types**	**Applications**
L. Nielsen et al. (1976) [57]	Coconut, olive, peanut, soybean, sunflower, safflower, whale, linseed and tung oils	Determining the unsaturation and average molecular weight
R. Sacchi et al. (1997) [58]	Virgin olive oil	Overview of quality assessment
Y. Miyake et al. (1998) [55]	Palm, olive, safflower, rapeseed, Soybean and corn oils	Determining unsaturated fatty acid
Y. Miyake et al. (1998) [56]	Olive oil, corn oil, rapeseed oil, soybean oil, safflower oil, palm oil, and hydrogenated oils	Determining iodine value
T. Igarashi et al. (2000) [41]	Fish oils	Determining DHA content and proportion of *n*-3 fatty acids

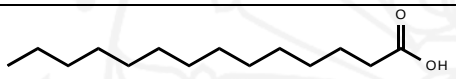
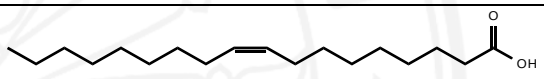
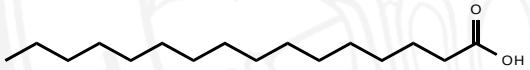
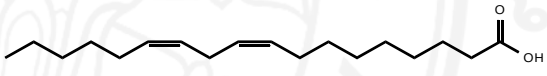
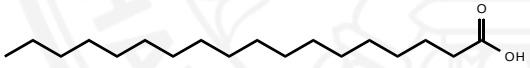
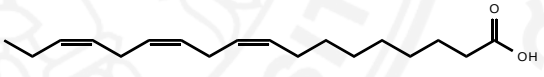
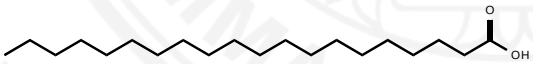
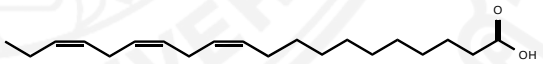
Note: DHA; Docosahexaenoic acid is one of *n-3* fatty acids type.

n-3 fatty acids is polyunsaturated fatty acid or linolenic acid.

2.3 Determination of fatty acid composition of oil by ^{1}H NMR

Fatty acids can be divided into two groups according to the type of bonds in the hydrocarbon chain including saturated fatty acid and unsaturated fatty acid. Moreover, fatty acids can be divided to monounsaturated and polyunsaturated fatty acid. Table 6 showed example of bond line structure of saturated fatty acids that include myristic acid, palmitic acid, stearic acid, arachidic acid and unsaturated fatty acids such as oleic acid, linoleic acid, linolenic acid and eicosatrinoic acid.

Table 6 Example of fatty acid molecular structures.

Saturated fatty acids	**Unsaturated fatty acids**
 Myristic acid (C14:0)	 Oleic acid (C18:1)
 Palmitic acid (C16:0)	 Linoleic acid (C18:2)
 Stearic acid (C18:0)	 Linolenic acid (C18:3)
 Arachidic acid (C20:1)	 Eicosatrienoic acid (C20:3)

R. Sacchi et al. (1993) [42] proposed a rapid method for the determination of *n*-3 polyunsaturated fatty acids (PUFAs) in fish lipids by ^{1}H NMR spectroscopy. The chemical shift of the methyl resonance of *n*-3 PUFAs could be observed at δ = 0.95 ppm and the methyl resonance of all other fatty acids also observed at δ = 0.86 ppm as shown in Fig. 8 with E labeling (or signal 10).

In 1997, R. Sacchi et al. [58] approached the applications of high-resolution ^{1}H and ^{13}C NMR spectroscopy in the analysis of virgin olive oil and performed the authentication and quality assessment of virgin olive oil.

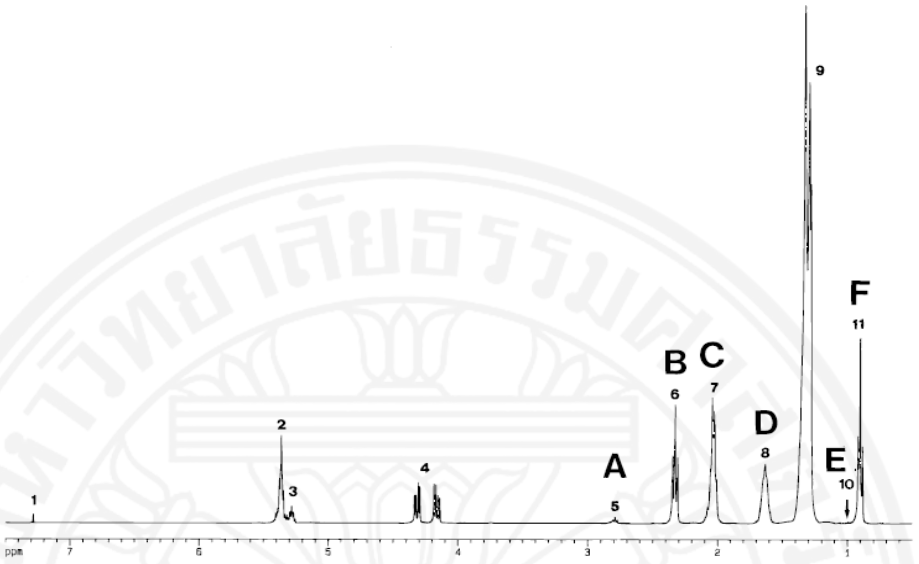


Figure 8 400 MHz ^{1}H NMR spectrum of a virgin olive oil. Assignments for signals 1-11(A-F) are given in Table 7 [58].

Table 7 Chemical shifts (δ) and assignment of the main resonances in the ^{1}H NMR spectrum of virgin olive oil (Fig. 8) [58].

		Assignment	
Peak	**δ (ppm)**	**Proton**	**Compound**
1	7.26	$CHCl_3$	Chloroform (solvent)
2	5.29	C**H**=CH	All unsaturated fatty acids
3	5.15	C**H**OCOR	Glycerol (triacylglycerols)
4	4.19	C$\mathbf{H}_2$OCOR	Glycerol (triacylglycerols)
5 (A)	2.76	CH=CHC$\mathbf{H}_2$CH=CH	Linoleyl and linolenyl (*bis*-methylene)
6 (B)	2.20	C$\mathbf{H}_2$COOH	All acyl chains (α-proton to acyl gr.)
7 (C)	2.02	C$\mathbf{H}_2$CH=CH	All unsaturated fatty acids
8 (D)	1.60	C$\mathbf{H}_2$CH$_2$COOH	All acyl chains (β-proton to acyl gr.)
9	1.20	$(C\mathbf{H}_2)_n$	All acyl chains
10 (E)	0.95	CH=CHCH$_2$C$\mathbf{H}_3$	Linolenyl
11 (F)	0.85	CH$_2$CH$_2$CH$_2$C$\mathbf{H}_3$	All acids except linolenyl

R. Sacchi et al.[58] proposed to determine sum of saturated fatty acid (SFA), *n*-9 and *n*-7 monounsaturated fatty acid (MUFA) and *n*-6 linoleic content which were relative to the *n*-3 linolenic content obtained from characteristic of methyl signal at 0.95 ppm (signal E in Fig. 8). According to the equation 1 and 2, methyl signals play an important role in these formulas representing proportion of methyl group of linolenic to methyl group of all fatty chains.

$$n\text{-3 linolenic} = E/(E+F) \quad (1)$$

$$\text{SFA} + \text{MUFA} + n\text{-6 linoleic} = F/(E+F) \quad (2)$$

The allylic protons signal at 2.02 ppm (signal C in Fig. 8) can be used to quantify the amount of MUFA and linoleic acids as twice in proportion to all fatty chains that represented as α-protons of acyl group and obtained from the intensity of signal B (Fig. 9) and defined as $2xCH_2$ of allylic protons to CH_2 of α-protons from acyl group in equation 3. Consequently, *n*-3 linolenic content from equation 1 was placed in equation 3 to reveal equation 4:

$$\text{MUFA} + n\text{-3 linolenic} + n\text{-6 linoleic} = C/2B \quad (3)$$

$$\text{MUFA} + n\text{-6 linoleic} = [C/2B] - [E/(E+F)] \quad (4)$$

R. Sacchi et al. (1997) proposed the diallylic protons at 2.73 ppm (signal A) to represent the amount of *n-6* linoleic content which was subtracted from the amount of *n*-3 linoleic content with no explanation as showed in equation 5. Therefore, the monounsaturated fatty acid chains (MUFA) can be determined by equation 6.

$$n\text{-6 linoleic} = 2A - [E/2(E+F)] \quad (5)$$

$$\text{MUFA} = \{[C/2B]-[E/(E+F)]\}- \{2A-[E/2(E+F)]\} \quad (6)$$

Finally, saturated fatty acids can be calculated as follows:

$$\text{SFA} = \text{C16: 0} + \text{C18: 0}$$

$$= [F/(E+F)]-[C/2B]-[E/(E+F)] \quad (7)$$

R. Sacchi et al. (1997) reported the results showed a good agreement with gas chromatographic analyses if this method gave detail less than gas chromatography technique [58].

To clarify equation 5, it could be suggested that there are some mistake in this paper because signal A is not in proportion to any signals and cannot be related to fraction of E/(E+F) at all. Signal A is accounting for *bis*-methylene of *n-6* linoleic protons and *bis*-methylene *n-3* linolenic protons, while E/2(E+F) is derived by linoleic and represented that an amount of *bis*-methylene of linolenic is 2-times more than methyl of *n-3* linolenic protons. However, full information has not been provided to clearify in details. *n*-6 Linoleic content was proposed by other research groups later with clearer explanation in relation to α-protons of acyl group.

M.D. Guillén and A. Ruiz (2003) [38] used ^{1}H NMR spectroscopy to assign the signal spectra of different types of protons with standard compound in vegetable oils and determined the signal areas. Then, they considered that the number of protons of each type in the sample is proportion to the area of the spectra. It can be calculated the iodine value and the proportion of different acyl groups from their data [59]. They studied on different types of oil that showed a broad range of composition. Fig. 9 shows the ^{1}H NMR spectrum of hazelnut oil and walnut oil samples. General assignment of the signals of these spectra is given in Table 8.

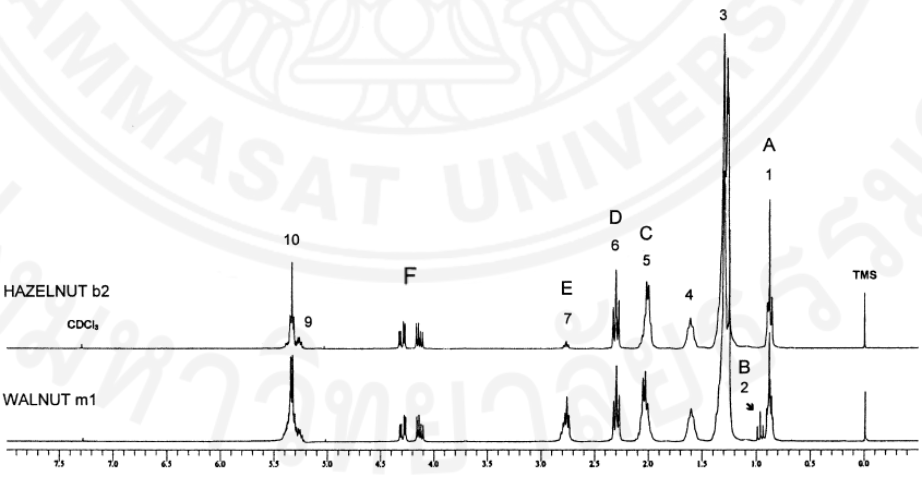


Figure 9 ^{1}H NMR spectrum of hazelnut and walnut oils [59].

Signal 1 is produced by overlapping of triplet signals of methyl group protons of saturated, *n*-9 (i.e., oleic), and *n*-6 (i.e., linoleic) acyl groups give a triplet signal between

0.83-0.93 ppm. Signal 2 is a triplet due to methyl protons of *n*-3 (i.e., linolenic) acyl groups and appears between 0.93-1.03 ppm. Signal 2 is a triplet due to methyl protons of *n*-3 (i.e., linolenic) acyl groups and appears between 0.93-1.03 ppm. The difference in chemical shifts between methyl proton signals is due to their proximity to the double bond of the chain. Signal 3 is due to protons of methylene groups in position β, or further, from olefinic groups, or in position γ, or further, from carboxyl groups inside the triglyceride molecule, and appears between 1.22- 1.42 ppm. Signals 4 and 6 are due to methylenic protons in β and α position, in relation to the carboxyl group, and appear between 1.52-1.70 ppm, and 2.23-2.36 ppm, respectively. Signal 5 is due to α methylenic protons in relation to a single double bond, also named allylic protons and appear between 1.94-2.14 ppm. Signal 7 appears approximately 2.74-2.84 ppm and is caused by overlapping of signals from α methylenic protons between two double bonds, also named bis-allylic protons. Signal 8 at 4.10-4.32 ppm is due to four protons on carbon atoms 1 and 3 of the glyceryl group. Signal 9 at 5.20-5.26 ppm is due to the proton on carbon atom 2 of the glyceryl group and overlaps slightly with signal 10 at 5.26-5.40 ppm assigned as olefinic protons of different acyl groups.

According to M.D. Guillén report, the number of protons of each fatty type in the sample is proportion to the area of the ^{1}H NMR spectra signals. In general, the integration of some signals of the spectra can be calculated the proportions of the different acyl groups in the samples. The determination of the proportions of oleic (O), linoleic (L), linolenic (Ln) and saturated (S) acyl groups can be obtained from the following equations:

$$Ln\ (\%) = 100[B/(A+B)] \quad (8)$$

$$L\ (\%) = 100\ [(E/D)-2[B/(A+B)]] \quad (9)$$

$$O\ (\%) = 100\ [(C/2D) - (E/D) + [B/(A+B)]] \quad (10)$$

$$S\ (\%) = 100\ [1-(C/2D)] \quad (11)$$

where **A** is the area of signal 1, corresponding to protons of terminal methyl groups in all acyl groups except Ln groups; **B** is the area of signal 2, corresponding to methyl protons of Ln groups; **C** is the area of signal 5, corresponding to methylenic protons at α position in relation to one double bond, also named allylic protons; **D** is the area of signal 6, corresponding to methylenic hydrogen atoms at α position in relation to the carboxyl group; **E** is the area of signal 7, corresponding to methylenic protons at α position of two double bonds, also named *bis*-allylic protons; and **F** is the area of signal 8, corresponding to protons on carbon atoms 1 and 3 of the glyceryl group.

Table 8 Assignment of signals of ^{1}H NMR spectra from edible oils. Signal number agrees with those in Fig. 9 [59].

Signal	**δ (ppm)**	**Proton**	**Attribution**
1 (A)	0.83-0.93	-C**H**$_3$	saturated, oleic and linoleic acyl chains
2 (B)	0.93-1.03	-CH$_2$CH$_2$C**H**$_3$	linolenic acyl chains
3	1.22-1.42	-(C**H**$_2$)n-	acyl chains
4	1.52-1.70	-OCO-CH$_2$-C**H**$_2$-	acyl chains (β-proton to acyl gr.)
5 (C)	1.94-2.14	-C**H**$_2$CH=CHCH$_2$CH=CHC**H**$_2$-	acyl chains (α-proton to double bond)
6 (D)	2.23-2.36	-OCO-C**H**$_2$-	acyl chains (α-proton to acyl gr.)
7 (E)	2.74-2.84	=CHC**H**$_2$CH=	acyl chains (*bis*-methylene)
8 (F)	4.10-4.32	-C**H**$_2$OCOR	glyceryl group
9	5.20-5.26	>C**H**OCOR	glyceryl group
10	5.26-5.40	-CH$_2$C**H**=C**H**CH$_2$-	acyl chains

M. D. Guillén and A. Ruiz (2003) [59] described for the first time a fast and simple methodology based on ^{1}H NMR spectroscopy to determine the proportion of the different acyl groups. This method can use to provide accurate results and used for the study of the composition of oil which is containing similarly acyl group. They revealed equation 9

with more practical approach to *bis*-methylene protons (signal E) in proportion to α-protons of acyl group (signal D). The proportion of *bis*-methylene protons of L groups was found in Ln groups in 2 positions. Therefore, equation 9 was subtracted by proportion of *bis*-methylene protons of Ln groups. Upon the α-methylene protons (signal C) of all presented double bonds, equation 10 is derived with α-protons of acyl group in a ratio of 1:2 (-OCO-C**H**$_2$- per -C**H**$_2$CH=CHCH$_2$CH=CHC**H**$_2$-) and subtracted by its proportion with *bis*-methylene protons of L and Ln groups, then added up with excess *bis*-methylene intensity of Ln groups. However, the low intensity of signal B can cause troublesome for the overall determination.

J. Sedman et al. (2010) [60] also used ^{1}H NMR spectroscopy to determine nutrition labeling compositional data (NLCD) components in oils and fats. They presented the different of *cis*- and *trans*- unsaturation in edible oils by this technique. The determination of NLCD components was carried out by using equations 12-17. The letters of following equation agree with signal letter in Table 9.

Equation 12-17 related to the proportion of CH_2 at C-2 position or α-methylene protons of acyl groups (signal G). The saturated content was determined by equation 12; summary of terminal methyl groups (signal A and B) and subtracted from allylic protons of *cis* double bonds (signal F). Mono-*cis* content was determined by terminal methyl in *n*-3 polyunsaturated (signal B) and allylic protons of *cis* double bonds (signal F). Because terminal methyl in *n*-3 polyunsaturated (signal B) was combined with allylic protons of *trans* double bonds (signal E) and diallylic protons (signal H), both signals were subtracted from terminal methyl in *n*-3 polyunsaturated (signal B) to calculate mono-*cis* content in equation 13. Mono-*trans* content was directly determined by allylic protons of *trans* double bonds (signal E) as shown in equation 14. Poly-*cis* component or equation 15 was obtained from apportioning between diene and triene components (equation 16-17). Dienes content was determined by diallylic protons (signal H) and double subtracted of terminal methyl in *n*-3 polyunsaturated (signal B) due to 2 positions diallylic protons in Ln as shown in equation 16. Terminal methyl in *n*-3 polyunsaturated (signal B) was used for trienes determination (equation 17).

Table 9 List of ^{1}H resonance peaks in $CDCl_3$ (700 µL) containing TMS (20 µL), their fixed integration limits, relaxation times, and assignments of some signal [60].

Peak	Integration limits (ppm)	T_1 range[a] (s)	Assignment	Intensity proportional to:
A	0.94-0.71	2.86-3.19	Terminal CH_3 in FA chains except *n*-3 polyunsaturated FAs	3 (saturated + monoene + diene)
B	1.05-0.94	4.83-4.95	Terminal CH_3 in *n*-3 polyunsaturated FA chains	3 (triene)
C	1.47-1.08	1.15-1.53	CH_2 between two other methylene groups except at C-3 position	
-	1.56-1.47	6.14	H_2O	
D	1.78-1.56	0.99	CH_2 at C-3 position in FA chains	2 (saturated + monoene + diene + triene)
E	1.98-1.92	n/a	CH_2 allylic to *trans* double bonds	4 (*trans*-monoene)
F	2.12-1.79	1.18-1.22	CH_2 allylic to *cis* double bonds	4 (*cis*-monoene + diene + triene)
G	2.46-2.23	0.93-1.04	CH_2 at C-2 position in FA chains	2 (saturated + monoene + diene + triene)
H	2.98-2.58	1.77	Diallylic CH_2	2 (diene) + 4 (triene)
I	4.48-3.94	0.61-0.62	CH_2 at α-C of glycerol backbone	
J	5.59-5.06	1.46	CH (double bonds and β-C of glycerol backbone)	

[a] ±5

Saturates	=	(4A+4B-3F)/6G	(12)
Mono-*cis*	=	(4B+3F-3E-6H)/6G	(13)
Mono-*trans*	=	E/2G	(14)
Poly-*cis*	=	(3H-2B)/3G	(15)
Dienes	=	(3H-4B)/3G	(16)
Trienes	=	2B/3G	(17)

In addition, M.D. Guillén and P.S. Uriarte (2012) [61] observed the evolution of extra virgin olive oil throughout the heating process by ^{1}H NMR as shown the original spectrum in Fig. 10. They studied on the changes in the composition of acyl groups, aldehydes formation, di- and monoglyceride formation (called modified acyl group, M), epoxide formation and the molar percentage of the several kinds of acyl groups with heating time.

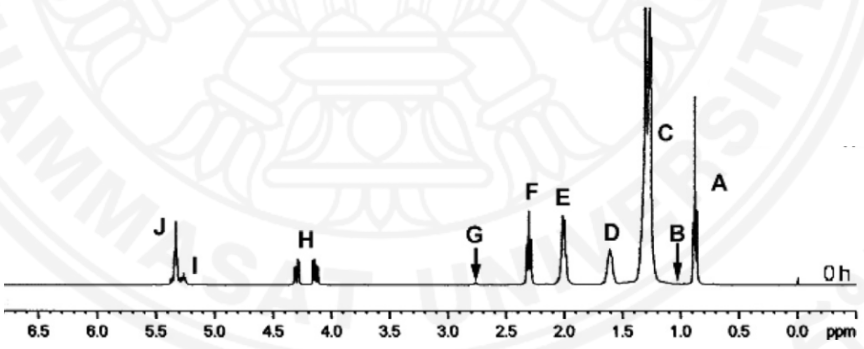


Figure 10 ^{1}H NMR spectrum of the original extra virgin olive oil [61].

They carried out the equations for calculating composition of acyl groups considering that amount of di- or mono-glycerides was much smaller than the amount of triglycerides and area of *bis*-allilic protons. Trilinolein and trilinolenin were used as standard compounds and the area (A) of the *bis*-allilic protons of linolenic (signal G_{Ln}) and linoleic (signal G_L) group can be determined separately. The letters of following equations (equation 18-20) agree with signal letter in Table 10.

$$Ln\ (\%) = 100[A_{GLn}/3A_H)] \quad (18)$$

$$L\ (\%) = 100[2A_{GL}/3A_H] \quad (19)$$

$$O\ (\%) = 100[(A_E - 2A_{GL} - A_{GLn})/3A_H] \quad (20)$$

Table 10 The assignment of the extra virgin olive oil from ^{1}H NMR spectrum. Analysis in deuterated chloroform contained 0.2% of non-deuterated chloroform and a small amount of tetramethylsylane (TMS) [61].

Signal	Chemical shift (ppm)	Functional group
A	0.83-0.93	–C**H**$_3$ (saturated, oleic and linoleic acyl group)
B	0.93-1.03	–CH$_2$C**H$_3$** (linolenic acyl group)
C	1.22-1.42	–(C**H**$_2$)n– (acyl group)
D	1.52-1.70	–OCO–CH$_2$–C**H**$_2$– (acyl group)
E	1.94-2.14	–C**H**$_2$–CH=CH– (acyl groups)
F	2.23-2.36	–OCO–C**H**$_2$– (acyl group)
G	2.70-2.84	=HC–C**H**$_2$–CH= (acyl groups), (*bis*-allylic protons)
H	4.10-4.32	–C**H**$_2$OCOR (glyceryl group)
I	5.20-5.26	>C**H**OCOR (glyceryl group)
J	5.26-5.40	–C**H**=C**H**– (acyl group)

According to trilinolenin, equation 18 is accounting to 12:4 protons of *bis*-allylic to methylene protons of glyceryl group per molecule. Similar to equation 19, a proportion of 6:4 was used to determine linoleic content, then both linoleic and linolenic fractions were used to subtract from all α-protons of double bonds to obtain equation 20.

Later on, A. Martínez-Yusta and M.D. Guillén (2014) [62] have been modified those saturated plus modified acyl groups (S + M) relate to the area of mono-allylic protons (signal A_E) and can be calculated by equation 21:

$$S+M\ (\%) = 100\ [1-(A_E/3A_H)] \quad (21)$$

2.4 Analysis of mono- or diglycerides components in oil

^{1}H NMR can be applied to observe the formation of mono- or diglycerides in degradation process and several authors have been reported their investigation as shown in Table 11. The authors studied in different types of oil. Therefore, the chemical shift of protons signal of mono- or diglycerides depended on oil nature, oil component and composition, etc. Those oil types included virgin olive oil, encapsulated marine cod liver oil, and soy bean oil. Sacchi et al. (1997) [58] analyzed glyceryl protons in different glycerides and Fig. 11 showed ^{1}H NMR spectrum of an underivatized of olive pomace oil. Sacchi et al. observed the signal of *sn*-1,2-diglycerides in *sn*-2 position at 5.07 ppm, *sn*-1 at 4.17 and 4.29 ppm and *sn*-3 at 3.66 ppm. The signal of *sn*-1,3-diglycerides in *sn*-1(3) and *sn*-2 position was recorded at 4.03 and 4.07 ppm, respectively.

Siddiqui et al. (2003) [63] used ^{1}H and ^{13}C NMR for detecting and quantifying the fatty acid (EPA: eicosopentaenoic acid; DHA: docosahexaenoic acid) and other components in marine cod liver oil supplement. In ^{1}H NMR study, Siddiqui et al. observed chemical shift of C**H**$_2$OH, *sn*-3 of *sn*-1,2-diglycerides at 3.66 ppm and ROC**H**$_2$, *sn*-1 at 4.17, 4.31 and C**H**OR, *sn*-2 at 5.07 ppm. Also, ROC**H**$_2$, *sn*-1,3 of *sn*-1,3-diglycerides was found at 4.03 ppm.

Jin et al. (2007) [64] identified the intermediates compound in the transesterification reaction of vegetable oil. The chemical shift and assignment of mono- and diglycerides consist of *sn*-1,2-diglycerides (C**H**OR, *sn*-2 at 5.0 ppm; C**H**$_2$OH, *sn*-3 at 3.78 ppm), *sn*-1,3-diglycerides (ROC**H**$_2$-, *sn*-1,3 at 4.05 ppm), 1-monoglycerides (-C**H**$_2$OH, *sn*-3 at 3.6 ppm; C**H**OH, *sn*-2 at 3.9 ppm; ROC**H**$_2$, *sn*-1 at 4.25 ppm). 2-Monoglycerides was not found under this reaction.

Compton, Vermillion, and Laszlo (2007) [65] studied kinetics of acyl migration from synthesized 2-monoacylglycerides to 1-monoacylglycerides. NMR was used for characterization of those compounds and represent chemical shift including 2-monoglycerides; C**H**$_2$OH, *sn*-1,3 at 3.85 ppm; C**H**OR, *sn*-2 at 4.95 ppm and 1-monoacylglycerides; C**H**$_2$OH, *sn*-3 at 3.66 ppm; C**H**OH, *sn*-2 at 3.95 ppm; ROC**H**$_2$, *sn*-1 at 4.21 ppm.

Table 11 ^{1}H NMR chemical shifts of protons signal of mono- or diglycerides given by several authors [61].

Compounds	**Chemical shifts (ppm)**	**Proton**
1,2-Diglycerides	4.17-4.29[a,b] (dd);	ROC**H**$_2$–CH(OR′)–CH$_2$OH
	4.25-4.35[c];	
	4.17-4.31[d]; 4.25[e]	
	5.07[a,b,d] (m); 5.0 [e]; 5.1 [c]	ROCH$_2$–C**H**(OR′)–CH$_2$OH
	3.66[a,b,d] (d); 3.76[c] (t); 3.78[e]	ROCH$_2$–CH(OR′)–C**H**$_2$OH
1,3-Diglycerides	4.07[a,b]; 4.18[c]	ROCH$_2$–C**H**(OH)–CH$_2$OR′
	3.96[e] (m); 3.99[a]; 4.18[c];	ROC**H**$_2$–CH(OH)–CH$_2$OR′
	4.03[b,d]; 4.05[e]	
1-Monoglycerides	3.66[f] (dd); 3.6[e]	ROCH$_2$–CH(OH)–C**H**$_2$OH
	3.9[e] (m); 3.95[f]	ROCH$_2$–C**H**(OH)–CH$_2$OH
	4.21[f] (dd); 4.25[e]	ROC**H**$_2$–CH(OH)–CH$_2$OH
2-Monoglycerides	3.85[f] (dd)	HOCH$_2$–CH(OR)–C**H**$_2$OH
	4.95[f] (m)	HOCH$_2$–C**H**(OR)–CH$_2$OH

[a] Sacchi et al. (1996) [66]; [b] Sacchi, Addeo, and Paolillo (1997) [58]; [c] Ayer and Pedras (1987); [d] Siddiqui et al. (2003) [63]; [e] Jin et al. (2007) [64]; [f] Compton, Vermillion, and Laszlo (2007) [65].

Likewise, it can be studied oil composition by ^{13}C NMR application. There are many studies on ^{13}C NMR for lipids composition including the characterization of lipids minor compounds of mono- and diglycerides for assessing the age and quality of lipids. ^{13}C NMR chemical shifts of carbon signal of mono- and diglycerides were studied by R. Sacchi et al. (1997) [58] and Siddiqui et al. (2003) [63], these spectrum are shown in Fig. 11a and 11b, respectively.

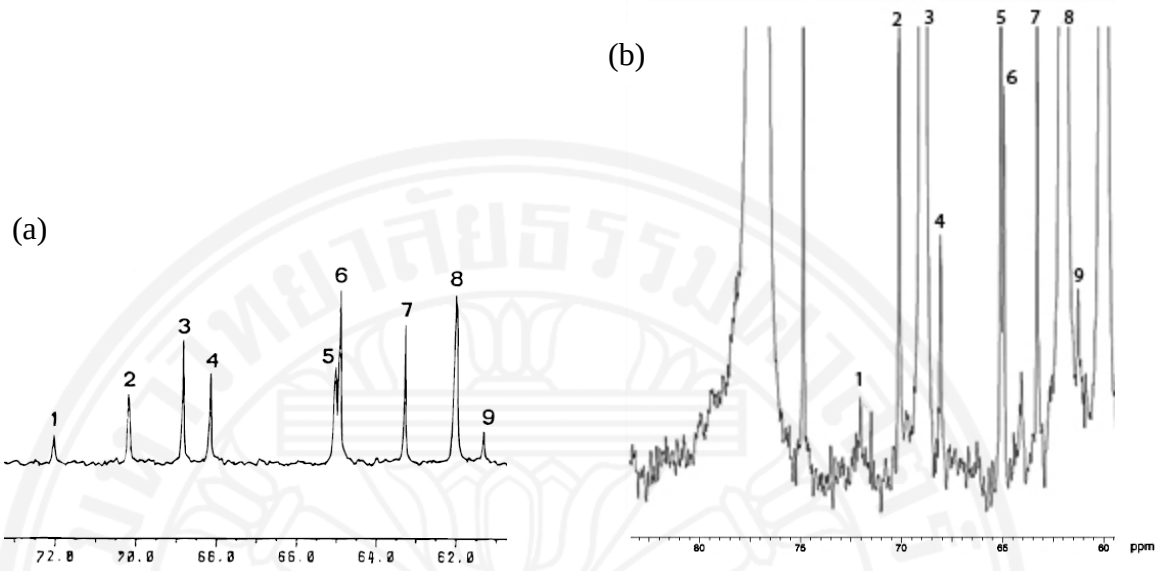


Figure 11 The enlargement ^{13}C NMR spectrum of (a) standard mixture of glycerides in olive oil [58] and (b) encapsulated marine (cod liver) oil supplements [63]. Assignment of signal is given in Table 12.

From Fig. 11a, signal 1 is appeared at 72.08 ppm and represented **C**HOR, *sn*-2 of *sn*-1,2-diglycerols. Signal 2 is produced by **C**HOH, *sn*-2 of *sn*-1-monoglycerols at 70.25 ppm. Signal 3 represented **C**HOH, *sn*-2 of triglycerols at 68.84 ppm. Signal 4 located at 68.30 ppm and show the signal of **C**HOH, *sn*-2 of *sn*-1,3-monoglycerols. Signal 5 is found at 65.05 ppm and represented **C**H_2OR, *sn*-1 of *sn*-1-monoglycerols. Signal 6 is produced by **C**H_2OR, *sn*-1,3 of *sn*-1,3-diglycerols at 64.99 ppm. Signal 7 represented **C**H_2OH, *sn*-3 of *sn*-1-monoglycerols at 63.34 ppm. Signal 8 is signal of **C**H_2OR, *sn*-1 of *sn*-1,2-diglycerols at 62.04 ppm. Signal 9 is appeared at 61.43 ppm and represented **C**H_2OH, *sn*-3 of *sn*-1,2-diglycerols. Moreover, Siddiqui et al. (2003) [63] also used ^{13}C NMR for detecting and quantifying the fatty acid and other components as mention above (Fig. 11b). The chemical shift from both authors is not obviously difference even they studied different oil type (see Table 12).

Table 12 ^{13}C NMR chemical shifts of carbons signal of mono- and diglycerides.

Peak	Carbon	Compounds	Chemical shifts (ppm)	
			R. Sacchi et al. (1997) [58]	Siddiqui et al. (2003) [63]
1	>**C**HOR, *sn*-2	*sn*-1,2-diglycerols	72.08	72.13
2	>**C**HOH, *sn*-2	*sn*-1-monoglycerols	70.25	70.19
3	>**C**HOH, *sn*-2	Triglycerols	68.84	68.93
4	>**C**HOH, *sn*-2	*sn*-1,3-diglycerols	68.30	68.14
5	-**C**H_2OR, *sn*-1	*sn*-1-monoglycerols	65.05	65.15
6	-**C**H_2OR, *sn*-1,3	*sn*-1,3-diglycerols	64.99	64.99
7	-**C**H_2OH, *sn*-3	*sn*-1-monoglycerols	63.34	63.34
8	-**C**H_2OR, *sn*-1	*sn*-1,2-diglycerols	62.04	62.04
9	-**C**H_2OH, *sn*-3	*sn*-1,2-diglycerols	61.43	61.32

Note: *sn*; related with position of acyl chain on triglycerides (such as *sn*-1 is represented proton at carbon 1 or the α-position of the glycerides; *sn*-2 is represented proton at carbon 2 or the β-position of the glycerides; *sn*-3 is represented proton at carbon 3 or the α-position from other end of the glycerides.)

2.5 Analysis of oxygenated components in oil

As mentioned above, the degradation of oil through oxidation reaction can be formed some unpleasant compounds and ^{1}H NMR can observe aldehydes, ketones, epoxides and other oxygenated compounds if oxidation occurred.

Moya Moreno et al. [28] reported thermo-oxidative reaction (up to 150°C) in edible oil and fats. They found the formation of hydroperoxide decomposed to secondary oxidation compound. Fig. 12a showed hydroperoxide signal at 8-8.5 ppm, when the hydroperoxide concentration reaches a certain level of second oxidation occurs with the formation of new carbonyl compounds, mainly aldehydes as showed in Fig. 12b at 9.4 and 9.6 ppm. The full molecular structures have not been provided (as shown in Fig. 12).

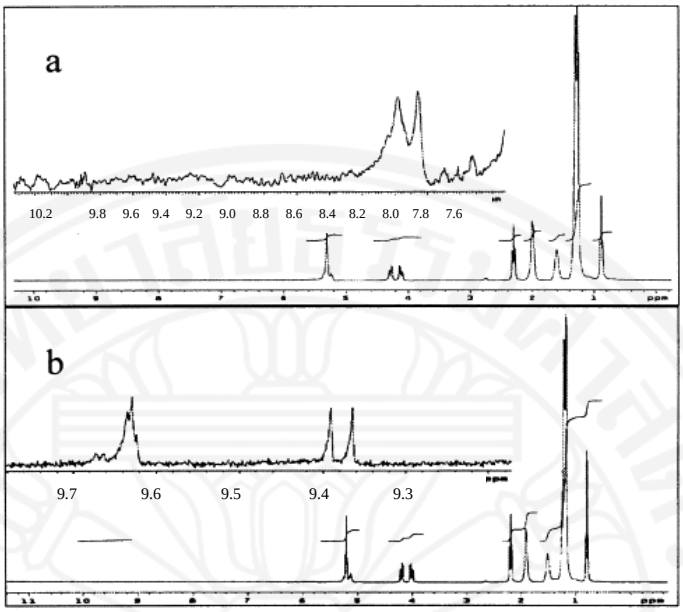
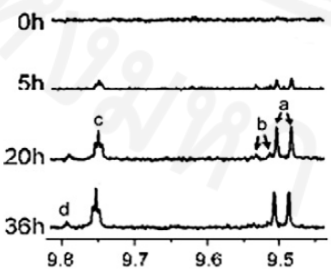
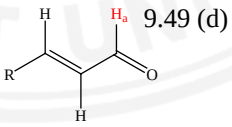


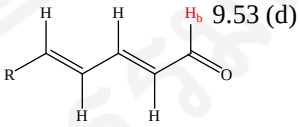
Figure 12 ^{1}H NMR spectra of olive oil (a) heated at 80°C for 20 min and (b) heated at 300°C for 40 min [28].

On the other hand, M.D. Guillén et al. [61] monitored the change of fried (190°C) extra virgin olive oil and observed the formation of aldehydes, di- and monoglycerides and epoxides but could not detected intermediate compounds such as hydroperoxide conjugated dienic compounds (As shown in Fig. 13) because of their formation very early or at lower temperature.

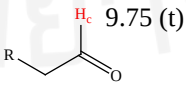




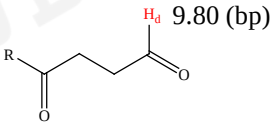
a) (*E*)-2-alkenals



b) (*E*,*E*)-2,4-alkadienals



c) alkanals



d) 4-oxo-alkanals

Figure 13 Enlargement of the ^{1}H NMR spectral regions of aldehydes of the original extra virgin olive oil and after 5, 20, and 36 hours of heating at 190 °C [61].

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials

3.1.1 Chemicals and reagents

Cold-pressed rice bran oil was collected in June 2013 from organic local cooperative of rice (Lopburi province, L-RBO and Yasothorn province, Y-RBO). Potassium hydroxide, Potassium iodide and Sodium thiosulfate were obtained from Ajax Finechem Pty Ltd. Ethanol, Glacial acetic acid and Wijs solution were purchased from RCI Labscan Ltd. Chloroform and phenolphthalein were purchased from Carlo erba reagent. Sodium sulphate anhydrous was obtained from RANKEM and hexane was obtained from QRëC. Methanol and acetonitrile were purchased from Merck. Deuterated chloroform ($CDCl_3$, Assay 99.8 atom %D) and hexadeuterodimethyl sulfoxide (DMSO-d_6, Assay 99.9 atom %D) were purchased from Sigma-aldrich. All chemicals and solvents used in this study were of analytical grade.

3.1.2 Apparatus

3.1.2.1 NMR spectroscopy

NMR analyze was performed using Bruker Avance 400-MHz Spectrometer equipped with 5 mm NMR tube. Deuterated chloroform ($CDCl_3$) and hexadeuterodimethyl sulfoxide (DMSO) were used as solvent and internal standard.

3.1.2.2 Gas chromatography-Mass spectroscopy (GC-MS)

The solution of methyl ester was prepared through esterification and measured by Shimadzu GC-2010 plus chromatograph equipped with mass spectrometer detector.

3.1.2.3 UV-Vis spectrophotometer

γ-Oryzanol contents can also be determined by using UV-Vis spectroscopy (Shimadzu UV-spectrometer, UV-1700) without oil extraction.

3.2 Methods and preparation

3.2.1 Oil analysis

The oil used in this research was cold-pressed rice bran oil that obtains from 2 different regions (Lopburi province, L-RBO and Yasothorn province, Y-RBO). Both oil samples were produced in 2013. Only one bottle of each RBO samples was freshly opened, stored in the darkness at room temperature (28-35°C) and analysed in every month or two months. The rest of both RBO samples were kept as sealed bottle provided and stored in the darkness at room temperature and in refrigerator below 15°C for 1 year. The quality of oil sample was evaluated by using classical method to determine acid value, peroxide value, iodine value. All chemical analyses were carried out according to the AOAC method [75].

- **Acid value (AV)**

Acid value is the number of mg of potassium hydroxide required to neutralize the free acids in 1.0 g of the substance. The acid value was calculated from

$$AV = \frac{5.61 \times V}{m}$$

Where V is the volume in ml of titrant and m is the mass of oil sample.

- **Iodine value (IV)**

The iodine value is a measure of the total number of double bonds present in the sample. It represents the quantity of iodine (in grams) that will react with the double bonds in 100 g of sample. The iodine value was calculated from

$$IV = \frac{1.269 \times (n_1 - n_2)}{m}$$

Where n_1 is the volume in ml of thiosulfate solution used for carry out a blank test, n_2 is the volume in ml of thiosulfate solution used for the titration, and m is the mass of oil sample

- **Peroxide value (PV)**

Peroxide value is the number that expresses, in milliequivalents of active oxygen, the quantity of peroxide contained in 1000 g of the substance. The peroxide value was calculated from

$$PV = \frac{1000 \times (V_1 - V_0) \times c}{m}$$

where V_1 is the volume in ml of thiosulfate solution used for the titration, V_0 is the volume in ml of thiosulfate solution used for carry out a blank, c the thiosulfate concentration, and m is the mass of oil sample. All samples was analyzed every month and carried out in triplicate.

3.2.2 NMR analysis

Each oil sample (approx. 70 mg per sample) was added to 5mm NMR tubes and dissolved in 600 µL of deuterated chloroform and a small proportion (a drop) of dimethyl sulfoxide-d_6. The ^{1}H NMR and ^{13}C NMR spectroscopy were performed on a Bruker Avance 400 MHz and 100 MHz spectrometer, respectively. The chemical shift was expressed in δ scale (ppm). Both ^{1}H NMR and ^{13}C NMR were used to identify the formation of fatty acid. All NMR data were analysed by NMR software and solvents were used as internal references.

^{1}H NMR was obtained under the following conditions: sweep width 6410.3 Hz; data points; 32768, pulse width; 6.0 µsec, pulse delay; 2.0 sec and number of scan; 4.

^{13}C NMR was recorded on the following conditions; sweep width 23980.8 Hz; data points; 65536, pulse width; 6.0 µsec, pulse delay; 1.50 sec and number of scan; 12288.

3.2.3 Fatty acid composition of oils

This section is to compare a result of fatty acid composition from ^{1}H NMR technique with gas chromatography technique. The fatty acid composition was determined by converting them to methyl esters through esterification reaction by heating oil (30 g) to 80°C. Then, 3%wt potassium hydroxide in methanol was added into reaction solution and stirred this solution for 30 min. After refluxing, the solution was cooled to room temperature and collected the upper layer and then separated glycerol out. The solution was neutralized by warm water and dried with sodium sulfate anhydrous. The methanol was evaporated after filtrated the sodium sulfate anhydrous. The Fatty acid methyl esters (FAMEs) were identified using a Shimadzu GC-2010 plus chromatograph equipped with a split/splitless capillary injector and mass spectrometer detector. Analytical separation was achieved on HP5-INNOWAX column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was He, which was supplied at a flow rate of 1.41 ml/min. The injected amount was 0.1μl and the split ratio was 1:60. Temperature settings were as follows: injector 230°C; the oven temperature was held at 160°C for 5 min. and then programmed to 210°C at 4°C/min, and held for 6 min at 210°C.

3.2.4 Oryzanol analysis

UV-Vis spectroscopy was applied to determine the oryzanol contents directly from oil sample without any extraction by weighing 10 mg of RBO and dissolving in hexane to make the volume up to 10 mL. The OD was measured in a 1-cm cell at 314 nm in a Shimadzu UV-1700 spectrophotometer. The oryzanol content in the oil was calculated by using the equation [18]

$$\text{oryzanol, g (\%)} = \frac{\text{OD of hexane solution}}{\text{weight (g) of oil} \times 10} \times \frac{100}{358.9}$$

Where OD is optical density and 358.9 is the specific extinction coefficient of oryzanol.

3.2.5 Statistical analysis

All experimental of conventional analysis and NMR analysis were carried out in triplicate and reported as mean values±SD. Data was subjected to analysis of variance (ANOVA) by Excel and revealed that these significant differences (p=0.05) with t-stat, t-critical in parentheses were between conditions (time and treatment).

Sample name are usually called as sample code and showed detail below.

L-RBO: RBO samples obtained from Lopburi province.

Y-RBO: RBO samples obtained from Yasothorn province.

RRO-1: RBO samples were freshly opened and analysed at time 0 month.

RRO-2: RBO samples were analysed at time 12 month.

RRO-3: RBO samples were kept as sealed bottle and stored in the dark at room temperature throughout the period of 1 year.

RRO-4: RBO samples were kept as sealed bottle and stored in the dark in refrigerator below 15°C throughout the period of 1 year.

All sample code was followed by month and year at time analysis.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Chemical properties of oil

Oil stability included oxidative stability, hydrolytic stability and heat stability which correspond to chemical properties. The iodine values (IV) was used to evaluate the number of unsaturated fatty acid in oil. High amount of IV indicated the oil consist of high unsaturated composition. It can be easily assessed oxidative rancidity which is lead to oil degradation. The factor that makes rancid oil was oxygen in the air. Lipase was also caused in a rancid and called hydrolytic rancidity. Due to the occurrence of oxidative rancidity, the amount of double bonds or unsaturation in fatty acid decreased as the IV decreased. There is caused by increase of peroxide values (PV) which indicated oxidative rancidity occurrence. Likewise, hydrolytic rancidity affected to increase of acid values (AV). In this investigation, RBO from 2 different regions were studied. Oil samples (L-RBO and Y-RBO) were first opened on October, 2013 and kept at room temperature in the dark for a period of time to evaluate the stability of oil every one-three months. The AV, IV and PV of RBO were shown in Table 13 and Fig. 14-16, respectively.

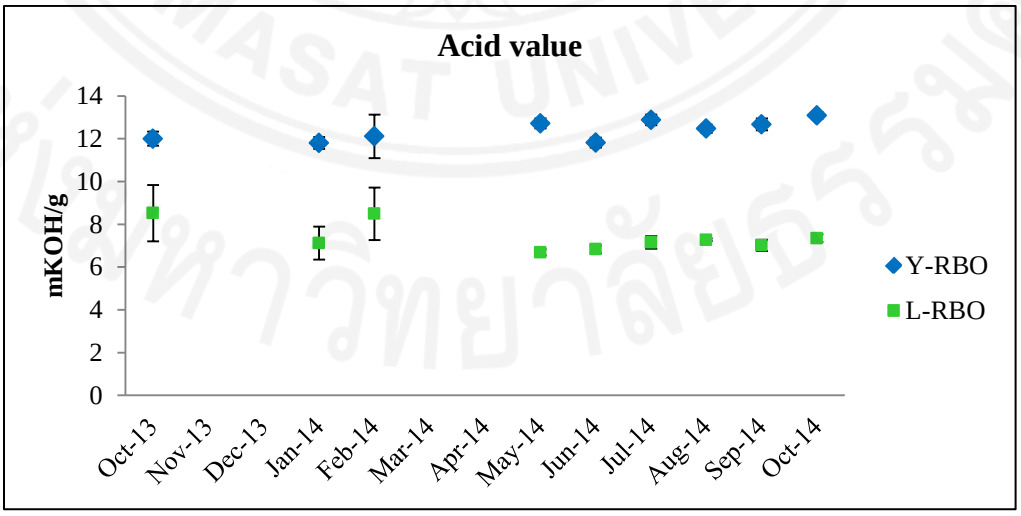


Figure 14 Acid values of L-RBO and Y-RBO over 1-year storage.

This study found that L-RBO over one year storage represented AV of 8.52±1.32, 7.12±0.77, 8.49±1.23, 6.69±0.14, 6.83±0.18, 7.14±0.29, 7.27±0.06, 7.02±0.26 and 7.35±0.18 mgKOH/g and Y-RBO over one year storage showed almost constant in AV of 12.00±0.33, 11.8±0.28, 12.11±1.01, 12.72±0.23, 11.82±0.23, 12.88±0.25, 12.47±0.19, 12.67±0.28 and 13.08±0.17 mgKOH/g. Normally, AV in RBO was not more than 0.5% (codex) in contrast with these results. This is may be caused by production process to packing process. As shown in Fig. 14, the overall trend of AV in RBO which submitted to air exposure and storage temperature increased when shelf-life increased.

In addition, the trend of IV for L-RBO was 31.45±0.18, 44.2±0.50, 40.08±3.81, 35.72±0.96, 32.17±1.24, 32.22±0.68, 41.94±0.76, 44.19±1.32 g/100g and for Y-RBO was 31.24±0.73, 41.57±1.51, 42.28±0.58, 36.37±0.65, 33.46±0.76, 32.78±2.11, 42.65±0.7, 43.56±1.15 g/100g. Generally, chemical properties of oil revealed the decrease of iodine value and the trend of acid value and peroxide value normally increased in oils submitted to exposure the air, shelf-life and storage temperature. Lower in iodine value as a result of the disappearance of the double bonds in acyl group caused by polymerization and/or oxidation at the double bond [61]. Also, IV of RBO normally in range 90-115 g/100g. At this point, the results of IV was decreased during Jan-Jul, 2014 and revealed the increase of IV over all (Fig. 15), which may be caused by the content of γ-oryzanol in RBO medium, acted progressively with the aging time as a consequence of the antioxidant property of γ-oryzanol to this medium, thus it prevented the loss of unsaturation [67].

Considering the PV that should be increase corresponding to IV decreased. The result showed that PV of L-RBO was 14.07±0.23, 17.05±1.39, 18.00±1.84, 27.86±3.80, 33.24±0.34, 32.97±3.98, 31.65±2.59, 33.41±1.14 and 58.76±0.83 meq/kg. PV of Y-RBO 17.32±1.14, 14.70±0.83, 14.22±0.30, 23.05±2.83, 23.81±0.28, 24.52±1.76, 35.96±0.99, 36.52±2.74 and 44.21±2.35 meq/kg. PV of the both RBO samples started to raise the value to higher than 20 meq/kg after six months of storage (Fig. 16).

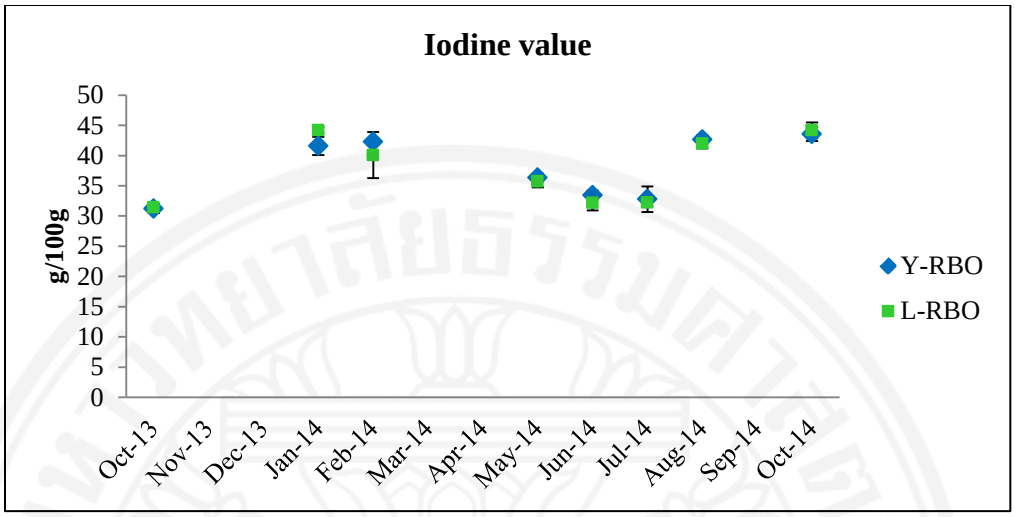


Figure 15 Iodine values of L-RBO and Y-RBO over 1-year storage.

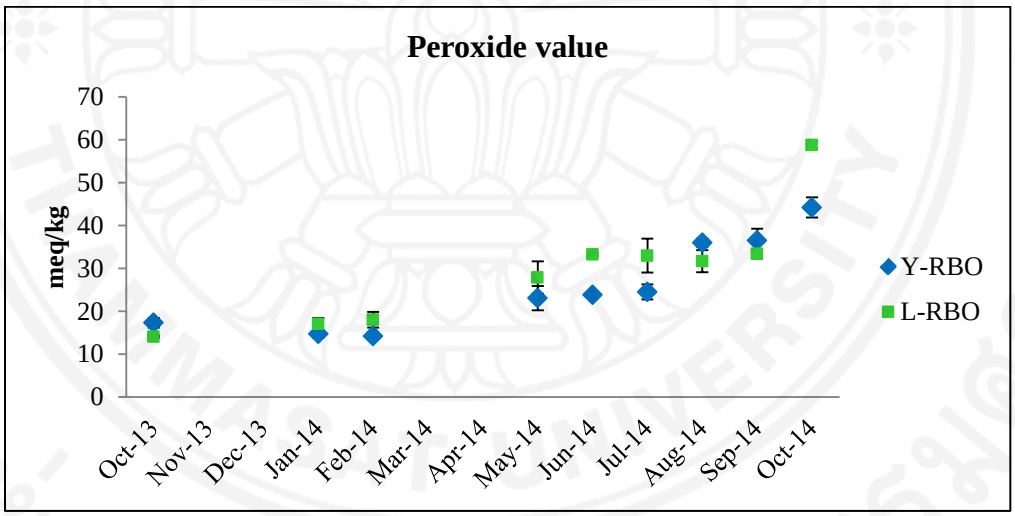


Figure 16 Peroxide values of L-RBO and Y-RBO over 1-year storage.

In addition, the effect of different storage conditions for chemical properties was evaluated as showed in Table 14. Chemical properties of RBOs from different regions in different storage conditions were compared in Fig. 17-19. It is known that the decrease of IV in oils caused by oxygen in the air, the light, the heat or the temperature and others. There affected to oil properties such as IV, PV and AV.

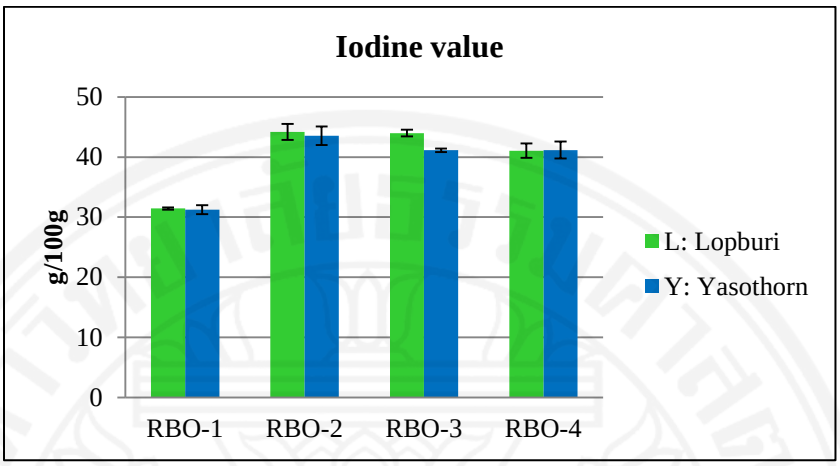
Table 13 Chemical properties of L-RBO and Y-RBO over one year storage.

Month	**AV**, mgKOH/g		**IV**, g/100g		**PV**, meq/kg	
	L-RBO	**Y-RBO**	**L-RBO**	**Y-RBO**	**L-RBO**	**Y-RBO**
Oct-13	8.52±1.32	12.00±0.33	31.45±0.18	31.24±0.73	14.07±0.23	17.32±1.14
Jan-14	7.12±0.77	11.8±0.28	44.2±0.50	41.57±1.51	17.05±1.39	14.70±0.83
Feb-14	8.49±1.23	12.11±1.01	40.08±3.81	42.28±0.58	18.00±1.84	14.22±0.30
May-14	6.69±0.14	12.72±0.23	35.72±0.96	36.37±0.65	27.86±3.80	23.05±2.83
Jun-14	6.83±0.18	11.82±0.23	32.17±1.24	33.46±0.76	33.24±0.34	23.81±0.28
Jul-14	7.14±0.29	12.88±0.25	32.22±0.68	32.78±2.11	32.97±3.98	24.52±1.76
Aug-14	7.27±0.06	12.47±0.19	41.94±0.76	42.65±0.7	31.65±2.59	35.96±0.99
Sep-14	7.02±0.26	12.67±0.28	-	-	33.41±1.14	36.52±2.74
Oct-14	7.35±0.18	13.08±0.17	44.19±1.32	43.56±1.15	58.76±0.83	44.21±2.35

Table 14 Chemical properties of L-RBO and Y-RBO at different condition of oil storage.

Property	**L-RBO**				**Y-RBO**			
	RBO-1[a]	**RBO-2**[b]	**RBO-3**[c]	**RBO-4**[d]	**RBO-1**[a]	**RBO-2**[b]	**RBO-3**[c]	**RBO-4**[d]
AV, mgKOH/g	8.52±1.32	7.35±0.18	7.58±0.22	5.34±0.18	12.00±0.33	13.08±0.17	12.44±0.08	10.29±0.03
IV,g/100g	31.45±0.18	44.19±1.32	43.98±0.56	41.06±1.19	31.24±0.73	43.56±1.55	41.13±0.29	41.16±1.42
PV, meq/kg	14.07±0.23	58.75±0.83	8.85±0.69	15.84±0.81	17.32±1.14	44.21±2.35	4.82±0.78	15.52±0.50

Note: All oil samples are the same batch and received on Oct 2013.; [a] tested in Oct 2013; [b] tested in Oct 2014; [c] Oil sample was kept in dark at room temperature (29-32°C) as sealed bottle and tested on Oct 2014; [d] Oil sample was kept in refrigerator (≤15°C) as sealed bottle and tested on Oct 2014.



Note: RBO-1 was tested in Oct 2013; RBO-2 was tested in Oct 2014; RBO-3 was kept in dark at room temperature (29-32°C) as sealed bottle and tested on Oct 2014; RBO-4 was kept in refrigerator (≤15°C) as sealed bottle and tested on Oct 2014.

Figure 17 Iodine values of L-RBO and Y-RBO in different storage conditions.

According to different storage conditions, Fig.17 showed that the IV of both RBOs after one-year storage revealed insignificantly different which may be subjected by complexity of crude cold-pressed RBO matrixes. L-RBO-1, L-RBO-2, L-RBO-3 and L-RBO-4 observed the IV of 31.45±0.18, 44.19±1.32, 43.98±0.56 and 41.06±1.19 g/100g, respectively. Y-RBO-1, Y-RBO-2, Y-RBO-3 and Y-RBO-4 observed the IV of 31.24±0.73, 43.56±1.55, 41.13±0.29 and 41.16±1.42 g/100g, respectively. When the different storage conditions were investigated through the year, all samples were appeared with sediment and colloid of micro particles which were suggested to free the unsaturated fatty chains and unbound double bonds. That was proposed as a reason that both freshly opened bottles obtained lower IV in L-RBO-1 (31.45±0.18 g/100g) and Y-RBO-1 (31.24±0.73 g/100g).

Freshly opened bottle of L-RBO-1 and Y-RBO-1 observed the PV of 14.07±0.23 and 17.32±1.14 meq/kg, respectively. From Fig.18, after year storage, both RBO obtained significantly difference of PV at P value of 0.05 except Y-RBO stored below 15°C (Y-RBO-4) was not significant different from fresh oil at 0.05. L-RBO-2, L-RBO-3

and L-RBO-4 observed the PV of 58.76±0.83, 8.85±0.63 and 15.84±0.81 meq/kg, respectively. Y-RBO-2, Y-RBO-3 and Y-RBO-4 observed the PV of 44.21±2.35, 4.82±0.78 and 15.52±0.50 meq/kg, respectively. The oil exposed to the air (RBO-2) obtained highest PV due to higher oxidation rate with oxygen molecules in the directly air exposure. Conversely, without air exposure oil storage below 15°C (RBO-4) showed almost maintained PV. Oil storage at room temperature reduced or retarded this oxidation process and revealed the decrease of PV after one year storage (RBO-3).

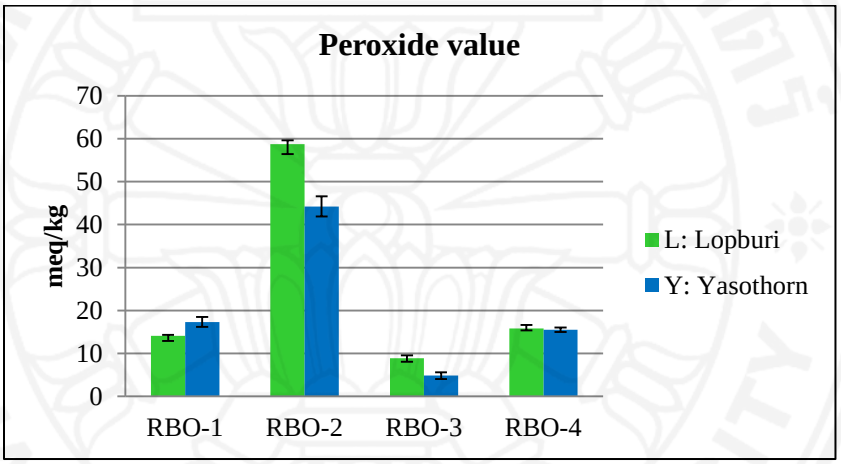


Figure 18 Peroxide values of L-RBO and Y-RBO in different storage conditions.

In addition, AV in Fig. 19 of both oils in all conditions was no significantly different at 0.05 in all samples. L-RBO-1, L-RBO-2, L-RBO-3 and L-RBO-4 observed the AV of 8.52±1.32, 7.35±0.18, 7.58±0.22 and 5.34±0.18 g/100g, respectively. Y-RBO-1, Y-RBO-2, Y-RBO-3 and Y-RBO-4 observed the AV of 12.00±0.33, 13.08±0.17, 12.44±0.08 and 10.29±0.03 g/100g, respectively. L-RBO and Y-RBO represented the AV range of 5.34-8.52 and 12.00-13.29 mgKOH/g, respectively. It can be indicated that after one year storage of oil was not affected to hydrolysis according to relatively constant of AV in every condition (p=0.05). Only oil storage below 15°C (RBO-4) showed slightly lowering in AV. At this point, it was suggested that hydrolysis may occur and transfer triglyceride to other compounds.

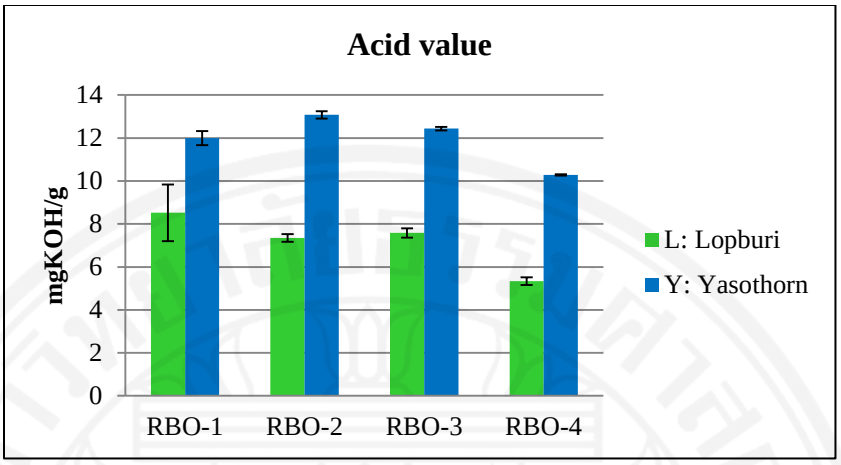


Figure 19 Acid values of L-RBO and Y-RBO in different storage conditions.

It was evident that the degradation reached by aging effect. Earlier research on olive oil storage has shown that bound phenolic compounds generally breakdown and lost over the course of storage, simultaneous to increase in phenolic alcohols [68-71]. Thus, it was suggested that, in RBO matrix, the decrease in steroidal phenolics possibly increases in phenolic acids (such as ferulic acids) which exhibit stronger radical scavenging activities than bound phenolic structures (such as cycloeucalenol ferulate) [72].

The results of different storage conditions related to chemical properties. Keeping oil at the dark in room temperature affected to the decrease of PV after one year storage of RBO. Providing storage below 15°C, PV was slightly increased. However, the different storage conditions did not affect strength to IV and AV though oil was stored in the dark or refrigerator. Therefore, it could be suggested that storage oil in refrigerator was good for the oil through one year.

4.2 ^{1}H NMR analysis of cold-pressed rice bran oil

Several techniques were used in oil analysis. ^{1}H NMR, which is one of rapid and easy technique, was used in this field for long time. Many researchers used ^{1}H NMR spectroscopy in oil and fats. Due to the important of oil stability on its quality, the stability of RBOs was then studied by ^{1}H NMR.

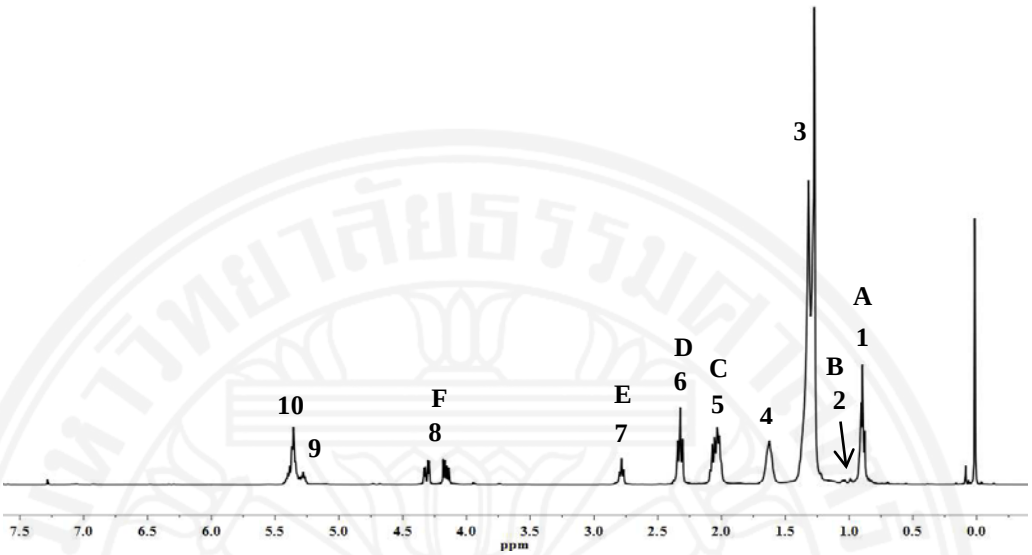


Figure 20 400 MHz ^{1}H NMR spectrum of L-RBO (Lopburi sampling on Oct-13). Labeled resonances are assigned in Table 15.

Data for L-RBO Oct-13, ^{1}H NMR (400 MHz, $CDCl_3$, DMSO): δ 5.28-5.43 (m, 2H, -CH_2C**H**=C**H**CH_2-), 5.22-5.28 (m, 1H, >C**H**OCOR), 4.10-4.35 (dd, 4H, -C**H**$_2$OCOR), 2.69-2.84 (t, 2H, =CHC**H**$_2$CH=), 2.22-2.39 (t, 2H, -OCO-C**H**$_2$-), 1.93-2.11 (bp, 4H, -C**H**$_2$CH=CHC**H**$_2$-), 1.51-1.71 (bp, 2H, -OCO-CH_2-C**H**$_2$-), 1.18-1.43 (bp, -(C**H**$_2$)$_n$-), 0.96-1.05 (m, 3H, -CH_2C**H**$_3$), 0.83-0.94 (m, 3H, -C**H**$_3$).

The common ^{1}H NMR signals of the major and some minor compounds together with their chemical shifts and their assignments to protons of the different functional groups were observed by following certain reference [58] as shown in Fig. 20 and Table 15.

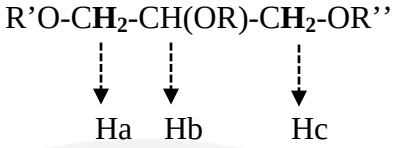
The ^{1}H NMR spectrum of RBO shows at ten signals of significant intensity. Signal of terminal methyl protons of saturated, oleic and linoleic acyl groups is called signal 1 or A at 0.83-0.94 ppm; this signal is due to the overlapping of the triplets of the methyl proton signals of the three above mentioned acyl groups. Signal 2 or B is a signal of terminal methyl protons of linolenic acyl group at 0.96-1.05 ppm. Signal 3 is found at 1.18-1.43 ppm and represented protons of methylene groups at β position or further in relation to double bonds, or at γ position in relation to carbonyl groups of triglyceride

molecule. Signal 4 is produced by methylene protons at β position of carbonyl group at 1.51-1.71 ppm. Signal 5 or C is signal of α methylene protons of single double bond, also named allylic protons and appear at 1.93-2.11 ppm. Signal 6 or D is found at 2.22-2.39 ppm meaning as methylene protons at α position of carbonyl group. Signal 7 or E is caused by overlapping of signals from α methylene protons between two double bonds, also named *bis*-allylic protons and represented at 2.69-2.84 ppm. Signal 8 or F is produced by four protons on carbon atoms 1 and 3 of the glyceryl backbone and appeared at 4.10-4.35 ppm; this signal is due to the coupling of two protons on two end carbons on the backbone. Signal 9 is represented a proton on carbon atom 2 of the glyceryl group at 5.22-5.28 ppm. Signal 10 is represented a signal of olefinic protons of different acyl groups at 5.28-5.43 ppm.

Table 15 Chemical shifts & integration and assignment of signals of the main resonances in the ^{1}H NMR spectrum of L-RBO (Oct-13). Signal number agrees with those in Fig 20.

			L-RBO Oct-13		
Signal	**Proton**	**Attribution**	**Sacchi R. et al. [58]**	**This study[a]**	**Integration[a]**
1 (A)	-C**H**$_3$	saturated, oleic and linoleic acyl chains	0.85	0.83-0.94(m)	10.44
2 (B)	-CHCH$_2$C**H**$_3$	linolenic acyl chains	0.95	0.96-1.05	0.79
3	-(C**H**$_2$)n-	acyl chains	1.20	1.18-1.43(bp)	70.23
4	-OCO-CH$_2$-C**H**$_2$-	acyl chains	1.60	1.51-1.71 (bp)	7.68
5 (C)	-C**H**$_2$CH=CHC**H**$_2$-	acyl chains	2.02	1.93-2.11(m)	10.57
6 (D)	-OCO-C**H**$_2$-	acyl chains	2.20	2.22-2.39(t)	7.05
7 (E)	=CHC**H**$_2$CH=	acyl chains	2.76	2.69-2.84(t)	2.00
8 (F)	-C**H**$_2$OCOR	glyceryl group	4.19	4.10-4.35(dd)	4.35
9	>C**H**OCOR	glyceryl group	5.15	5.22-5.28	1
10	-CH$_2$C**H**=C**H**CH$_2$-	acyl chains	5.29	5.28-5.43	6.59

[a] from this study using NMR software



The basic structure of triglyceride is also shown above. R'CO, RCO and R''CO are the acyl groups. The four protons attached to glycerol carbons of glyceryl backbone. All four protons are non-equivalent and couple with each other. Notice that the coupling constant, J_{ab} and J_{bc} are the largest ($J_{ab} = J_{bc} = 11.9$ Hz). The germinal coupling constant, J_{aa} and J_{cc} ($J_{aa} = 6$ Hz and $J_{cc} = 4.3$ Hz), are so small that it can only be seen by close inspection of the signals for Ha and Hc.

4.3 Determination of fatty acid composition

4.3.1 ^{1}H NMR technique

As mention above, the number of proton of each type in the sample is proportion to the area of the ^{1}H NMR spectra signals. Therefore, the integration of some signals of the spectra allows calculating the proportions of the different acyl groups in the samples. The determination of the proportions of oleic (O), linoleic (L), linolenic (Ln) and saturated (S) acyl groups can be obtained from ^{1}H NMR technique and carried out by the equations 23-38. The parameter A-E is the integration of some signals of the spectra as shown in Fig. 20. The fatty acid composition was studied by four methods. Method I [59] based on proportional methyl group in sample and method II [73] based on the methylenic proton in α position. Method III [61] based on methylenic proton of glyceryl group and method IV was modified from method I-III.

- **Method I [59]**

$$\text{Ln (\%)} = 100\,[B/(A+B)] \quad (23)$$

$$\text{L (\%)} = 100\,[(E/D)-2[B/(A+B)]] \quad (24)$$

$$\text{O (\%)} = 100\,[(C/2D) - (E/D) + [B/(A+B)]] \quad (25)$$

$$\text{S (\%)} = 100\,[1-(C/2D)] \quad (26)$$

Method I based on the proportion of methyl proton of Ln. The %Ln group was determined by using equation 23. Where A is integration area of methyl proton from saturated, oleic, linoleic groups and B is integration area of methyl proton from linolenic groups. The %L group was determined by using equation 24 where E is integration area of *bis*-allylic protons and D is integration area of methylene protons at α position of carbonyl group. The E signal from L groups can be found in Ln groups. Therefore this Ln group was subtracted from L. The %O group can be determined by using equation 25 where C is integration area of methylene protons next to one double bond (α-proton to double bond), E is integration area of *bis*-allylic protons and D is integration area of methylene protons at α position of carbonyl group. Because this C signal is found in Ln and L group, the equation 25 should subtract another area. The %S group was calculated by using equation 26, which is made by its difference from unsaturated groups.

- **Method II [73];** [a]do not provide in this method

$$\text{Ln (\%)} = 100\,[2B/3D] \quad (27)$$

$$\text{L (\%)} = 100\,[(3E-4B)/3D] \quad (28)$$

$$\text{S (\%)} = 100\,[(4A+4B-3C)/6D] \quad (29)$$

$$\text{O (\%)} = 100-[\text{Ln}+\text{L}+\text{S}]^{a} \quad (30)$$

Method II is depended on each acyl chains in relation to signal D which is signal of methylene protons at α position of carbonyl group. The determination of %Ln group is derived from terminal methyl proton of Ln (signal B). There are 3 protons of signal B and 2 protons of methylene protons at α position of carbonyl group (signal D). Therefore %Ln group can be calculated by using equation 27. For determining %L group, *bis*-allylic protons (signal E) is used. As mentioned in method I, the *bis*-allylic protons can be also found in Ln group. Due to number of *bis*-allylic protons in Ln group, this signal is subtracted by twice Ln group as shown in equation 28. The determination of %S group, is derived from the summary of terminal methyl groups of Ln and L also subtracted the methylene protons next to one double bond (α-proton to double bond) (signal C) as shown in equation 29. In addition, the %O group, which is not included in

this reference method II, can be determined by its difference from summarizing %Ln, %L and %S groups as shown in equation 30.

- **Method III [61]**

$$\text{Ln (\%)} = 100\,[E_{Ln}/3F] \quad (31)$$

$$\text{L (\%)} = 100\,[2E_{L}/3F] \quad (32)$$

$$\text{O (\%)} = 100\,[(C-2E_{L}-E_{Ln})/3F] \quad (33)$$

$$\text{S (\%)} = 100\,[1-(C/3F)] \quad (34)$$

From method III, all equations are based on the amount of di- or monoglycerides which are much smaller than triglycerides in quantity. This method proposed that all triglyceride consist of the same acyl group and signal F, represented 4α-protons on carbon atoms 1 and 3 of the glyceryl group was chosen as the main part in the method. The determination of %Ln group is derived from integration area of *bis*-allylic protons of Ln group. There are 12 protons of *bis*-allylic protons of Ln group and 4 protons of 4α-protons on glyceryl group. Therefore %Ln group can be calculated by using equation 31. Also, the L group represented 6 protons of *bis*-allylic protons and can be determined by using equation 32. The determination of %O and %S group indicated similarly idea from equation 25 and equation 26, respectively. Nevertheless, this research cannot separate the different signal of *bis*-allylic protons between Ln and L group. The determination of both groups used only E signal for equation 31-33. It shows as a major limitation of the method.

- **Method IV**

$$\text{Ln (\%)} = 100\,[E/2D] \quad (35)$$

$$\text{L (\%)} = 100\,[E/D] \quad (36)$$

$$\text{O (\%)} = 100\,[(C-3E)/2D] \quad (37)$$

$$\text{S (\%)} = 100\,[1-(C/2D)] \quad (38)$$

Method IV is presently modified from method II and III. This method depended on methylene protons at α position of carbonyl group (signal D) and proposed that all triglyceride consist of the same acyl group. The determination of %Ln and %L group are derived from *bis*-allylic protons (signal E) for %Ln and %L group. As known that *bis*-allylic protons for Ln are twice of L group, both groups can be calculated by equation 35 and 36, respectively. The determination of %O group which is similar to equation 33 used methylene protons next to one double bond (α-proton to double bond) follow with subtraction of Ln and L group as shown in equation 37. The %S group is the same as method I and showed in equation 38.

Due to the difference of each equation from all method, all data are put in equation 23-38. The result of the percentage of fatty acid composition (unsaturated fatty acids and total saturated fatty acids) from example of RBO, which is kept at refrigerator or RBO-4, is shown in Table 16. %Ln group from method I and II obtained nearly reference from CODEX more than from method III and IV because of integration area of signal B or terminal methyl proton of Ln. It can be indicated that this signal is specific for determination of Ln group. In this research, intensity of signal E cannot be separated to Ln clearly because signal E also found in L group. Therefore, %Ln from method III and IV that used signal E or *bis*-allylic protons observed higher Ln composition. In fact, triglycerides consist of different fatty acid can called mixed triglycerides. Method III that proposed all triglycerides consist of the same acyl group. There is as a result %Ln inaccurate in method III and also other fatty acid composition from this method.

The determination of %L group from method I and II was obtained with signal E or *bis*-allylic protons and subtracted by twice of Ln group. Therefore, %L group from both methods was similar. Method III and IV have the same problem with determination of Ln. Although L group can be separated to Ln, the result is not accurate as a mention above.

Table 16 Fatty acid composition (%) of L-RBO-4 and Y-RBO-4 (Nov-14) by ^{1}H NMR technique.

Oil	Method	Saturated Fatty Acids (%)				Unsaturated Fatty Acids (%)			
		C14:0	**C16:0**	**C18:0**	**Total Sat. (S)**	**C18:1 (O)**	**C18:2 (L)**	**C18:3 (Ln)**	**Total Unsat.**
RBO	CODEX [76]	ND-0.1	14-23	0.9-4.0	-	38-48	21-42	0.1-2.9	-
L-RBO-4 (Nov-14)	I	+	+	+	32.95±0.07	45.33±0.37	18.11±0.65	3.59±0.36	67.03±1.38
	II	+	+	+	33.56±0.73	44.83±1.66	18.06±0.72	3.55±0.46	66.44±2.84
	III	+	+	+	17.24±0.17	36.01±0.22	31.17±0.11	15.58±0.05	82.76±0.38
	IV	+	+	+	32.95±0.07	29.17±0.12	25.25±0.12	12.63±0.06	67.05±0.30
Y-RBO-4 (Nov-14)	I	+	+	+	27.54±0.25	47.85±0.18	22.08±0.04	2.59±0.13	72.52±0.35
	II	+	+	+	36.57±0.80	39.06±0.78	21.55±0.20	2.82±0.17	63.43±1.15
	III	+	+	+	15.10±0.27	37.11±0.23	31.86±0.14	15.93±0.07	84.90±1.05
	IV	+	+	+	27.54±0.5	31.67±0.16	27.32±0.16	13.60±0.08	72.59±0.40

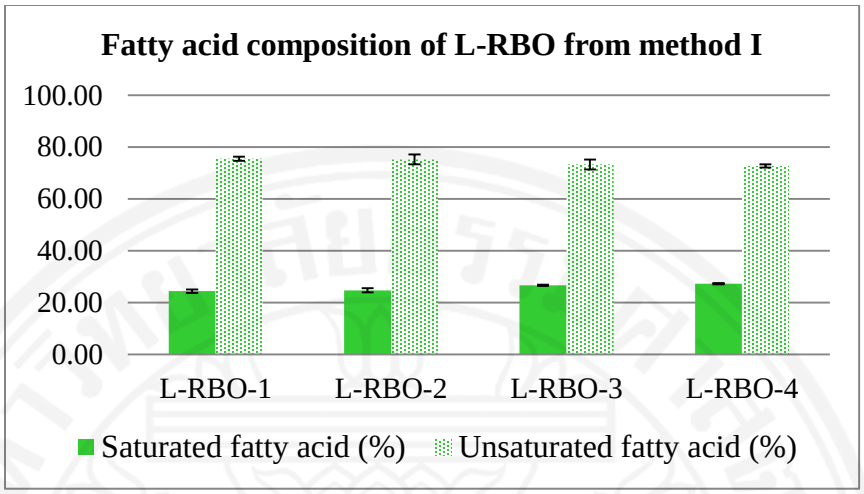
C14:0-myristic acid, C16:0-palmitic acid, C18:0-stearic acid, C18:1-oleic acid (O), C18:2-linoleic acid (L), C18:3-linolenic acid (Ln), ND: non detectable, defined as $\leq$ 0.05%, (+) cannot be calculated from the equation. The results are represented in terms of mean values±SD of three determinations.

All method except method II has the same idea for determination of %O group. %O group obtained from method I and IV depended on methylene protons next to one double bond (α-proton to double bond) (signal C) and methylene protons at α position of carbonyl group (signal D) following subtracted by L and Ln group of each method. The difference between method II and other was determined by subtracting from the summarization of Ln, L and S group. The determination of %S group from method I, III and IV which obtained the same idea, was calculated by its different from unsaturated group. As above mention, the most problem for all determination was signal E because intensity of signal E cannot be separated clearly. Therefore, the determination that used signal E or *bis*-allylic protons observed higher or lower value for some composition. Also, method III proposed all triglycerides consist of the same acyl group but in contrast to the fact. Some fatty acid composition from this method was incorrect.

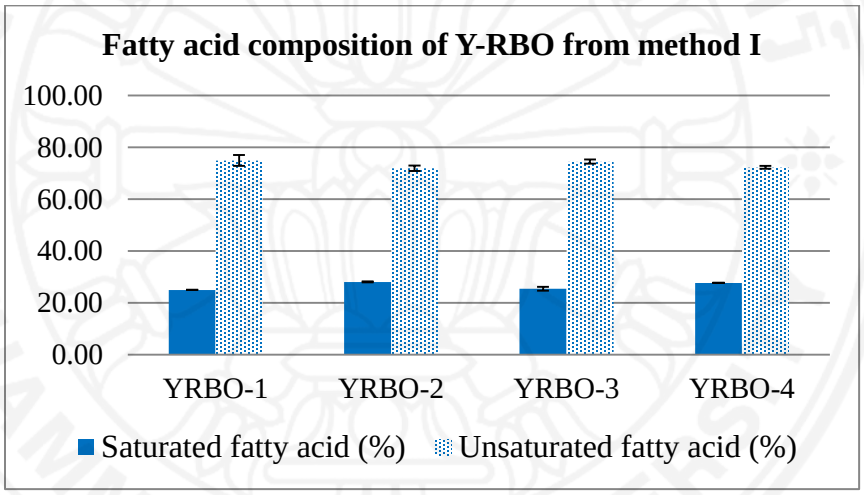
In addition, the fatty acid composition was analyzed in different storage conditions of oil and derived from method I. The reason of method I was chosen because this method obtained from proportional of methyl group in sample also %Ln group derived from terminal methyl proton of Ln. It was expected to obtain a near to fact of fatty acid composition. The result was summarized into saturated fatty acid and unsaturated fatty acid as shown in Fig. 21.

From Fig. 21a, %saturated fatty acid of L-RBO is not significant difference at 0.05 after one year storage (1.32, 2.77) even though stored in the dark at room temperature (7.5, 2.77) or kept in refrigerator (9.48, 2.77). After one year storage, %saturated fatty acid of Y-RBO kept at room temperature is significant different at P value of 0.05 (25.19, 2.77) but this is not significant different at P value of 0.05 in the dark at room temperature (1.16, 2.77) and kept in refrigerator (43.53, 2.77) as shown in Fig. 21b.

(a)



(b)



Note: RBO-1 was tested in Oct 2013; RBO-2 was tested in Oct 2014; RBO-3 was kept in dark at room temperature (29-32°C) as sealed bottle and tested on Oct 2014; RBO-4 was kept in refrigerator (≤15°C) as sealed bottle and tested on Oct 2014.

Figure 21 The fatty acid composition of (a) L-RBO and (b) Y-RBO in different storage conditions derived from ^{1}H NMR spectrum and calculated by method I.

4.3.2 Gas chromatography technique

Gas chromatography was used for determination of fatty acid composition as the conventional method. In this research, RBO was converted to methyl ester through esterification reaction by using 3%wt KOH in methanol as catalyst. Conversion of the esterification reaction was achieved 80-95% and obtained by ^{1}H NMR data.

Fatty acid composition was determined the proportion of acyl groups that contained in oil sample by gas chromatography-mass (GC-MS) spectroscopy as shown in Table 17. GC-MS provided fatty acid composition of RBO including myristic acid, palmitic acid, steric acid, oleic acid, linoleic acid and linolenic acid. Fatty acid composition of L-RBO was obtained 0.75±0.02, 20.89±0.14, 4.60±0.17, 26.22±0.33, 42.22±0.03, 29.31±0.53 and 2.25±0.03, respectively. Fatty acid of Y-RBO was obtained 0.65±0.10, 20.57±0.45, 4.47±0.03, 25.69±0.58, 44.15±1.56, 28.02±0.87 and 2.13±0.14, respectively.

Table 17 Fatty acid composition (%) of L-RBO-4 and Y-RBO-4 (Feb-15) in triplicate determinations by GC-MS technique.

	CODEX [76]	**L-RBO-4 (Feb-15)**	**Y-RBO-4 (Feb-15)**
Saturated (%)			
C14:0	ND-0.1	0.75±0.02	0.65±0.1
C16:0	14-23	20.87±0.14	20.57±0.45
C18:0	0.9-4.0	4.60±0.17	4.47±0.03
Total	-	26.22±0.22	25.69±0.46
Unsaturated (%)			
C18:1	38-48	42.22±0.03	44.15±1.56
C18:2	21-42	29.31±0.53	28.02±0.87
C18:3	0.1-2.9	2.25±0.03	2.13±0.14
Total	-	73.78±0.53	74.3±1.79

C14:0-myristic acid, C16:0-palmitic acid, C18:0-stearic acid, C18:1-oleic acid, C18:2-linoleic acid, C18:3-linolenic acid), ND -non detectable, defined as $\leq$ 0.05%

The determination of fatty acid composition by using ^{1}H NMR technique obtained a good result compared with reference standard composition. However, the preparation oil to volatile compound required a lot of time and chemicals for this technique.

Table 18 Fatty acid composition (%) of L-RBO-4 and Y-RBO-4 (Sep-15) by ^{1}H NMR technique and gas chromatography technique.

Oil	Method	Saturated Fatty Acids (%)				Unsaturated Fatty Acids (%)			
		C14:0	C16:0	C18:0	Total Sat. (S)	C18:1 (O)	C18:2 (L)	C18:3 (Ln)	Total Unsat.
L-RBO-4 (Sep-15)	I	+	+	+	23.30	49.21	25.34	2.14	76.69
	II	+	+	+	38.53	34.31	24.69	2.47	61.47
	III	+	+	+	17.99	34.49	31.68	15.84	82.01
	IV	**+**	**+**	**+**	**23.30**	**32.25**	**29.63**	**14.81**	**76.69**
	GC-MS	**0.71**	**20.76**	**4.81**	**26.28**	**40.77**	**30.49**	**2.45**	**73.71**
Y-RBO-4 (Sep-15)	I	+	+	+	25.44	47.56	24.46	2.54	74.56
	II	+	+	+	39.82	33.54	23.73	2.91	60.18
	III	+	+	+	17.12	33.61	32.85	16.42	82.88
	IV	**+**	**+**	**+**	**25.44**	**30.24**	**29.55**	**14.78**	**74.57**
	GC-MS	**0.67**	**20.66**	**4.77**	**26.10**	**41.22**	**30.03**	**2.33**	**78.58**

C14:0-myristic acid, C16:0-palmitic acid, C18:0-stearic acid, C18:1-oleic acid (O), C18:2-linoleic acid (L), C18:3-linolenic acid (Ln), (+) cannot be calculated from the equation.

4.3.3 Comparing ^{1}H NMR technique with gas chromatography technique

RBO was analyzed the fatty acid composition by ^{1}H NMR technique compare with GC-MS technique. RBO storage in refrigerator was used for this. According to Table 18, %S and %L group which is derived from method IV obtained the content of both oils similarly to GC-MS technique but % Ln group of both oils from method I and II presented approximately content to GC-MS technique. %O group from ^{1}H NMR technique which obtained the content close to GC-MS technique was from method II for L-RBO and method I for Y-RBO. However, this research proposed method IV could achieve a result than method I-III. All fatty composition of Y-RBO from ^{1}H NMR technique is not significant different from GC-MS technique at 0.05 (0.01, 3.18). Also, Lopburi obtained non-significant different from GC-MS technique at 0.05 (2.01, 3.18).

4.4 Mono- and diglycerides components analysis

Fig. 22 showed the enlargement at 3.5-5.3 ppm region in the ^{1}H NMR spectrum of a fresh L-RBO (Fig.22a) compared to 12 month old L-RBO (Fig. 22b). Some of these chemical shifts were corresponded to ^{1}H-NMR signals of compound involved in hydrolytic and oxidative of RBO over 12 months old. Signal of glycerol proton of triglycerides at 4.1-4.3 ppm was decreased and some new peaks in Fig. 22b were appeared. These signals presented increasingly in comparison with characteristic fresh L-RBO signals (Fig. 22a), indicating that the oxidative degradation could take place as shown the signal at 4.07 ppm (Fig.22b), assigned to the proton of the glycerol group of *sn*-1,3-diglycerides [58], was indicative of the loss of quality and freshness of L-RBO.

In addition, Yasothorn sample revealed the enlargement of ^{1}H NMR spectrum regions of mono-, diglycerides of Y-RBO in April-2014 (counting 6 months, Fig. 23) between 3.6-5.3 ppm and assignment of ^{1}H NMR chemical shifts of protons signal of mono- or diglycerides were followed previous study [61], according to Table 11 and analyzed by NMR software. Chemical shifts were given in Table 19.

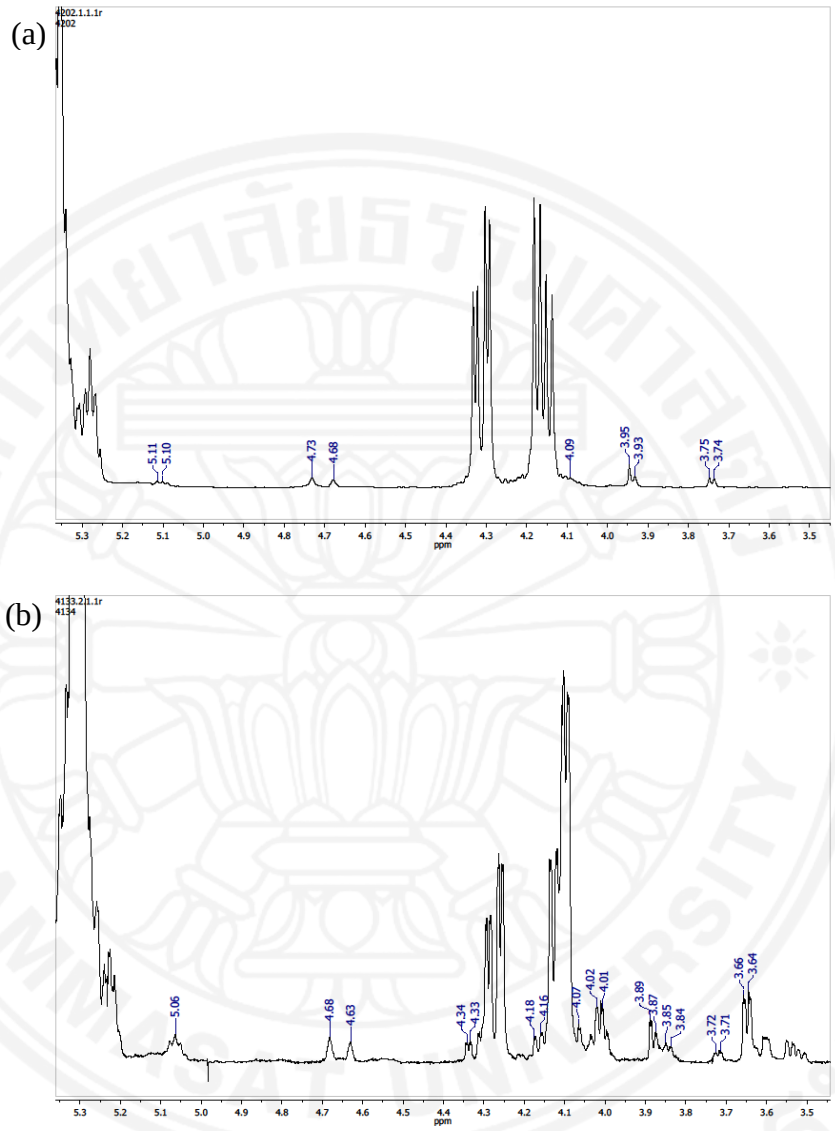


Figure 22 The enlargement at 3.5-5.3 ppm region in the ^{1}H NMR spectrum of (a) a fresh L-RBO and (b) 12 month old L-RBO.

From Fig. 23, there was given signal of mono- or diglycerides when one or two acyl groups were migrated from triglycerides. Chemical shift of glyceryl proton was shifted to high field because of losing the electron density of acyl group.

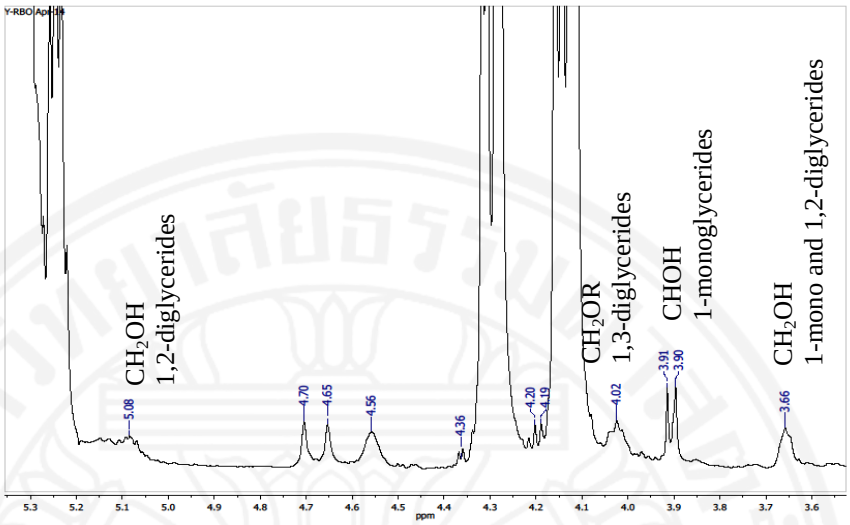


Figure 23 The enlargement at 3.6-5.3 ppm region in the ^{1}H NMR spectrum of Y-RBO (Apr-14). Labeled resonances are assigned in Table 19.

Table 19 Chemical shifts and assignment of signal of mono- and diglycerides in the ^{1}H NMR spectrum of Y-RBO (Apr-14).

Compounds	**Proton**	**Chemical shift (ppm)**			
		Sacchi R. et al. [58]	**Jin F. et al. [64]**	**Compton D. et al. [65]**	**Y-RBO Apr-14[a]**
sn-1,2-diglycerides	C**H**$_2$OR	4.29	4.25	-	4.10-4.20
	>C**H**OCOR	5.07	5.0	-	5.08
	C**H**$_2$OH	3.66	3.78	-	3.68
sn-1,3-diglycerides	C**H**$_2$OR	4.03	4.05	-	4.02
	C**H**OH	4.07	-	-	
sn-1-monoglycerides	C**H**$_2$OH	-	3.60	3.66	3.68
	>C**H**OH	-	3.90	3.95	3.90-3.91
	C**H**$_2$OR	-	4.25	4.21	4.10-4.20
sn-2-monoglycerides	C**H**$_2$OH	-	-	3.85	-
	>C**H**OCOR	-	-	4.95	-

[a] from this study using NMR software

RBOs used in this study contains CH_2OH, *sn*-3 of *sn*-1,2-diglycerides after 6-month observation, as found at 3.65 ppm of its ^1H NMR spectrum. This signal was shifted to high field compared to CH_2OR, *sn*-3-triglycerides (4.10-4.35 ppm). Also, CH_2OH, *sn*-2 of *sn*-1,2-diglycerides was appeared in a broad peak at 5.08 ppm. In term of CH_2OH, *sn*-3 of 1-monoglycerides, the chemical shift was assigned at 3.66 ppm due to slightly low field to CH_2OH, *sn*-3 of *sn*-1,2-diglycerides. The CH_2OR, *sn*-1 of *sn*-1,2-diglycerides and *sn*-1-monoglycerides was assigned at the same position at 4.10-4.20 ppm and appeared nearly CH_2OR, *sn*-1(3) of *sn*-1,3-diglycerides signal that observed at 4.02 ppm. It could be a reason of peak height at 4.10 ppm in Fig. 22b. The signal of *sn*-2 of *sn*-1-monoglycerides was assigned at 3.90-3.91 ppm.

The formation of *sn*-1,3-diglycerides is suggested to involve triglycerides which underwent hydrolytic reactions. Despite the fact that signals of *sn*-1,3-diglycerides revealed very small in the spectrum of the RBOs. Also, most of di- and monoglycerides showed overlap of those α,β-protons in glycerides.

The occurrence of free fatty acids can be formed in hydrolytic processes. The concentration of free fatty acids is very small proportion in the original oil. These compounds are observable in the spectrum through a part of its triplet signal of their $–CH_2–$ protons at α position in relation to the carboxylic group (indicated at 2.22-2.39 ppm by signal 6 in Fig. 20); this signal overlaps with the triplets of the same kind of protons of acyl chains of triglycerides. Due to the small abundance of bounded fatty acids, this signal cannot be used for a correct quantification of its concentration. ^{13}C NMR data would be studied and carried out to accomplish the results.

4.5 ^{13}C NMR analysis of cold-pressed rice bran oil

The ^{13}C NMR spectrum of Y-RBO in April-2014 was shown in Fig. 24. The assignment of ^{13}C NMR chemical shifts of carbons signal was accordance with previous reports [58] and analyzed by NMR software. Chemical shifts were given in Table 20.

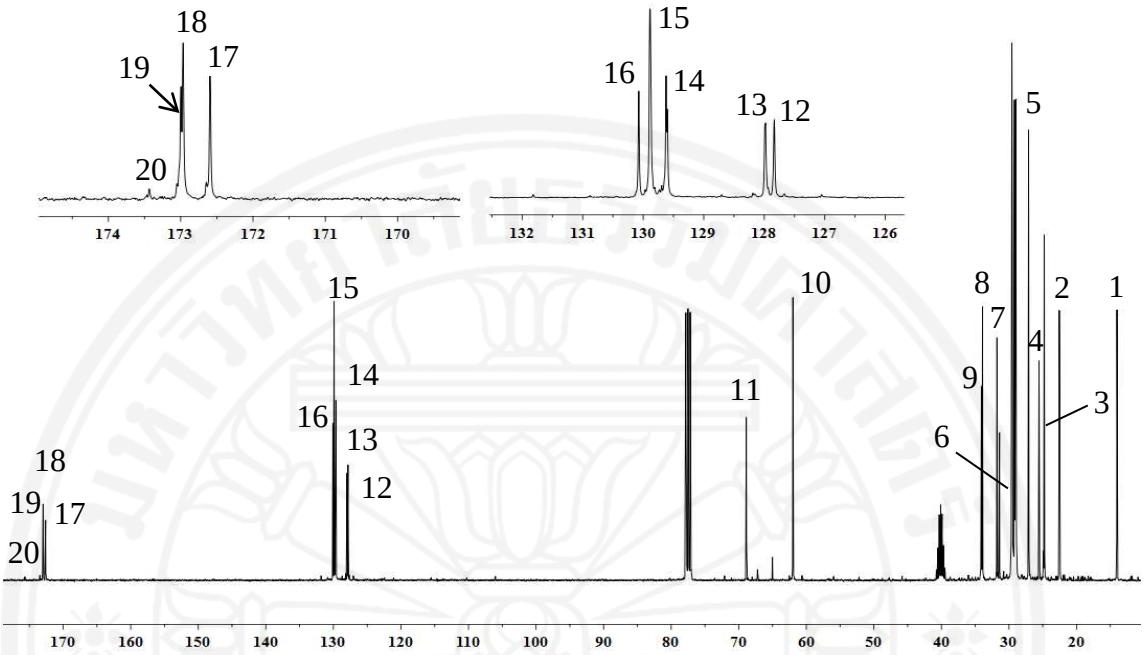


Figure 24 ^{13}C NMR spectrum of Y-RBO (Apr-14). Labeled resonances are assigned in Table 20.

Data for Y-RBO Oct-13, ^{13}C NMR (100 MHz, $CDCl_3$, DMSO): δ *C* 173.4, 173.0, 172.97, 172.59; *C*H 130.07, 129.89, 129.60, 127.98, 127.84, 68.88; *C*H_2 61.98, 34.08-34.15, 33.92, 31.78-31.80, 28.92-29.65, 27.07-27.10, 25.55, 24.78-24.90, 22.44-22.55; *C*H_3 13.97-14.06.

In previous studied, R. Zamora et al. (2002) investigated the ^{13}C NMR technique in the analysis of 109 vegetable oils to evaluate the potential of use oil fractions and ^{13}C NMR spectroscopy obtained much more information from the enriched polar oil fractions than from the original oils [74]. From F. Jin et al. (2007) [64] studied, applying the NMR technique to identify the intermediate compounds in transesterification of vegetable oil such as *sn*-1,2, 1,3-diglycerides and *sn*-1 , *sn*-2-monoglycerides.

Table 20 Chemical shifts and assignment of signals of the main resonances in the ^{13}C NMR spectrum of Y-RBO (Apr-14).

Signal	Carbon		Compounds	Chemical shift (ppm)	
				Sacchi R. et al. [58]	Y-RBO (Apr-14)[a]
1	-**C**H_3	*n*-1	saturated, oleic and linoleic acyl chains	14.15	13.97-14.06
2	-**C**H_2-	*n*-2	acyl chains	22.65	22.44-22.55
3	-OCO-CH_2-**C**H_2-		acyl chains	24.84	24.78-24.90
4	=CH**C**H_2CH=	diallylic	acyl chains	25.81	25.55
5	-**C**H_2CH=CH**C**H_2-	allylic	acyl chains	27.16	27.07-27.10
6	-(**C**H_2)n-		acyl chains	29.1-29.8	28.92-29.65
7	-**C**H_2-	*n*-3	saturated *n*-9, *n*-6 acids	31.88	31.78-31.80
8	-OCO-**C**H_2-	*sn*-1,3	acyl chains	34.02	33.92
9	-OCO-**C**H_2-	*sn*-2	acyl chains	34.18	34.08-34.15
10	-**C**H_2OCOR	*sn*-1,3	glyceryl group	62.18	61.98
11	>**C**HOR	*sn*-2	glyceryl group	68.92	68.88
12	-CH=**C**H-	C12	linoleyl group	127.86	127.84
13	-CH=**C**H-	C10	linoleyl group	128.06	127.98
14	-**C**H=CH-	C9	oleyl group	129.67	129.60
15	-**C**H=**C**H-	C10	oleyl group	129.98	129.89
16	-**C**H=CH-	C9	linoleyl group	130.02	130.07
17	-O-O**C**-R	*sn*-2	triacylglycerols	172.81	172.59
18	-O-O**C**-R	*sn*-1,3	triacylglycerols	173.26	172.97
19	-O-O**C**-R	C1	linolenyl group	173.04	173.00
20	H-O-O**C**-R		free fatty acid	174-176	173.43

[a] from this study using NMR software

^{13}C NMR data is then purposed in this research due to the wider chemical shift spread of the ^{13}C NMR resonances, the different carbon chemical environments and ultimately to the α-, β-position of fatty chains on the glycerol backbone (indicated by expanding signal at 74-58 ppm in Fig. 25 and Table 21), according to Table 12 [58].

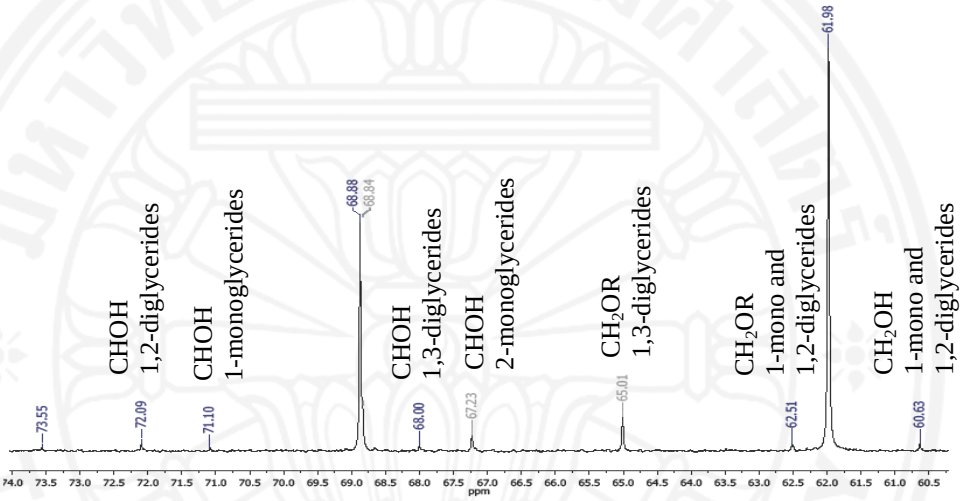


Figure 25 Enlargement of ^{13}C NMR spectrum region of mono- or diglycerides of Y-RBO (Apr-14). Labeled resonances are assigned in Table 21.

Fig.25 showed the enlargement at 60-74 ppm regions in ^{13}C NMR spectrum of mono- and diglycerides of Y-RBO in April-2014. The assignment of more carbon signals which were in agreement of literature data given in Table 21.

First, the 2 high peak height at 61.98 and 68.88 ppm were assigned to glyceryl carbon at *sn*-1,3 and *sn*-2 position of triglycerides, respectively. Other peak at 65.01 and 68.00 ppm were assigned to glyceryl carbon at *sn*-1,3 and *sn*-2 of *sn*-1,3-diglycerides, respectively. For *sn*-1,2-diglycerides, glyceryl carbon atom at *sn*-1, *sn*-2 and *sn*-3 position of *sn*-1,2-diglycerides was found carbon chemical shift at 62.51, 72.09 and 60.63 ppm, respectively. Also, *sn*-1-monoglycerides at *sn*-1 and *sn*-3 position was assigned to the same signal of *sn*-1,2-diglycerides. Carbon atom at *sn*-2 of *sn*-2-monoglycerides and *sn*-1-monoglycerides were noticeable found at 67.23 and 71.10 ppm, respectively.

Table 21 Chemical shifts and assignment of signal of mono- or diglycerides in the ^{13}C NMR spectrum of Y-RBO (Apr-14) (Fig. 25).

Compounds	Carbon	Chemical shift (ppm)			
		Sacchi R. et al. [58]	Siddiqui N. et al.[63]	Jin F. et al. [64]	Y-RBO Apr-14[a]
sn-1,2-diglycerides	**C**H_2OR	62.04	62.04	-	62.51
	>**C**HOCOR	72.08	72.13	71.93	72.09
	CH_2OH	61.43	61.32	61.10	60.63
sn-1,3-diglycerides	**C**H_2OR	64.99	64.99	64.84	65.01
	CHOH	68.30	68.14	68.78	68.00
sn-1-monoglycerides	**C**H_2OH	63.34	63.34	64.98	60.63
	>**C**HOH	70.25	70.19	70.04	71.10
	CH_2OR	65.09	65.15	63.20	62.51
sn-2-monoglycerides	**C**H_2OH	-	-	-	-
	>**C**HOCOR	-	-	-	67.23

[a] from this study using NMR software

According to Table 22, monitoring the change of chemical shift and intensity of oil over one year storage, the fresh extracted or cold-pressed oils from rice bran contained the native *sn*-1,2-diglycerides (shown clearly in Y-RBO with signals at 61.40, 62.28-62.33 and 72.62 ppm) and *sn*-1,3-diglycerides (shown in Y-RBO with signals at 65.05 and 68.10 ppm). The *sn*-3 position of *sn*-1,2-diglycerides and *sn*-1-monoglycerides was assigned in the same signal. For L-RBO, these signals was found at 61.57 ppm and shifted to high field at 61.75 ppm with high intensity from 0.02 to 0.03 after a year. For Y-RBO, these signals was found at 61.40 ppm and shifted to high field at 61.65 ppm with low intensity from 0.03 to 0.01. Both of RBOs showed a high field of the carbon at *sn*-1 position of *sn*-1,2-diglycerides and *sn*-1-monoglycerides signal with intensity unchanging after a year.

Table 22 ^{13}C NMR chemical shifts (in ppm) and int. (intensity) of carbon signal of mono- or diglycerides in both RBOs

Carbon	Compounds	L-RBO, Nov-13		L-RBO, Nov-14		Y-RBO, Nov-13		Y-RBO, Nov-14	
		ppm	int.	ppm	int.	ppm	int.	ppm	int.
CH_2OH, *sn*-3	*sn*-1,2-diglycerides	61.57	0.02	61.75	0.03	61.40	0.03	61.65	0.01
CH_2OR, *sn*-1	*sn*-1,2-diglycerides	62.27-62.34	0.03	62.37-62.42	0.02	62.28-62.33	0.04	62.35	0.04
CH_2OH, *sn*-3	*sn*-1-monoglycerides	61.57	0.02	61.75	0.03	61.40	0.03	61.65	0.01
CH_2OR, *sn*-1,3	*sn*-1,3-diglycerides	65.05	0.04	64.97	0.08	65.05	0.1	64.98	0.16
CH_2OR, *sn*-1	*sn*-1-monoglycerides	62.27-62.34	0.03	62.37-62.42	0.02	62.28-62.33	0.04	62.35	0.04
CH_2OH, *sn*-1,3	*sn*-2-monoglycerides	-		-		-		-	
>**C**HOR, *sn*-2	*sn*-2-monoglycerides	67.11	0.01	67.50	0.03	67.22	0.04	67.64	0.08
>**C**HOH, *sn*-2	*sn*-1,3-diglycerides	68.19	0.02	68.00	0.02	68.10	0.04	68.03	0.02
>**C**HOH, *sn*-2	*sn*-1-monoglycerides	70.25	0.01	72.07	0.02	70.91	0.06	70.94	0.01
>**C**HOR, *sn*-2	*sn*-1,2-diglycerides	72.08	0.01	73.55	0.01	72.62	0.04	72.13	0.04

Note: chemical shift and intensity from this study using NMR software

sn; related with position of acyl chain on triglycerides (such as *sn*-1 is represented proton at carbon 1 or the α-position of the glycerides; *sn*-2 is represented proton at carbon 2 or the β-position of the glycerides; *sn*-3 is represented proton at carbon 3 or the α-position from other end of the glycerides.

In Y-RBO, the signal of *sn*-1,3-diglycerides at *sn*-1,3 position shifted to low field and revealed higher intensity after a year from 0.1 to 0.16 with signal at 64.98 ppm. At the same position in L-RBO, this signal was found at 65.05 ppm and then shifted to low field at 64.97 ppm with the higher intensity from 0.04 to 0.08.

The *sn*-2 position of *sn*-1,3 and *sn*-1,2-diglycerides of L-RBO were found at 68.19 and 72.08 ppm, respectively. After storage for one year, the *sn*-1,3-diglycerides signal appeared at 68.00 ppm with constant intensity at 0.02 and *sn*-1,2-diglycerides signal that appeared at 73.55 ppm also revealed constant intensity at 0.01. In contrast, *sn*-1,3-diglycerides signal of Y-RBO showed low intensity from 0.04 to 0.02 and shifted from 68.10 to 68.03 ppm. *sn*-1,2-diglycerides signal was assigned at 72.62 ppm then shifted to 72.13 ppm with constant intensity at 0.04 after a year.

The glyceryl carbon atoms at *sn*-2 position of *sn*-2 and *sn*-1-monoglycerides of Y-RBO were noticeable found at 67.22 and 70.91 ppm, respectively. After a year, the *sn*-2-monoglycerides signal appeared at 67.64 ppm with higher intensity from 0.04 to 0.08 and *sn*-1-monoglycerides signal appeared at 70.94 ppm with lower intensity from 0.06 to 0.01. On the other hand, L-RBO revealed higher intensity in both signals. *sn*-2-monoglycerides signals appeared at 67.11 (then shifted to 67.50) ppm with higher intensity from 0.01 to 0.03 and *sn*-1-monoglycerides signal appeared at 70.25 (then shifted to 72.07) ppm with higher intensity from 0.01 to 0.02. The changes in intensity are due to disappearance of the acyl group in different media of both RBOs as shown in Fig.22. From literature [58], Larger amount of *sn*-1,3-diglycerides was found when the olive oil was neutralized. These trends increased with olive oil storage due to *sn*-1,2 to *sn*-1,3 isomerization which found in contrary for RBOs in this study.

The amount of free fatty acids and the corresponding mono- and diglycerides profile can also be used to define the degree of lipolytic alteration which is correlated with the quality of organic RBO. Since the ^{13}C NMR spectrum usually observes with decoupling technique, this is may affect to the intensity or peak area which is not proportional to the amount of nuclei directly. Therefore, the amount of non-equivalent carbons cannot determine from integration of carbon peak in the spectrum. The amount

of mono- and diglycerides or free fatty acid by ^{13}C NMR data will be studied in terms of a ratio. The *sn*-1 or *sn*-2-monoglycerides and the *sn*-1,2 or *sn*-1,3-diglycerides ratio can be easily determined by the integration using ^{13}C NMR spectroscopy technique. As shown in Table 22, these parameters were studied. It was noted that the ratio between *sn*-1,2 and *sn*-1,3-diglycerides at *sn*-2 position showed to be correlated with the quality-freshness of RBO [58]. From Fig. 26 revealed hydrolytic degradation of RBOs by showing the ratio of *sn*-1,3-diglycerides to total diglycerides and *sn*-1-monoglycerides to total monoglycerides of RBO. Total diglycerides was summarized from intensity of *sn*-1,2 and *sn*-1,3-diglycerides. Total monoglycerides was similarly summarized from *sn*-1 and *sn*-2-monoglycerides, according to Table 22.

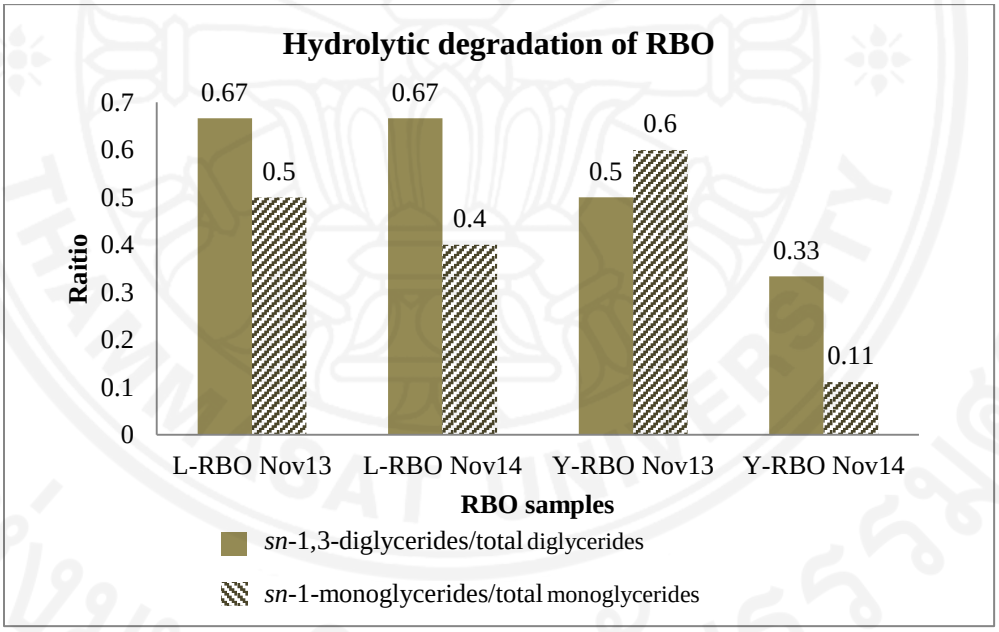
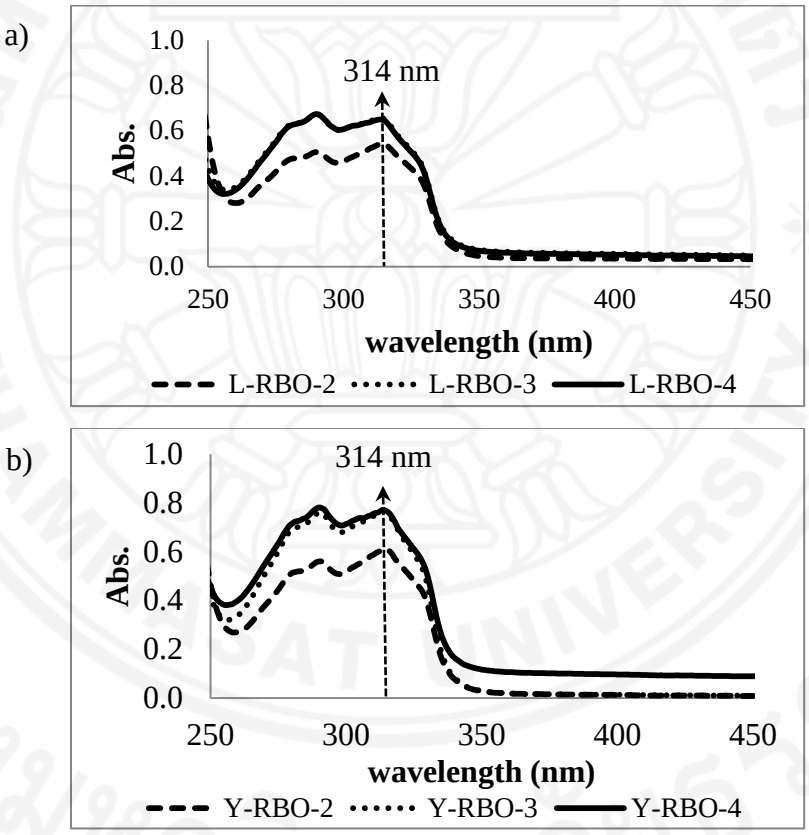


Figure 26 The ratio of *sn*-1,3-diglycerides to total diglycerides and *sn*-1-monoglycerides to total monoglycerides of RBO samples (L-RBO and Y-RBO).

The ratio of *sn*-1,3-diglycerides to total diglycerides of L-RBO was found at 0.67 in both period of time in contrast with Y-RBO, this ratio decreased from 0.5 to 0.33. In addition, *sn*-1-monoglycerides to total monoglycerides of L-RBO was observed slightly decreased ratio from 0.5 to 0.4 compared with Y-RBO that decreased from 0.6 to 0.11.

The ratio of *sn*-1-monoglycerides to total monoglycerides of RBO can provide a good discrimination of quality freshness by revealing decrease in *sn*-1-monoglycerides. However, the ratio of *sn*-1,3-diglycerides to total diglycerides of RBO could not be in agreement with the studies of olive oils to indicate quality of RBO. The structure-specific analysis of different compositions indicates the quality of the product as a rapid and reliable methodology.

4.6 Oryzanol contents



Note: RBO-2 was kept at room temperature (29-32°C) and first opened on Oct-13; RBO-3 was kept in dark at room temperature as sealed bottle; RBO-4 was kept in refrigerator (≤15°C) as sealed bottle.

Figure 27 The UV spectra of RBO samples a) L-RBO and b) Y-RBO.

The oil which kept in different storage conditions was analyzed the oryzanol content by UV spectroscopy. All samples were investigated at the same time. %Oryzanol of L-RBO-2, L-RBO-3, and L-RBO-4 was presented 1.39±0.02, 1.50±0.21 and 1.57±0.05, respectively. Y-RBO revealed %oryzanol of 1.69±0.05, 1.72±0.0 and 1.89±0.10 for Y-RBO-2, Y-RBO-3 and Y-RBO-4, respectively. The result indicated that both oils obtained higher oryzanol content if kept oil in refrigerator. After opened oil bottle, the oryzanol was decreased. Also, the oryzanol content of L-RBO was significantly different from Y-RBO at P value of 0.05 (9.16, 4.30).

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

In this research, cold-pressed rice bran oil (RBOs) obtained from Lopburi province and Yasothorn province were studied. The stability of RBOs after year storage in different conditions has been approved. It was found that all chemical properties parameters (acid value, iodine value and peroxide value) of both oil revealed significantly different at 0.05 except L-RBO obtained no significantly different of acid value at 0.05. The use of NMR methods can be applied to determine the fatty acid composition of RBOs. The fatty acid composition in terms of %oleic and %linoleic group of Y-RBO was significantly different through storage for one year and %oleic, %linoleic and %linolenic group of L-RBO was also significantly different. The NMR methods performed as an alternative method or method IV to GC-MS technique.

In addition, the different storage conditions such as kept as sealed bottle in the dark and in refrigerator affected to chemical properties. Especially, acid value of both oils was different. It can be indicated that temperature parameter related to acid value. However, all chemical properties of RBOs from Lopburi showed no significant difference from Yasothorn at P value of 0.05. Also, the fatty acid composition from all ^{1}H NMR method was not significantly different from GC-MS at 0.05 and %linolenic group from method I showed a good agreement with GC-MS. For the best result, %oleic group was suggested to derive from its difference from the summary of both %saturated and %linolenic from method I and %linoleic group from method IV.

Moreover, the formation of some compounds during storage such as mono- or diglycerides was also studied by ^{13}C NMR technique. After one year storage, L-RBO revealed constant ratio of *sn*-1,3-diglycerides to total diglycerides but Y-RBO showed the decrease of this ratio. Also, *sn*-1-monoglycerides to total diglycerides ratio of both oils decreased when stored for one year. In contrast, the fact that *sn*-1,3-diglycerides was increased due to age of storage and neutralization process etc. The formation of epoxide compound has not been found in this investigation. In addition, the different storage

conditions were affected to oryzanol content of RBOs. The oil maintained oryzanol content if keeping oil as sealed bottle in refrigerator.

As above, it can be concluded that the data detail from NMR spectrum was found to be linearly correlated with chemical properties such as peroxide value, iodine value and acid value. For instance, the oxidative compound region of the ^{1}H NMR spectrums can be corresponds to peroxide value. The NMR methods for determining the fatty acid composition in term of the amount of unsaturated fatty acid can be used for evaluate the iodine value. In addition, the lipid hydrolysis which was studied by ^{13}C NMR as the ratio of *sn*-1,3-diglycerides to total diglycerides and *sn*-1-monoglycerides to total diglycerides related to acid value of oil.

In future work, oryzanol content and its other properties in RBOs will be studied for completing the information of cold-pressed RBOs. NMR was proposed as a new method for determining the degree of unsaturation of oils, hydrolytic degradation and oxidation reaction in place of the conventional method. The NMR analysis of cold-pressed RBOs will be study to investigate the fatty acid composition to obtain more accuracy and precision.

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APPENDICES

APPENDIX A

^{1}H and ^{13}C NMR spectrum of RBOs

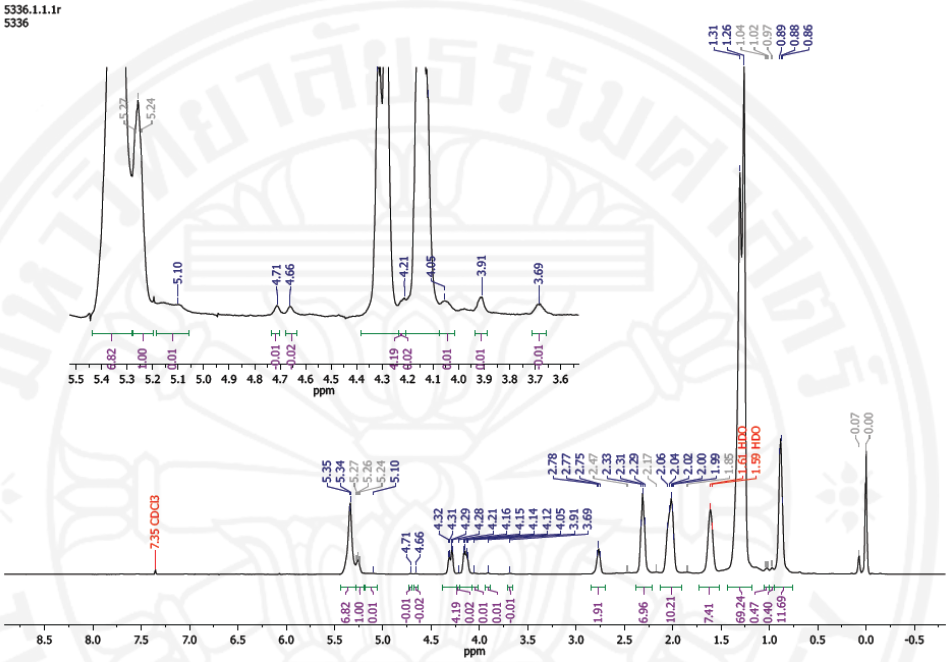


Figure 1A ^{1}H NMR spectrum of L-RBO on November 2014.

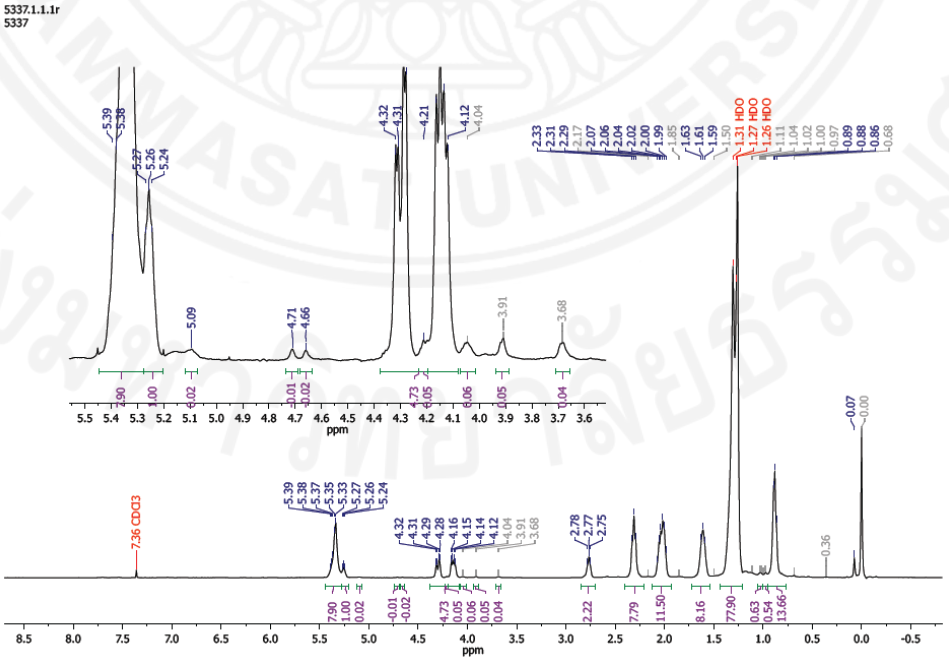


Figure 2A ^{1}H NMR spectrum of Y-RBO on November 2014.

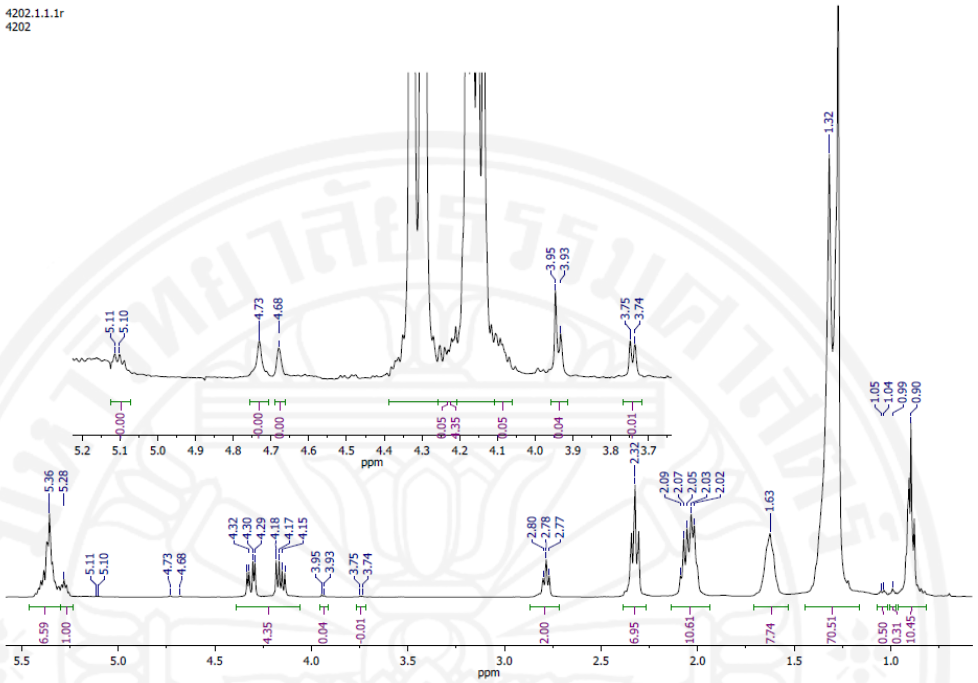


Figure 3A ^{1}H NMR spectrum of L-RBO-1.

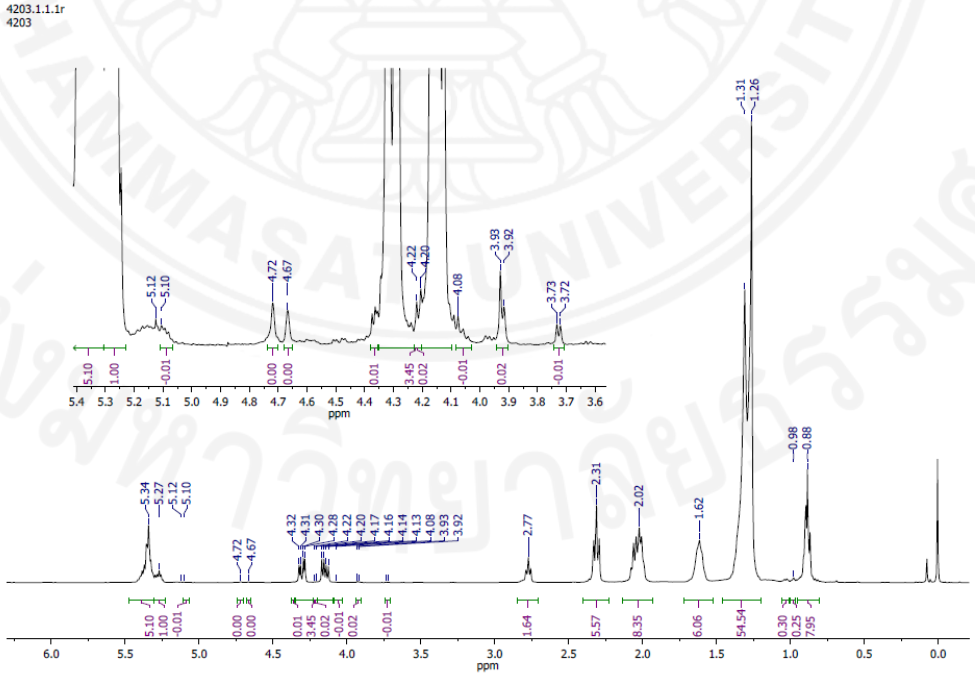


Figure 4A ^{1}H NMR spectrum of Y-RBO-1.

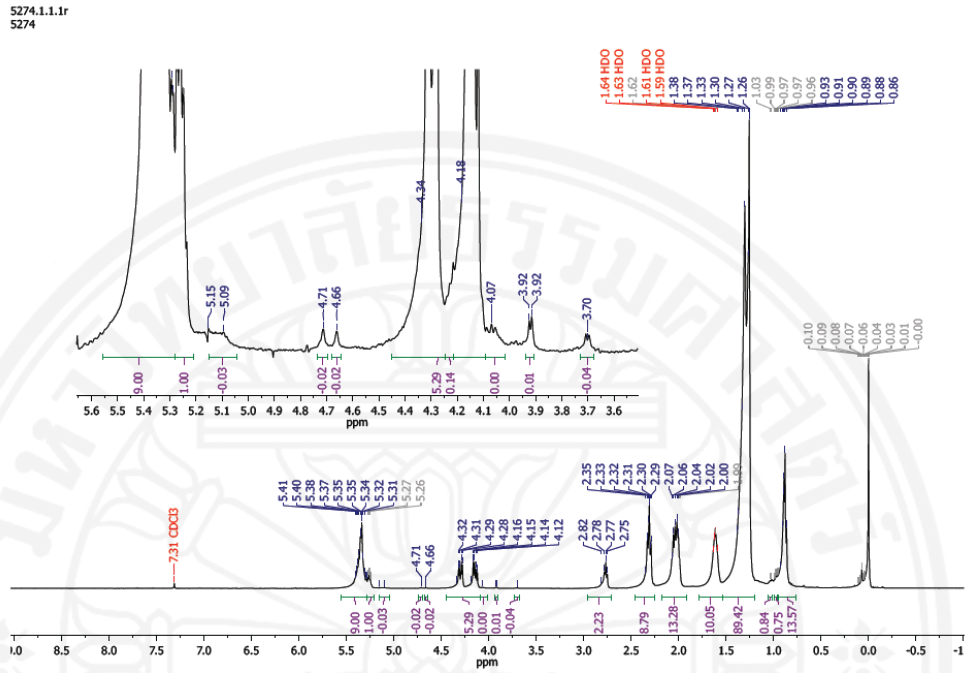


Figure 5A ^{1}H NMR spectrum of L-RBO-2.

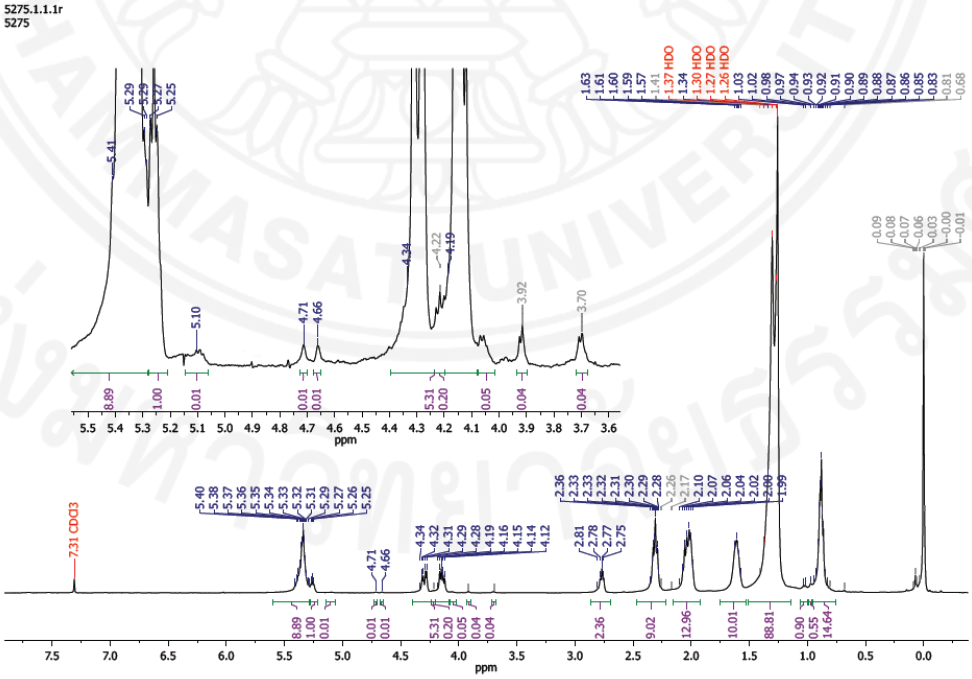


Figure 6A ^{1}H NMR spectrum of Y-RBO-2.

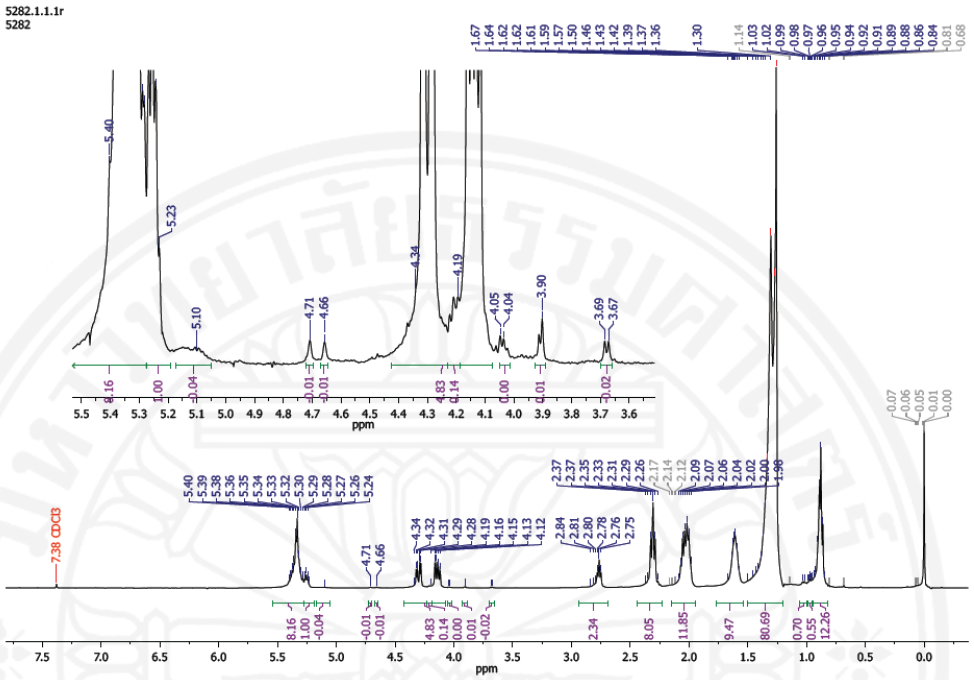


Figure 7A ^{1}H NMR spectrum of L-RBO-3.

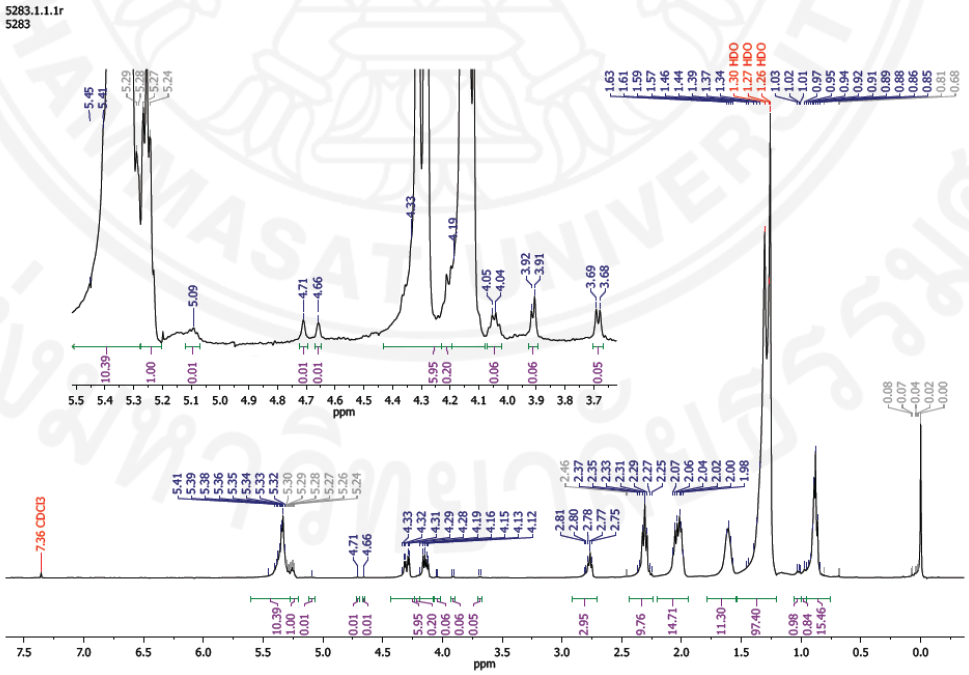


Figure 8A ^{1}H NMR spectrum of Y-RBO-3.

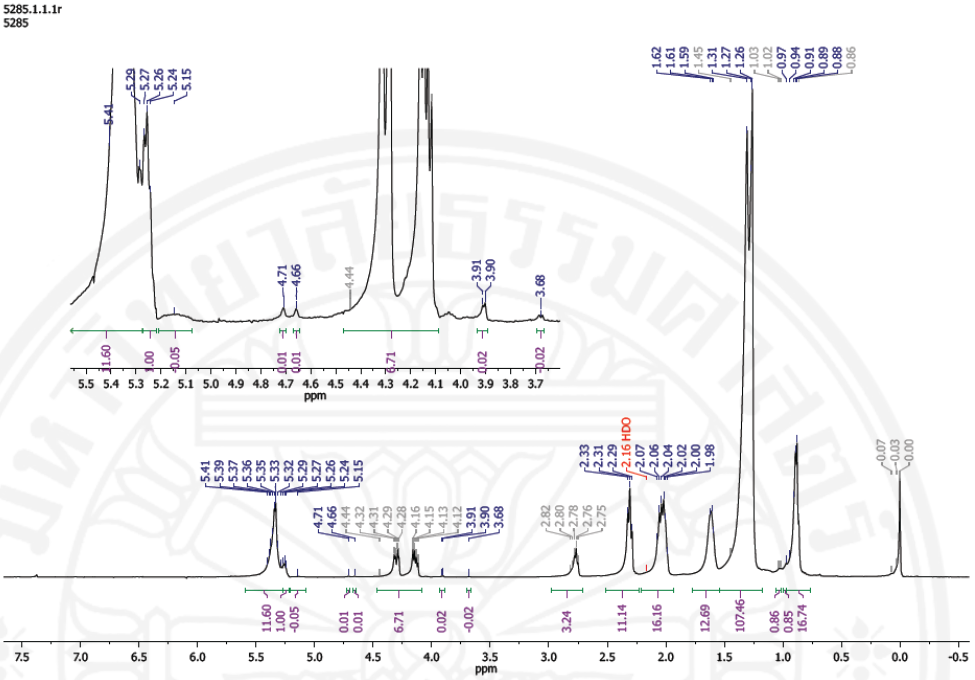


Figure 9A ^{1}H NMR spectrum of L-RBO-4.

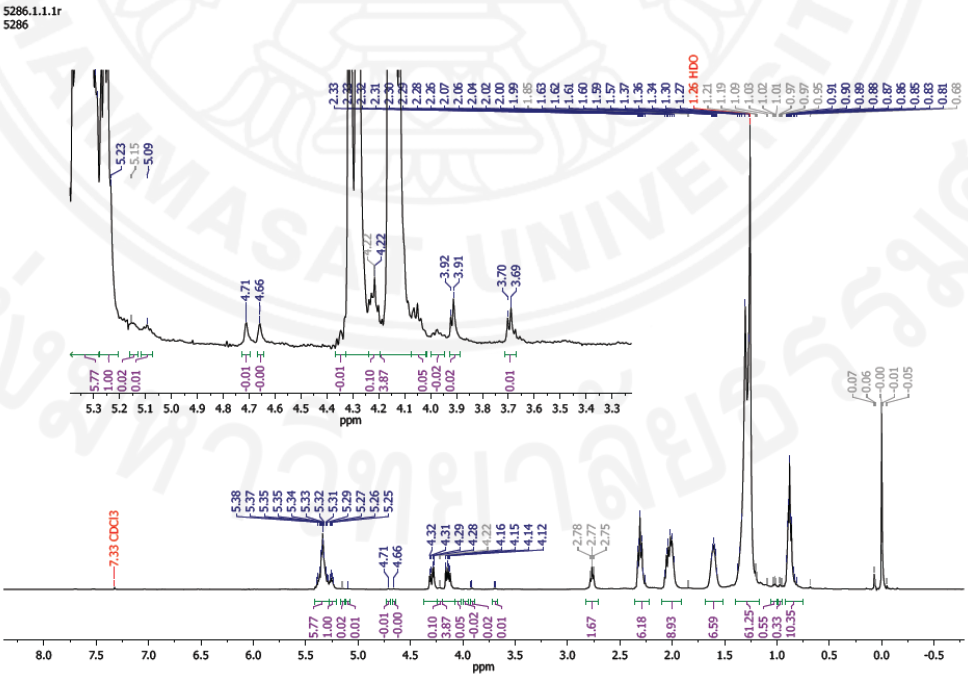


Figure 10A ^{1}H NMR spectrum of Y-RBO-4.

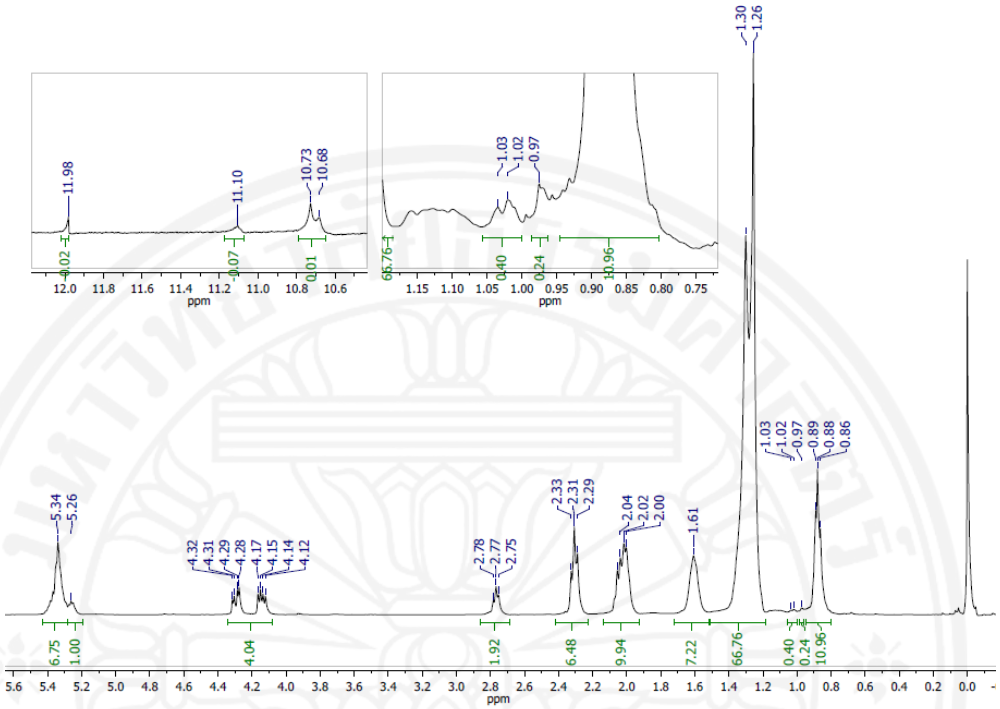


Figure 11A ^{1}H NMR spectrum of L-RBO on September 2015.

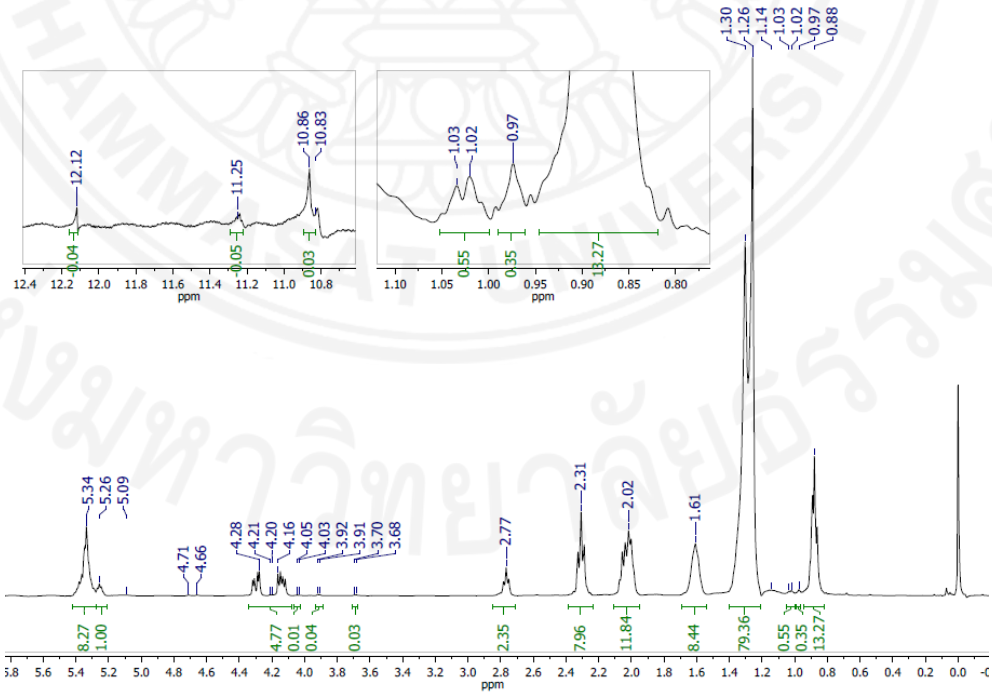


Figure 12A ^{1}H NMR spectrum of Y-RBO on September 2015.

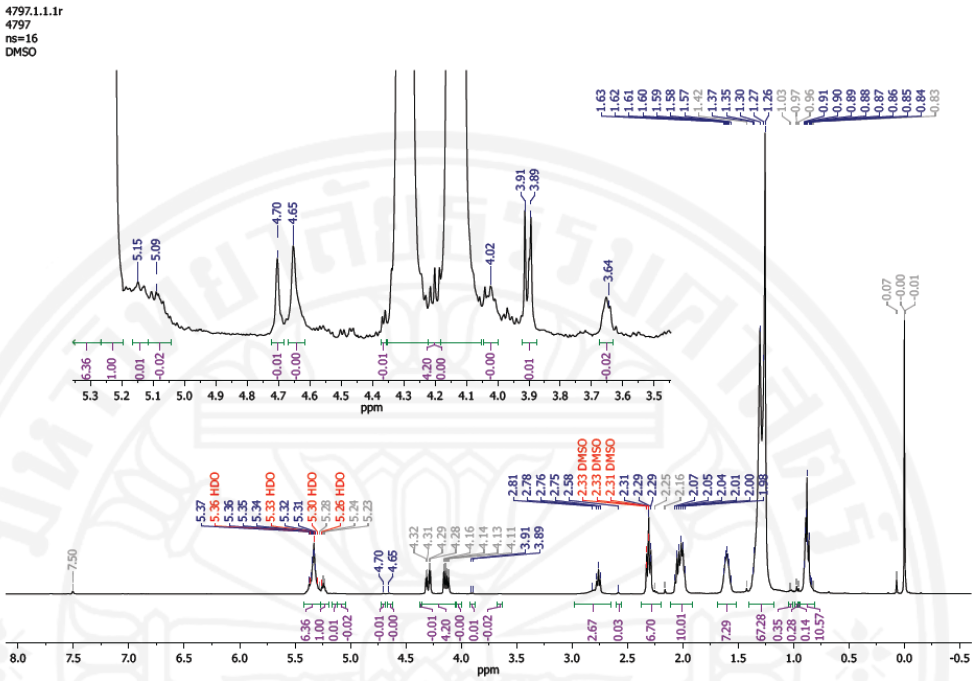


Figure 13A ^{1}H NMR spectrum of L-RBO on April 2014.

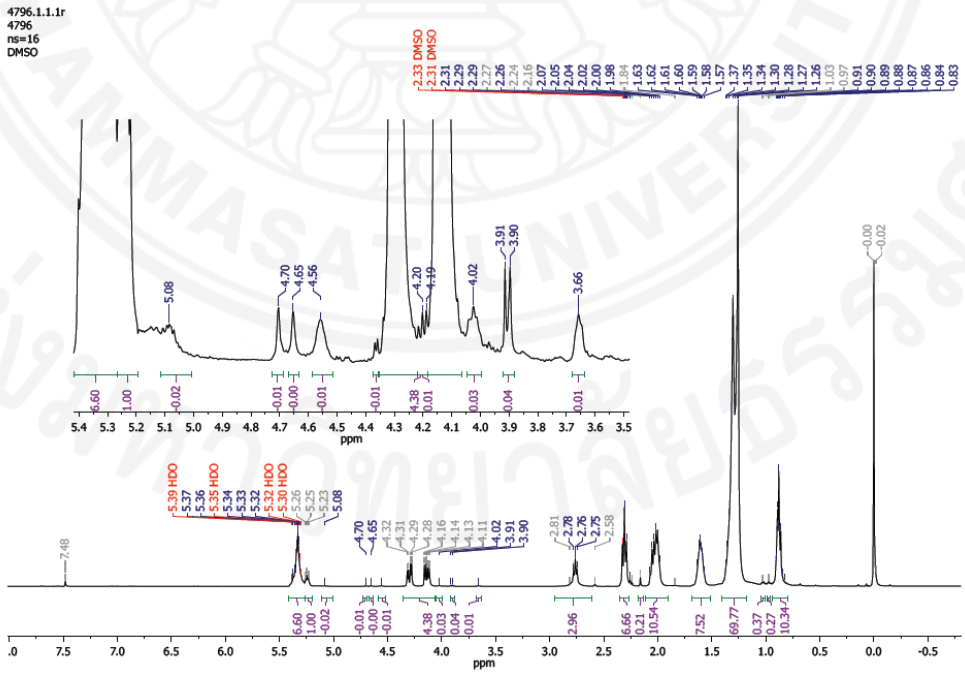


Figure 14A ^{1}H NMR spectrum of Y-RBO on April 2014.

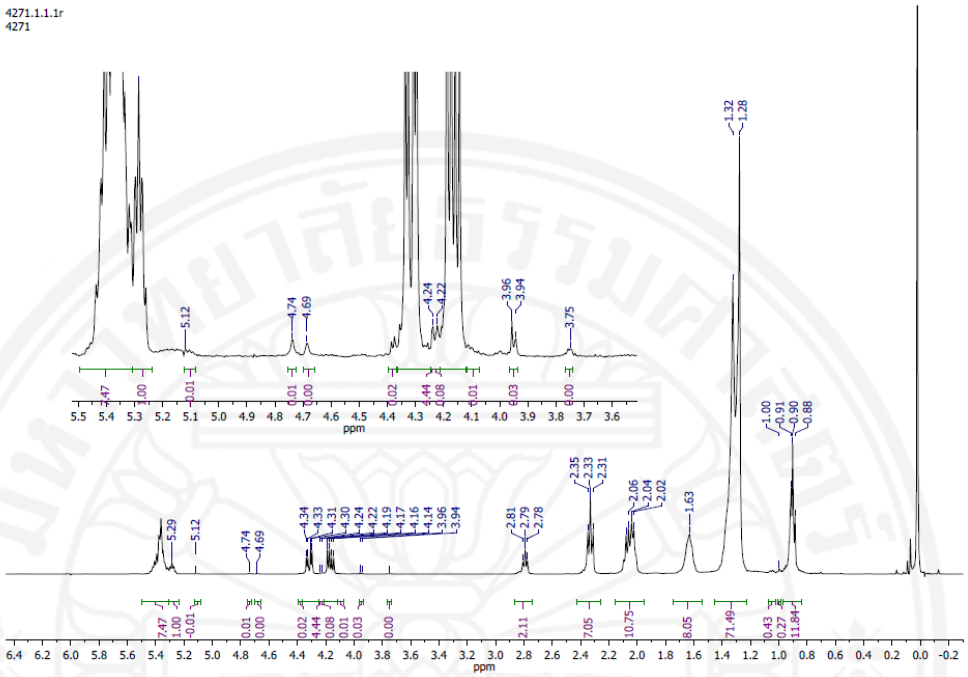


Figure 15A ^{1}H NMR spectrum of L-RBO on November 2013.

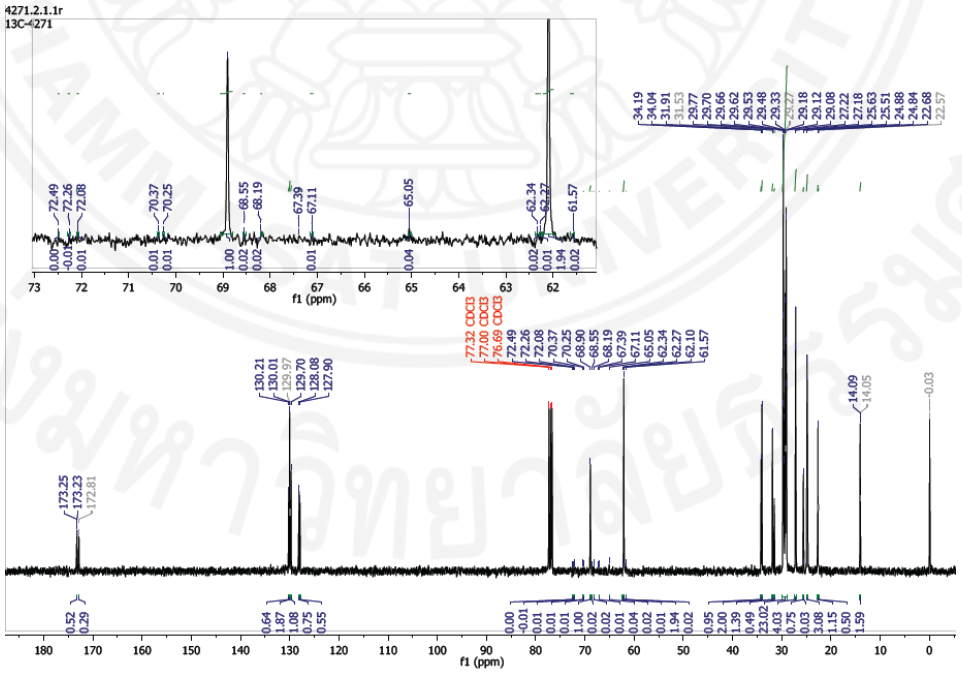


Figure 16A ^{13}C NMR spectrum of L-RBO on November 2013.

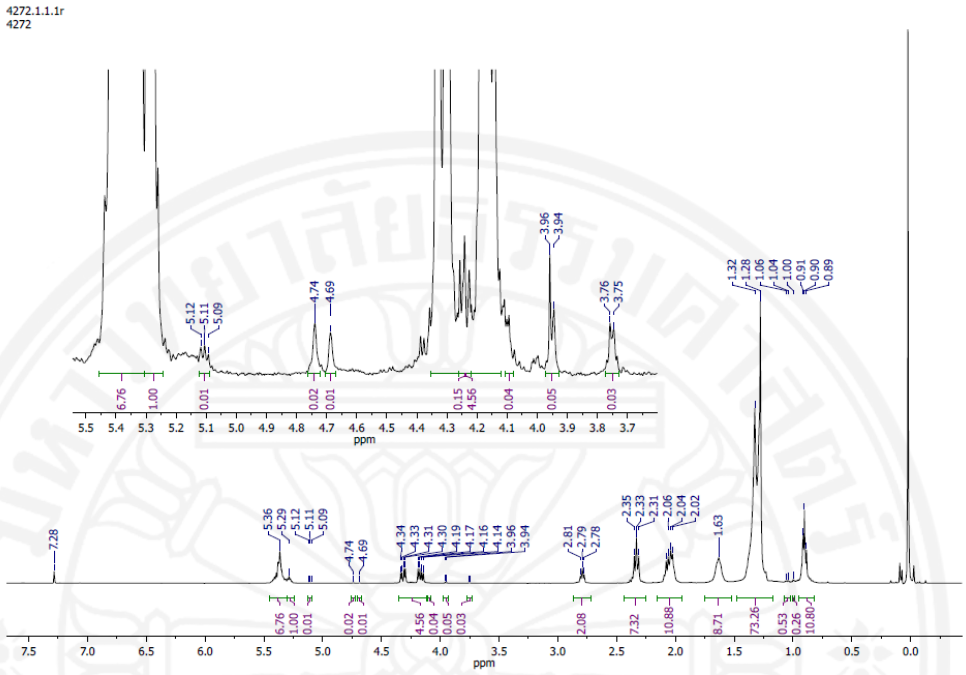


Figure 17A ^{1}H NMR spectrum of Y-RBO on November 2013.

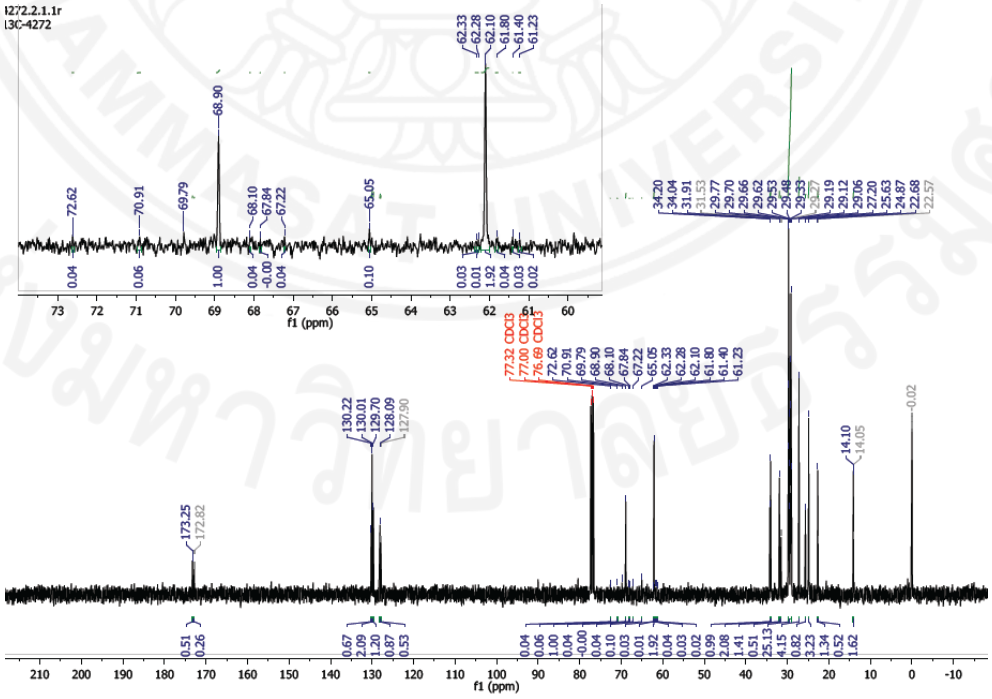


Figure 18A ^{13}C NMR spectrum of Y-RBO on November 2013.

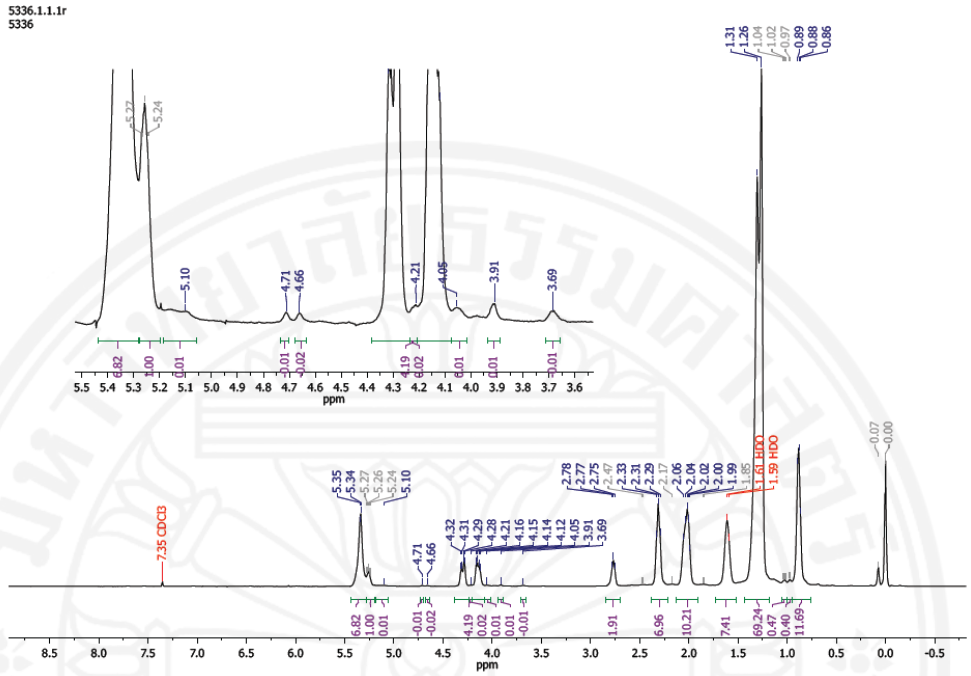


Figure 19A ^{1}H NMR spectrum of L-RBO on November 2014.

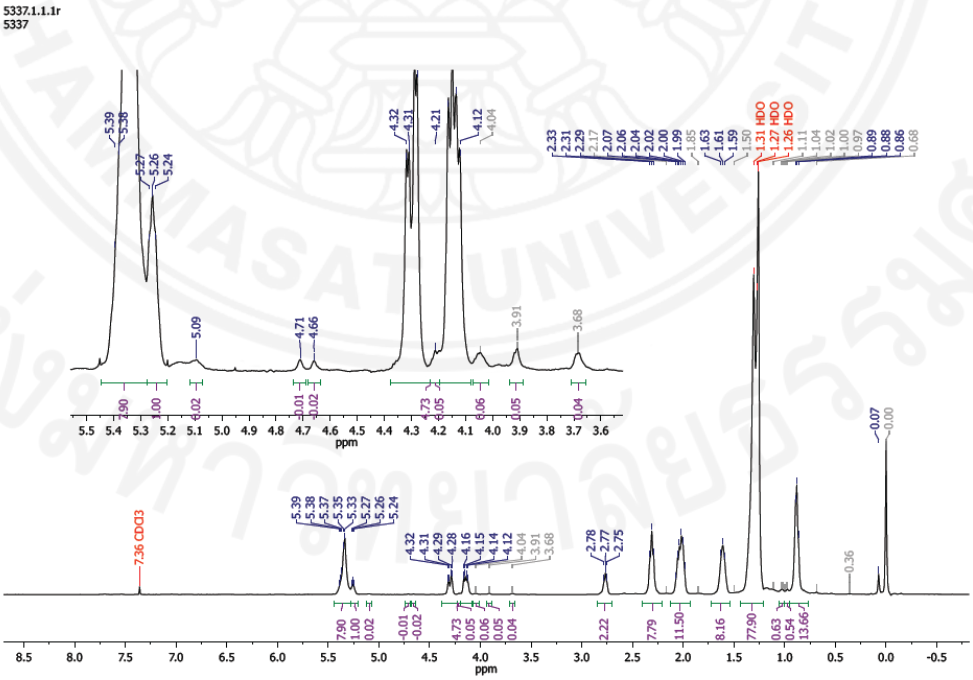


Figure 20A ^{1}H NMR spectrum of Y-RBO on November 2014.

APPENDIX B

Conversion of transesterification reaction

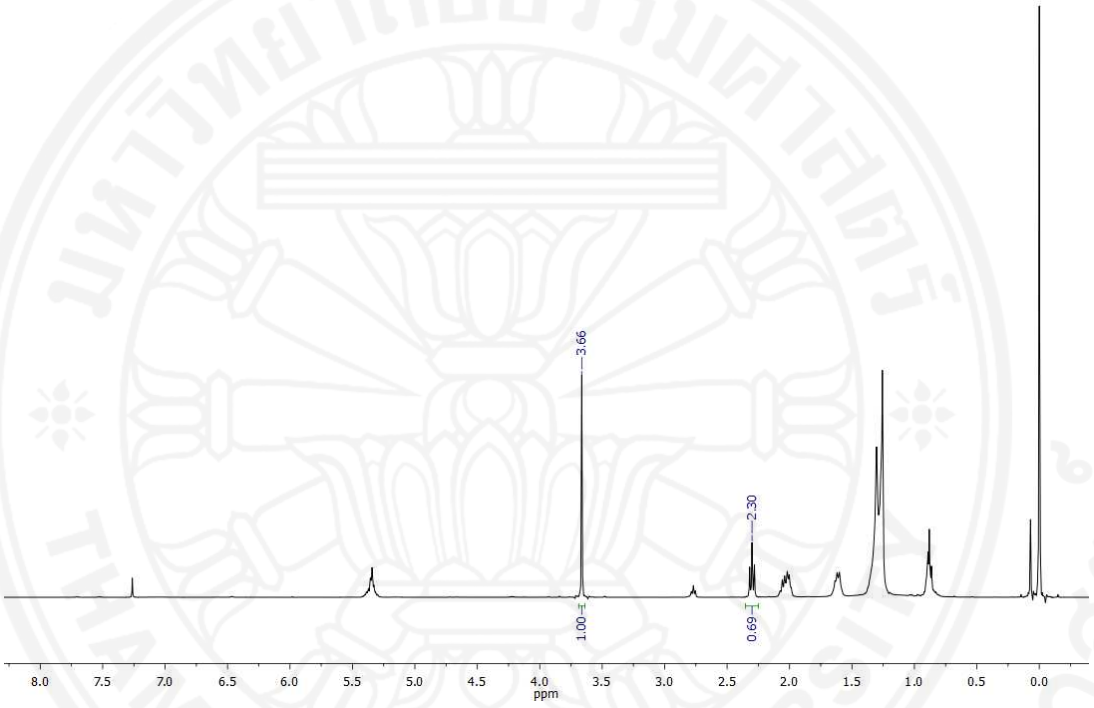


Figure 1B ^{1}H NMR spectrum of methyl ester of L-RBO

$$\%\text{conversion} = \frac{2\times \text{ integration area of methoxyl group}}{3\times \text{ integration area of } \alpha \text{ proton of carbonylgroup}} \times 100$$

$$= \frac{2\times 1.0}{3\times 0.69} \times 100$$

$$= 96.62\%$$

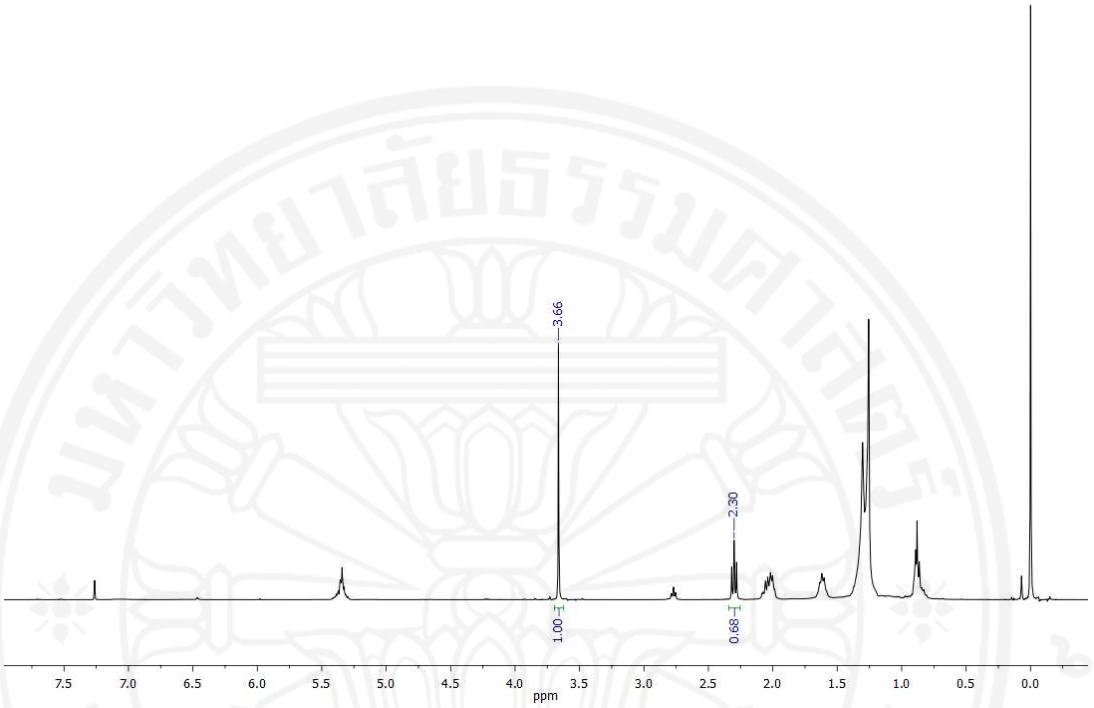


Figure 2B ^{1}H NMR spectrum of methyl ester of Y-RBO

$$\%\text{conversion} = \frac{2\times \text{ integration area of methoxyl group}}{3\times \text{ integration area of } \alpha \text{ proton of carbonylgroup}} \times 100$$

$$= \frac{2\times 1.0}{3\times 0.68} \times 100$$

$$= 98.04\%$$

APPENDIX C

Chromatogram of gas chromatography-mass spectroscopy

The oil samples was prepared to fatty acid methyl ester in triplicate and run all samples at the time.

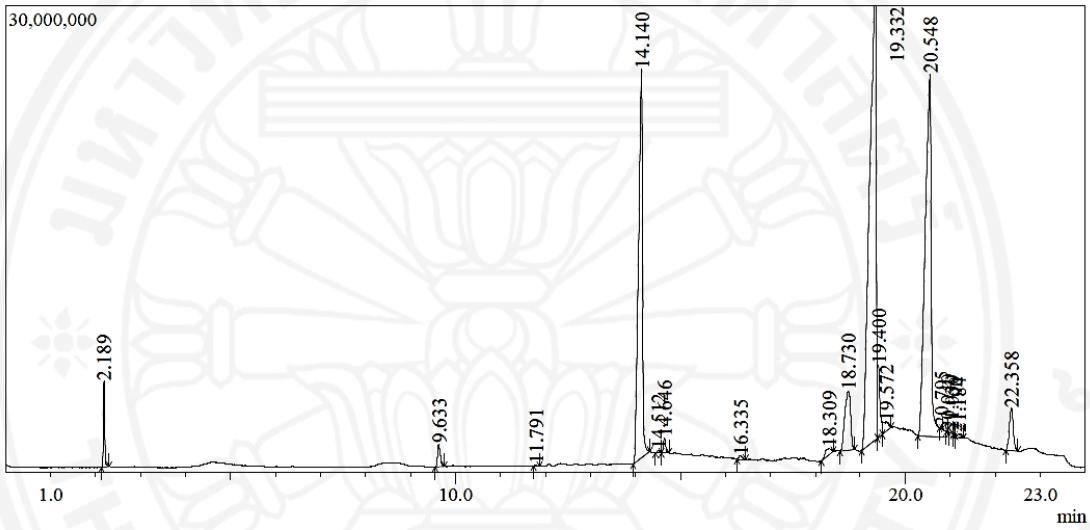


Figure 1C Chromatogram of L-RBO-4 (Feb-15) by GC-MS.

Peak report

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	2.189	2.140	2.300	11930129	1.54	5549017	5.59	2.15	Ethanol (CAS) Ethyl alcohol
2	9.633	9.550	9.755	5522206	0.71	1447725	1.46	3.81	Tetradecanoic acid, methyl ester (CAS) Methyl myristate
3	1.791	11.735	11.875	384254	0.05	104533	0.11	3.68	Pentadecanoic acid, methyl ester
4	4.140	13.955	14.320	154373589	19.93	24939296	5.14	5.19	Hexadecanoic acid, methyl ester (CAS) Methyl palmitate
5	4.512	14.450	14.585	723305	0.09	185645	0.19	3.90	9-Hexadecenoic acid, methyl ester, (Z)-
6	4.646	14.585	14.745	3626370	0.47	984868	0.99	3.68	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
7	6.335	16.265	16.450	1172444	0.15	211981	0.21	5.53	Heptadecanoic acid, methyl ester
8	8.309	18.135	18.400	4060523	0.52	413020	0.42	9.83	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
9	8.730	18.550	18.875	33310998	4.30	3830290	3.86	8.70	Octadecanoic acid, methyl ester (CAS) Methyl stearate
10	9.332	19.030	19.380	295438297	38.14	29697314	9.93	9.95	9-Octadecenoic acid, methyl ester, (E)-
11	9.400	19.380	19.485	16936075	2.19	4651990	4.69	3.64	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
12	9.572	19.485	19.685	5462755	0.71	617332	0.62	8.85	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
13	0.548	20.280	21.290	223002439	28.79	23293478	3.48	9.57	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS) Methy
14	0.795	20.760	20.820	169481	0.02	92192	0.09	1.84	10,13-Eicosadienoic acid, methyl ester (CAS) METHYL 10,
15	0.940	20.910	20.955	115667	0.01	76064	0.08	1.52	
16	1.030	20.960	21.060	329760	0.04	141466	0.14	2.33	10,13-Eicosadienoic acid, methyl ester (CAS) METHYL 10,
17	1.100	21.060	21.115	203363	0.03	75371	0.08	2.70	
18	1.184	21.115	21.290	973263	0.13	174833	0.18	5.57	9,15-Octadecadienoic acid, methyl ester (CAS) METHYL 9,
19	2.358	22.230	22.505	16894863	2.18	2725011	2.75	6.20	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-
				774629781	100.00	99211426	0.00		

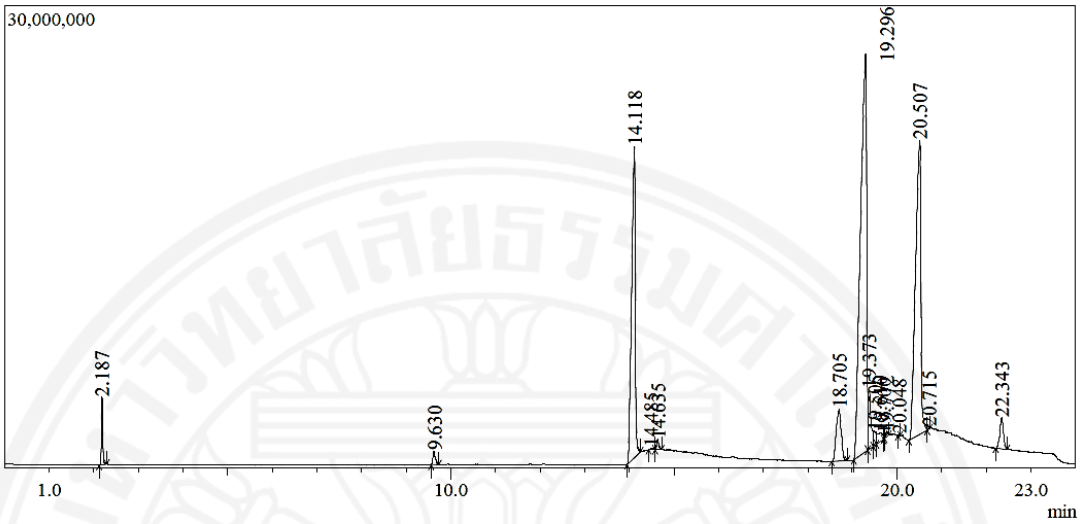


Figure 2C Chromatogram and peak report of Y-RBO-4 (Feb-15).by GC-MS.

Peak report

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	2.187	2.120	2.290	9407524	1.58	4377023	5.29	2.15	Ethanol (CAS) Ethyl alcohol
2	9.630	9.555	9.725	3226545	0.54	885209	1.07	3.64	Tetradecanoic acid, methyl ester (CAS) Methyl myristate
3	4.118	3.950	14.250	117535383	19.69	20186946	4.40	5.82	Hexadecanoic acid, methyl ester (CAS) Methyl palmitate
4	4.485	4.440	14.575	478992	0.08	111529	0.13	4.29	METHYL 15-ACETYLHYDROXYPALMITATE
5	4.635	4.575	14.720	2492518	0.42	652353	0.79	3.82	9-Hexadecenoic acid, methyl ester, (Z)- (CAS) Methyl palmi
6	8.705	8.535	18.880	25811349	4.32	3345517	4.04	7.72	Octadecanoic acid, methyl ester (CAS) Methyl stearate
7	9.296	9.025	19.345	238767797	40.01	25816273	1.20	9.25	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
8	9.373	9.345	19.460	17173981	2.88	3906312	4.72	4.40	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
9	9.505	9.460	19.540	4206284	0.70	900418	1.09	4.67	10-Undecenoic acid, methyl ester (CAS) Methyl 10-undecen
10	9.610	9.540	19.690	5031273	0.84	627702	0.76	8.02	Dodecanedioic acid (CAS) 1,12-Dodecanedioic acid
11	9.700	9.690	19.720	528342	0.09	332398	0.40	1.59	
12	9.772	9.720	19.820	1251568	0.21	308609	0.37	4.06	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
13	0.048	20.020	20.070	162121	0.03	89614	0.11	1.81	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
14	0.507	20.265	20.670	158412112	26.54	19075907	3.05	8.30	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS) Methy
15	0.715	20.670	20.730	515901	0.09	116821	0.14	4.42	5-Heptadecene, 1-bromo- (CAS) 1-BROMOHEPTADEC-5-
16	2.343	22.210	22.470	11806679	1.98	2011195	2.43	5.87	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-
				596808369	100.00	82743826	0.00		

Table 3C Fatty acid composition from GC-MS technique.

FFA	Saturated (%)			Unsaturated (%)		
	C14:0	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
LRBO-4-1, Feb-15	0.74	20.71	4.47	41.91	29.91	2.26
LRBO-4-2, Feb-15	0.74	20.98	4.53	42.57	28.89	2.27
LRBO-4-3, Feb-15	0.78	20.93	4.79	42.17	29.12	2.21
mean	**0.75**	**20.87**	**4.6**	**42.22**	**29.31**	**2.25**
SD	0.02	0.14	0.17	0.33	0.53	0.03
YRBO-4-1, Feb-15	0.55	20.19	4.43	45.57	27.22	2.03
YRBO-4-2, Feb-15	0.66	20.46	4.5	44.41	27.9	2.06
YRBO-4-3, Feb-15	0.75	21.06	4.47	42.48	28.95	2.29
mean	**0.65**	**20.57**	**4.47**	**44.15**	**28.02**	**2.13**
SD	0.1	0.44	0.03	1.56	0.87	0.14

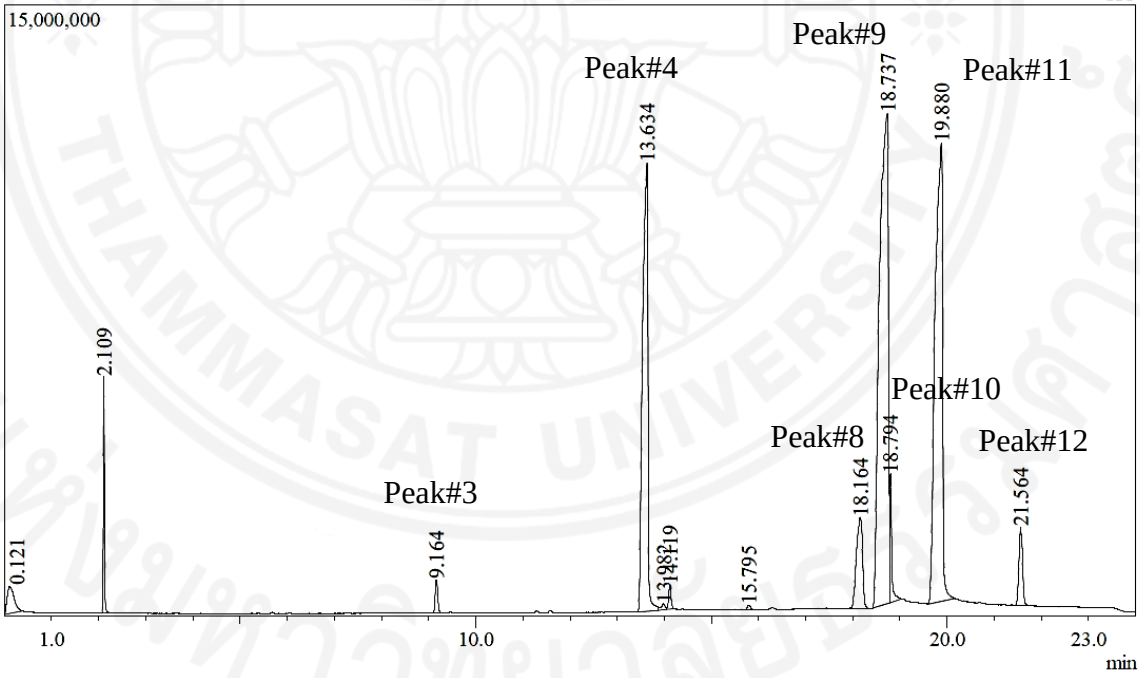
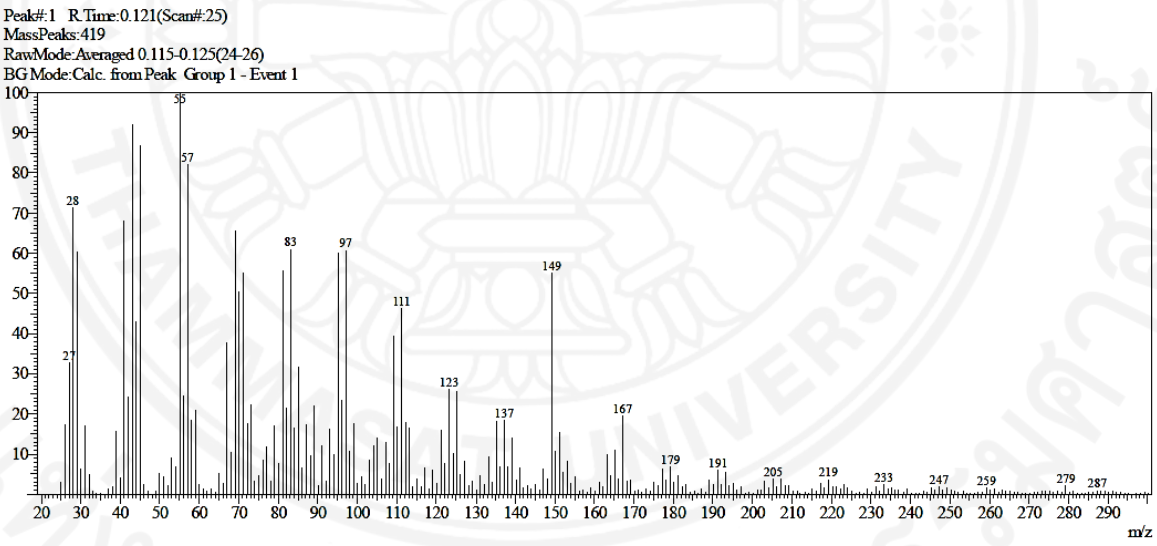


Figure 3C Chromatogram of L-RBO-4 (Sep-15) by GC-MS.

Peak report of L-RBO-4 (Sep-15)

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	0.121	0.010	0.380	6799109	1.60	664346	1.33	10.23	Oxirane, tetradecyl-
2	2.109	2.065	2.215	9452097	2.22	792419	1.64	1.63	Acetic acid, ethyl ester (CAS) Acetic acid ethyl ester (CAS)
3	9.164	9.085	9.280	2892556	0.68	824657	1.66	3.51	Tetradecanoic acid, methyl ester (CAS) Methyl myristate
4	3.634	3.415	3.865	4215195	9.79	011204	2.12	7.65	Hexadecanoic acid, methyl ester (CAS) Methyl palmitate
5	3.982	3.865	4.050	969209	0.23	151789	0.30	6.39	9-Hexadecenoic acid, methyl ester, (Z)- (CAS) Methyl palmi
6	4.119	4.050	4.225	2277893	0.54	601213	1.21	3.79	9-Hexadecenoic acid, methyl ester, (Z)-
7	5.795	5.725	5.880	428815	0.10	103858	0.21	4.13	Heptadecanoic acid, methyl ester
8	8.164	7.960	8.330	9487938	4.58	233996	4.49	8.72	Octadecanoic acid, methyl ester
9	8.737	8.410	8.775	5832996	6.62	042562	4.19	12.94	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
10	8.794	8.775	9.030	9539249	2.24	162864	6.35	3.02	11-Octadecenoic acid, methyl ester
11	9.880	9.595	0.165	3650503	9.06	259874	2.62	10.98	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS) Methy
12	1.564	1.445	1.705	9949092	2.34	927840	3.87	5.16	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-
				5494652	0.00	776622	100.00		

Spectrum of L-RBO-4 (Sep-15)



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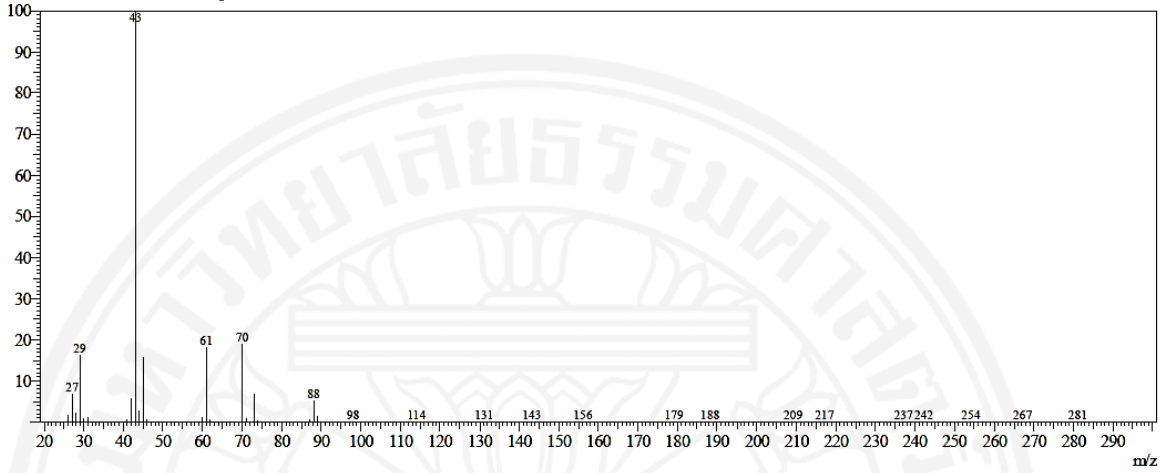
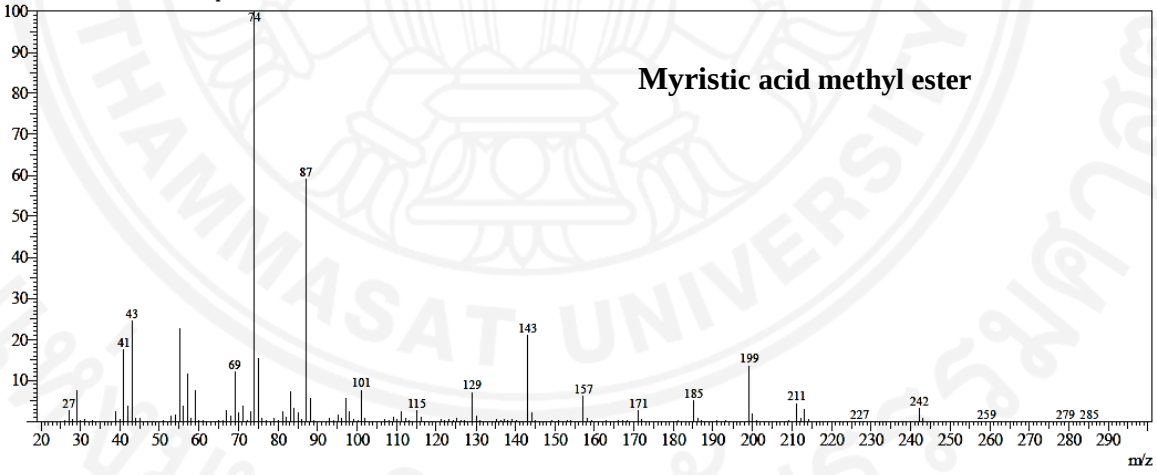
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:3 R.Time:9.164(Scan#:1834)
MassPeaks:361
RawMode:Averaged 9.160-9.170(1833-1835)
BG Mode:Calc. from Peak Group 1 - Event 1

Myristic acid methyl ester



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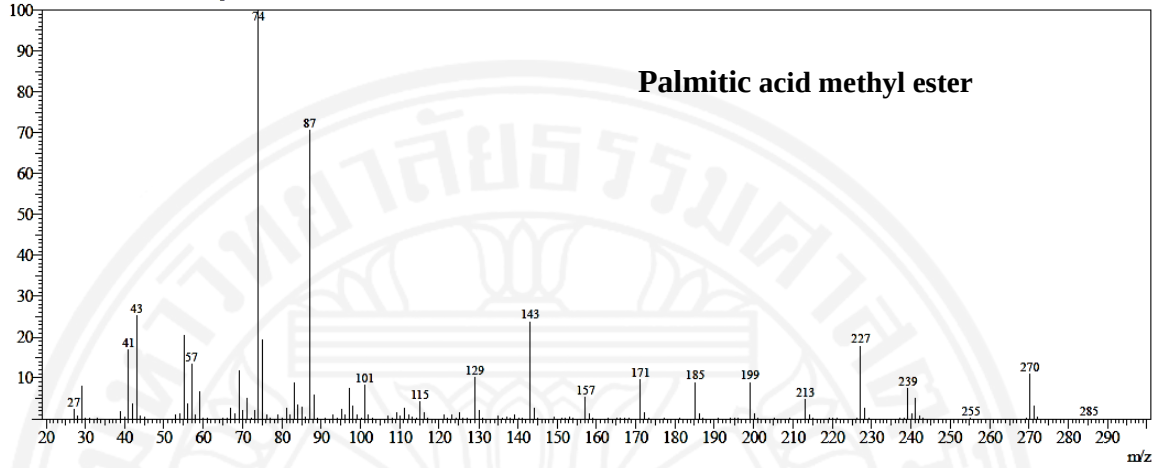
MassPeaks:312
RawMode:Averaged 13.630-13.640(2727-2729)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:5 R.Time:13.982(Scan#:2797)
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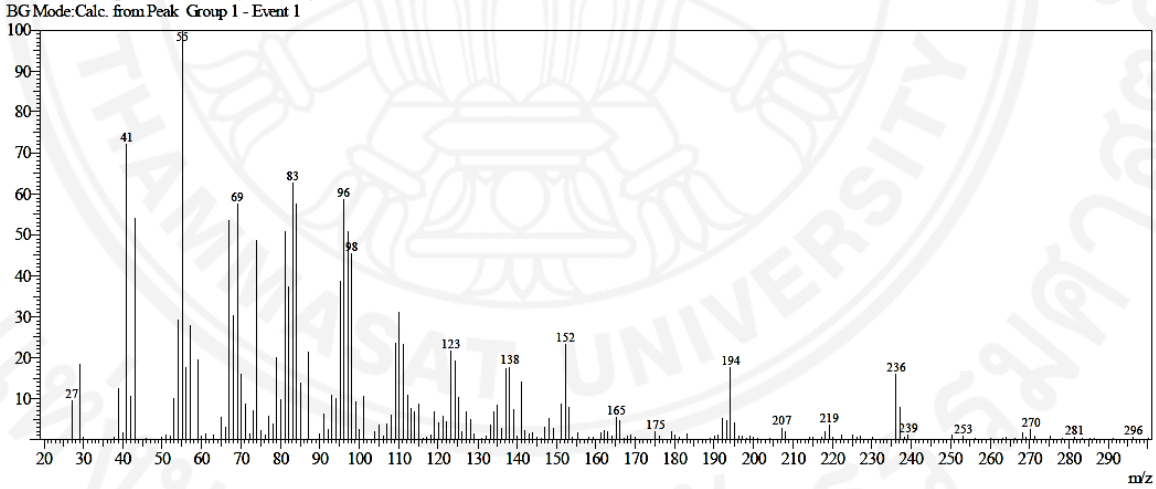
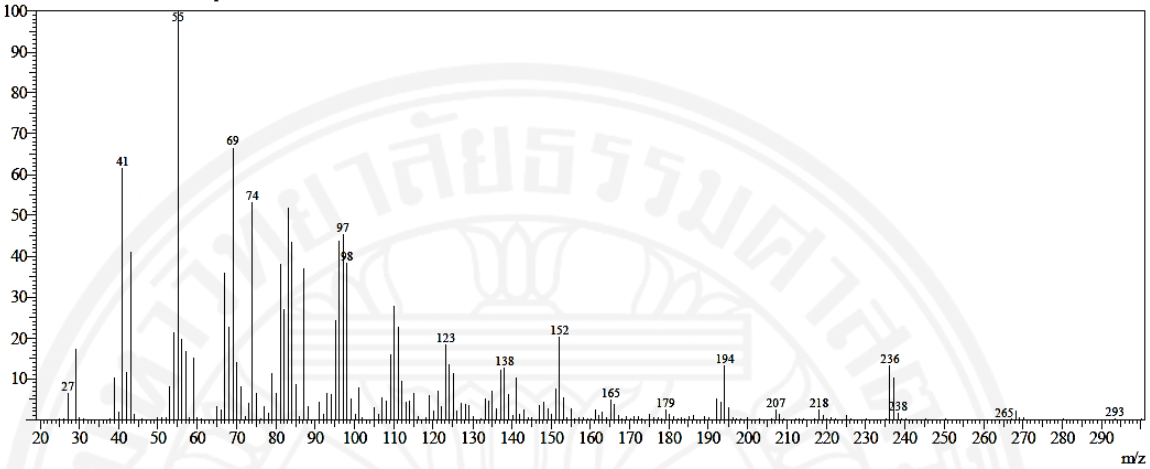
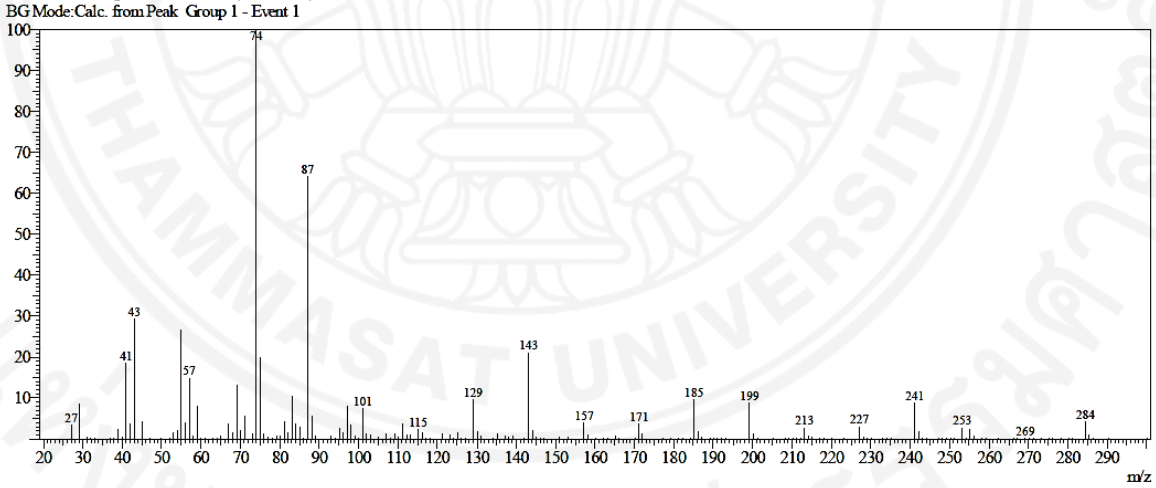
BG Mode:Calc. from Peak Group 1 - Event 1



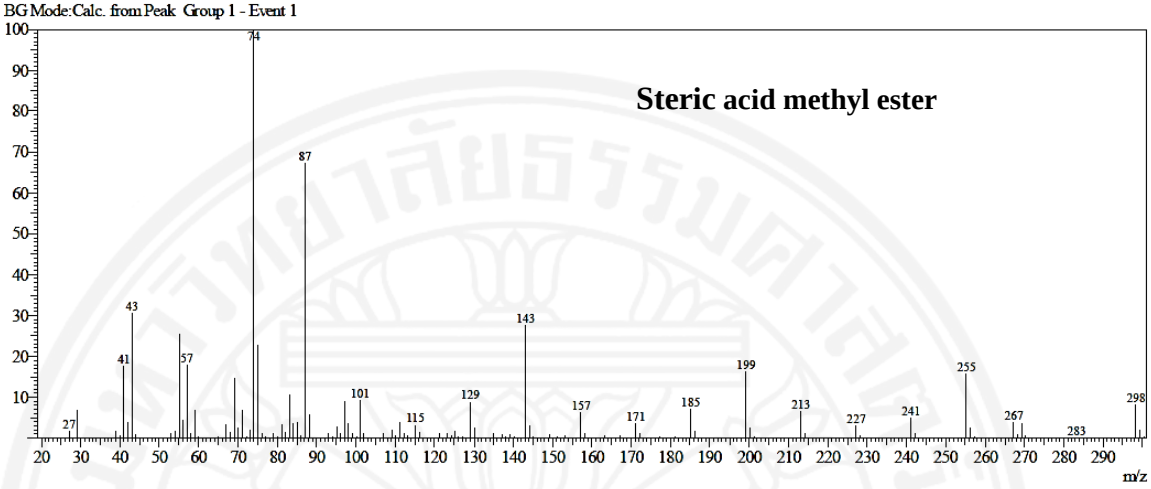
Peak#:6 R.Time:14.119(Scan#:2825)
MassPeaks:322
RawMode:Averaged 14.115-14.125(2824-2826)
BG Mode:Calc. from Peak Group 1 - Event 1



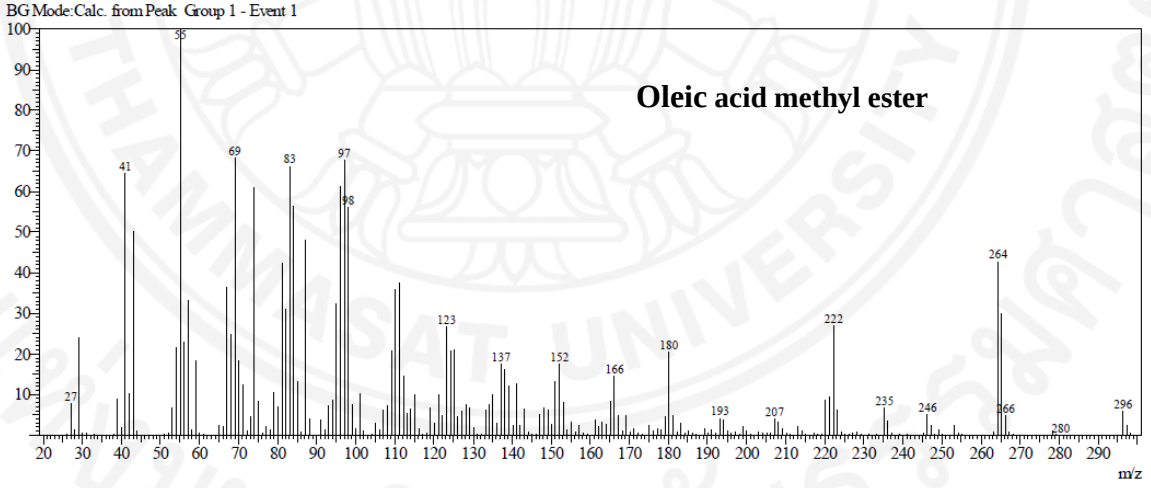
Peak#:7 R.Time:15.795(Scan#:3160)
MassPeaks:333
RawMode:Averaged 15.790-15.800(3159-3161)
BG Mode:Calc. from Peak Group 1 - Event 1



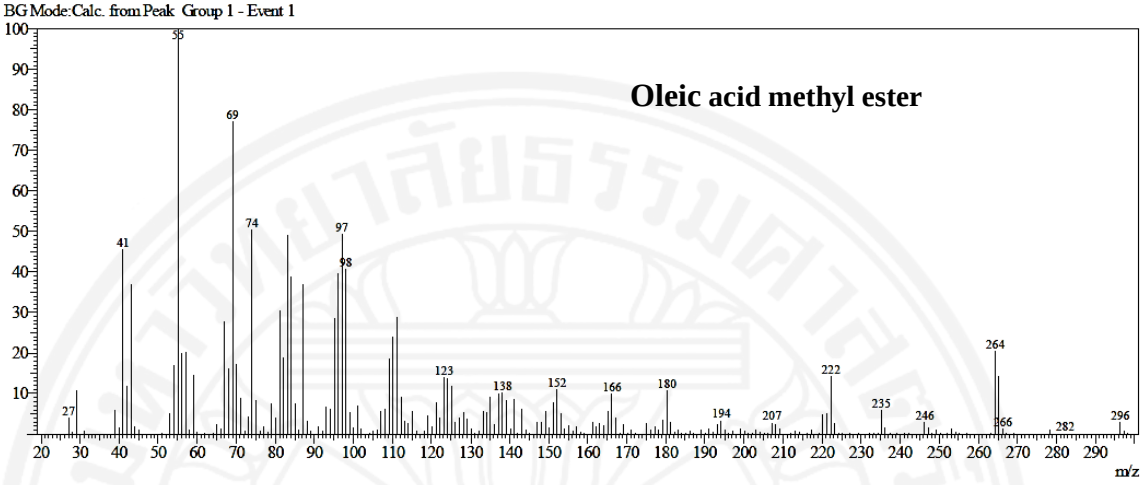
Peak#:8 R.Time:18.164(Scan#:3634)
MassPeaks:362
RawMode:Averaged 18.160-18.170(3633-3635)
BG Mode:Calc. from Peak Group 1 - Event 1



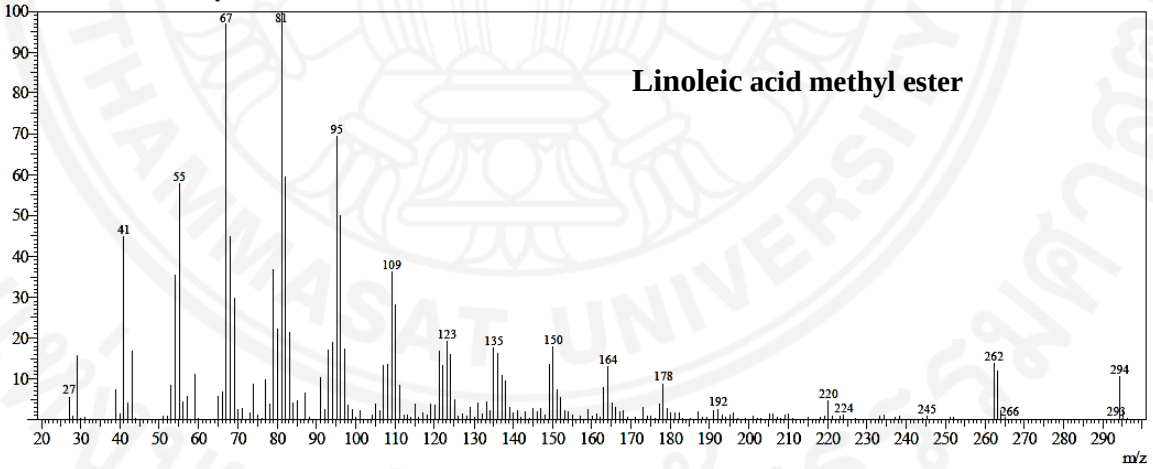
Peak#:9 R.Time:18.737(Scan#:3748)
MassPeaks:356
RawMode:Averaged 18.730-18.740(3747-3749)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:10 R.Time:18.794(Scan#:3760)
MassPeaks:326
RawMode:Averaged 18.790-18.800(3759-3761)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:11 R.Time:19.880(Scan#:3977)
MassPeaks:349
RawMode:Averaged 19.875-19.885(3976-3978)
BG Mode:Calc. from Peak Group 1 - Event 1



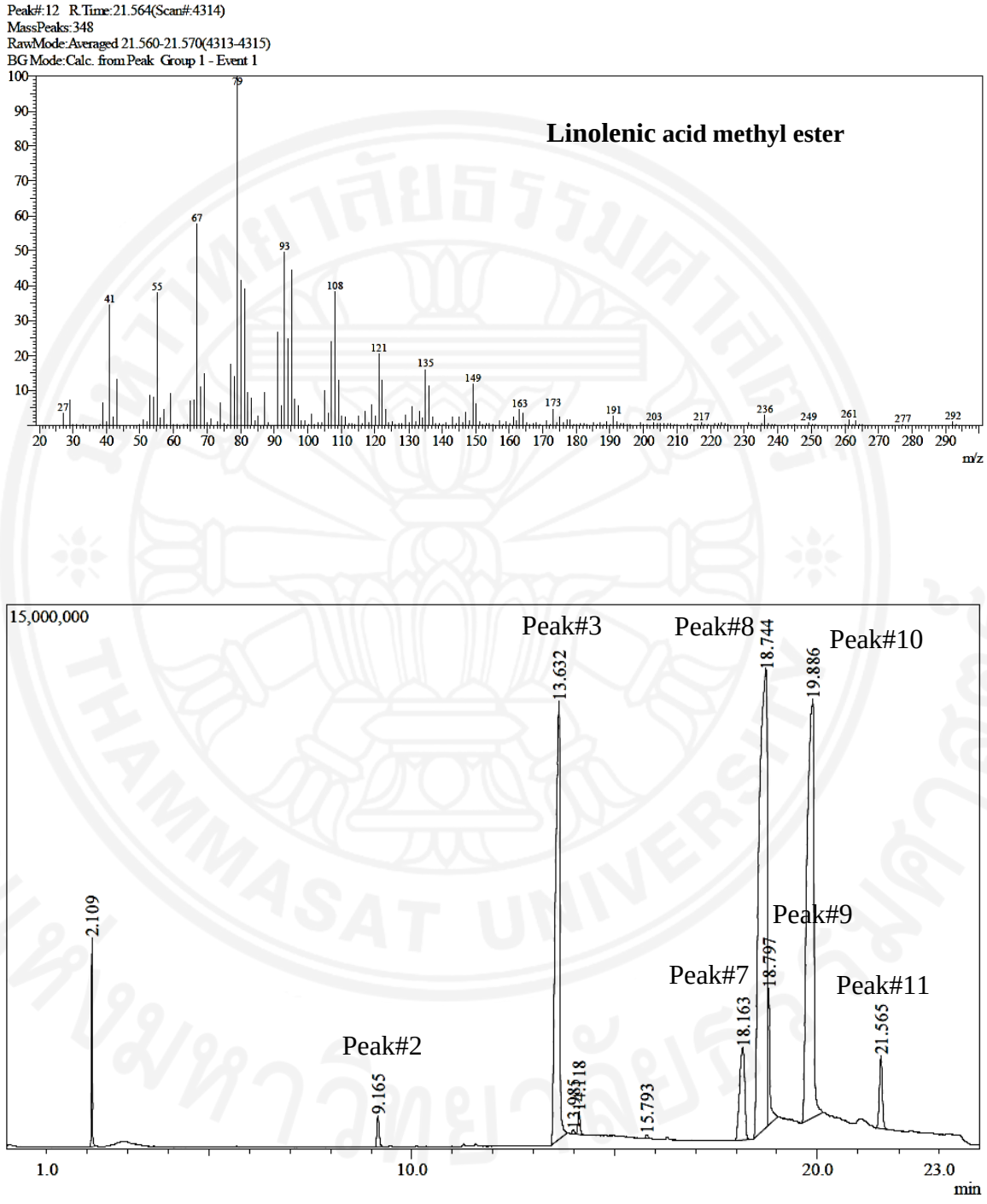
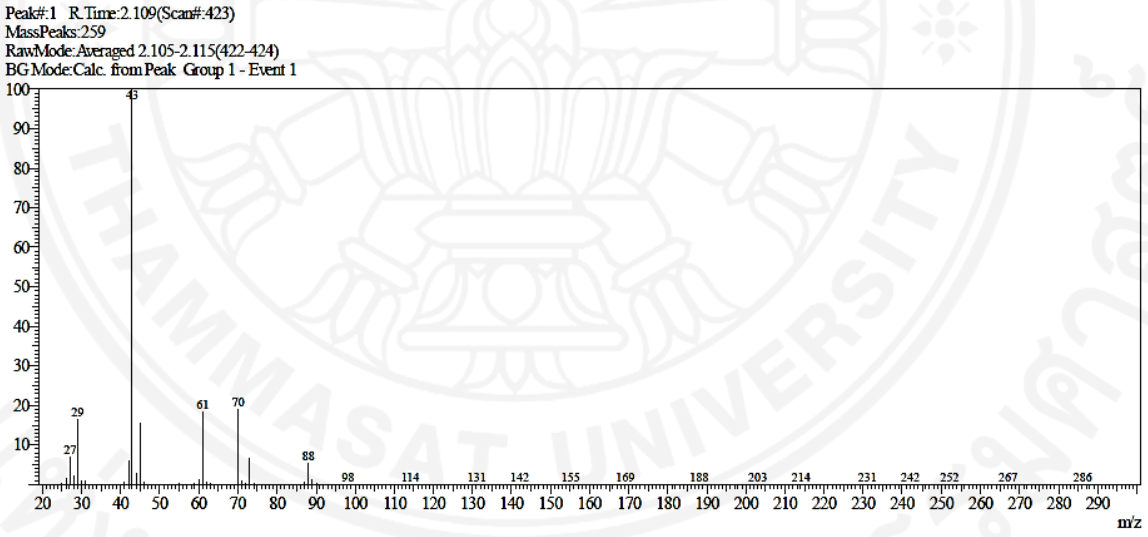


Figure 4C Chromatogram of Y-RBO-4 (Sep-15) by GC-MS.

Peak report of Y-RBO-4 (Sep-15)

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	2.109	2.070	2.215	9310698	2.06	5745991	11.07	1.62	Acetic acid, ethyl ester (CAS) Acetic acid ethyl ester (CAS)
2	9.165	9.085	9.275	2922943	0.65	854278	1.65	3.42	Tetradecanoic acid, methyl ester (CAS) Methyl myristate
3	3.632	3.430	13.840	0969737	0.10	12041588	23.21	7.55	Hexadecanoic acid, methyl ester (CAS) Methyl palmitate
4	3.985	3.915	14.050	415437	0.09	110435	0.21	3.76	9-Hexadecenoic acid, methyl ester, (Z)- (CAS) Methyl palmi
5	4.118	4.050	14.245	2248450	0.50	634453	1.22	3.54	9-Hexadecenoic acid, methyl ester, (Z)-
6	5.793	5.725	15.895	464610	0.10	108303	0.21	4.29	Heptadecanoic acid, methyl ester
7	8.163	7.960	18.335	2368158	4.94	2544207	4.90	3.79	Octadecanoic acid, methyl ester
8	8.744	8.420	18.780	9831128	7.53	12569168	24.22	3.51	9-Octadecenoic acid (Z)-, methyl ester (CAS) Methyl oleate
9	8.797	8.780	19.025	1576985	2.56	3759090	7.25	3.08	11-Octadecenoic acid, methyl ester
0	9.886	9.605	20.135	2178137	9.21	11515982	22.20	1.48	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS) Methy
1	1.565	1.440	21.690	0280513	2.27	2001662	3.86	5.14	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-
				2566796	0.00	51885157	100.00		

Spectrum of Y-RBO-4 (Sep-15)



Peak#:2 R.Time:9.165(Scan#:1834)
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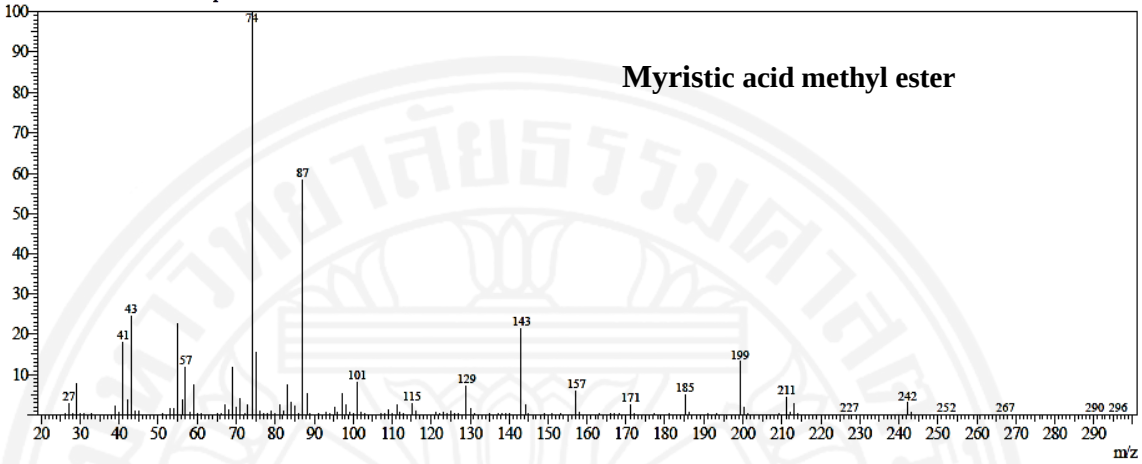
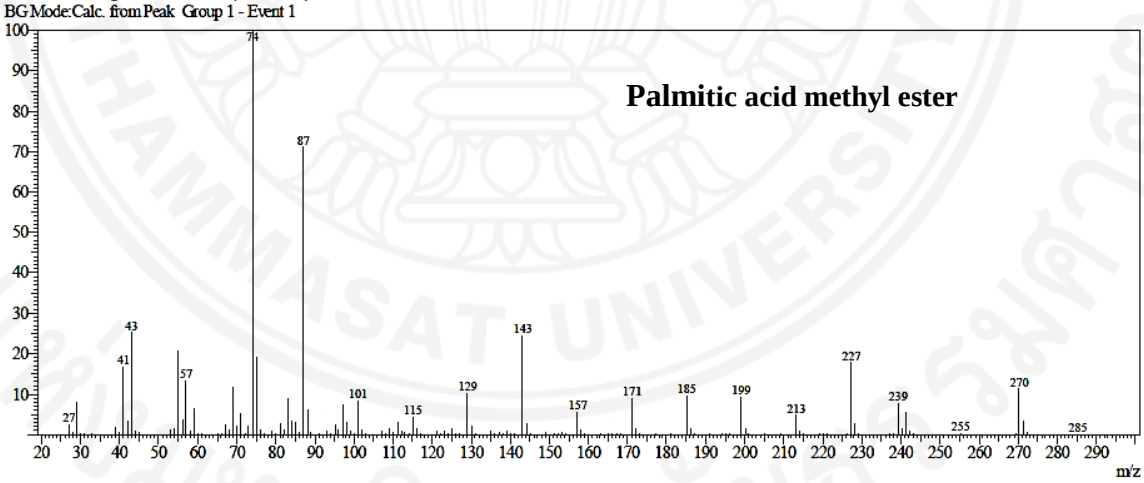
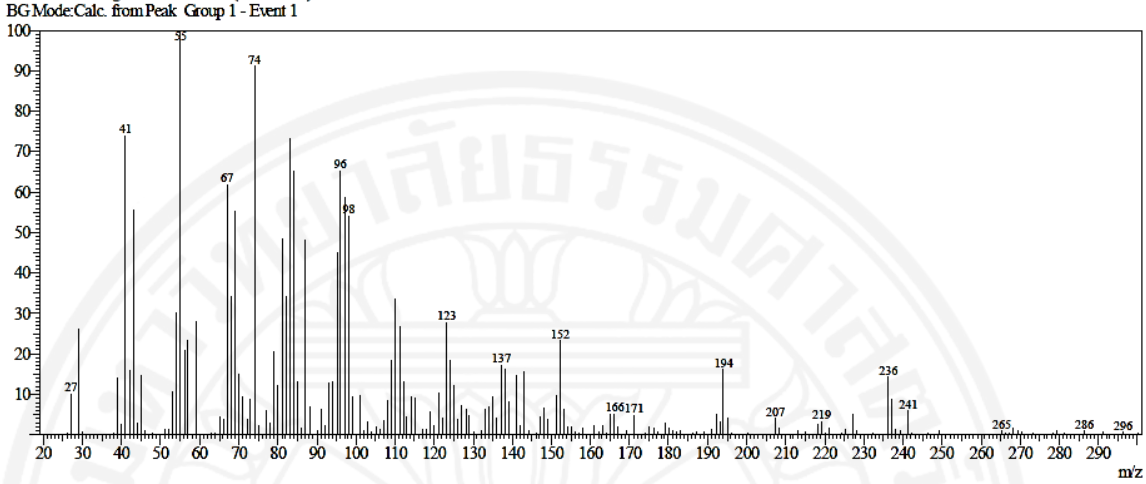
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:3 R.Time:13.632(Scan#:2727)
MassPeaks:310
RawMode:Averaged 13.625-13.635(2726-2728)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:4 R.Time:13.985(Scan#:2798)
MassPeaks:282
RawMode:Averaged 13.980-13.990(2797-2799)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:5 R.Time:14.118(Scan#:2825)
MassPeaks:361
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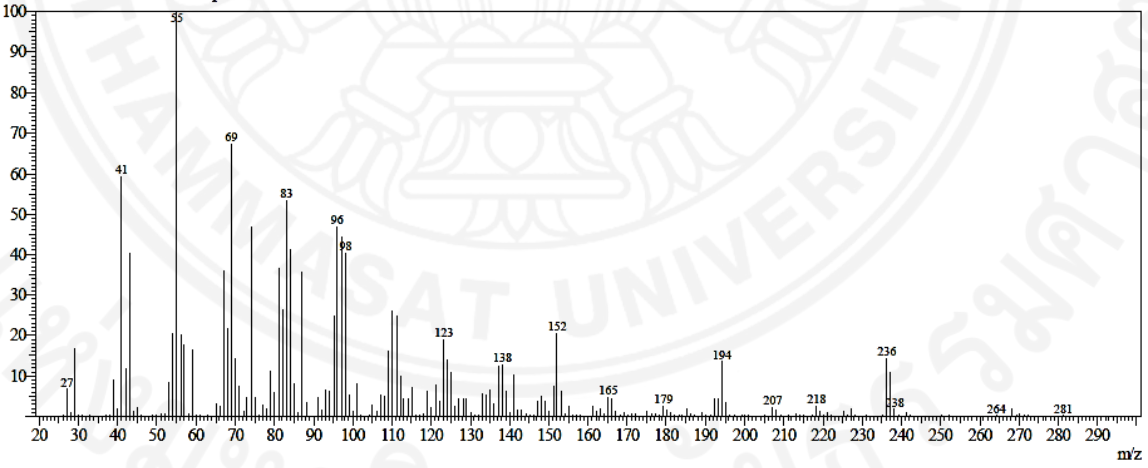
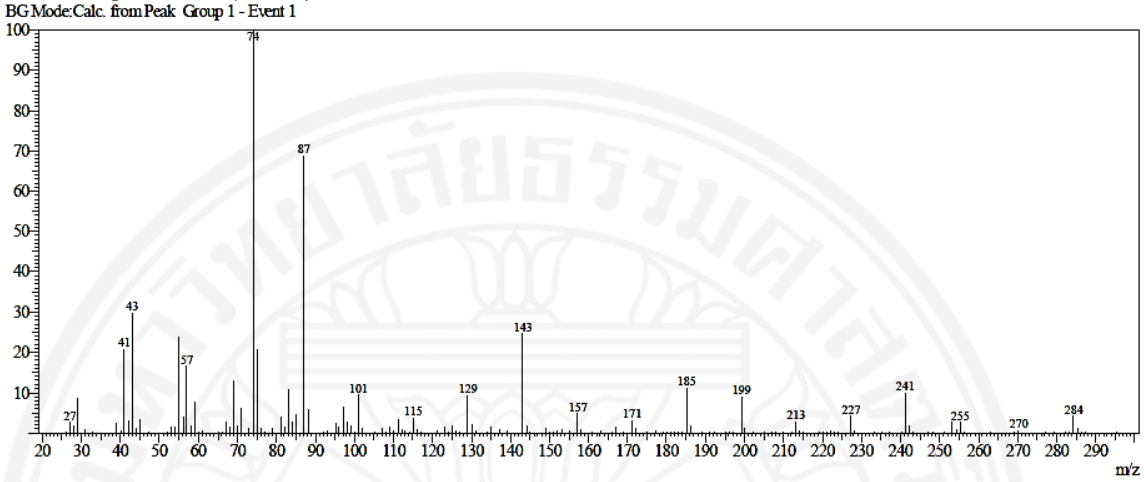
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:6 R.Time:15.793(Scan#:3160)
MassPeaks:280
RawMode:Averaged 15.790-15.800(3159-3161)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:7 R.Time:18.163(Scan#:3634)
MassPeaks:370
RawMode:Averaged 18.160-18.170(3633-3635)
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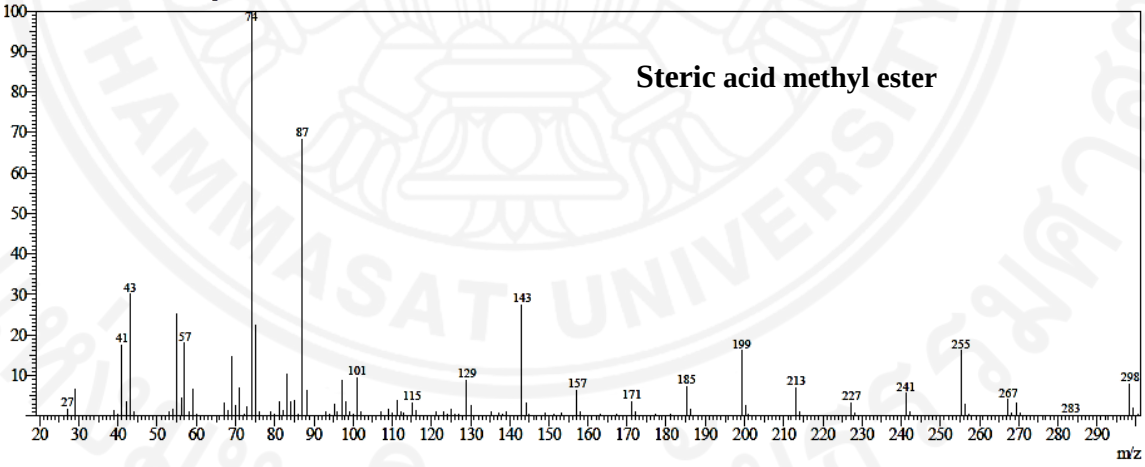

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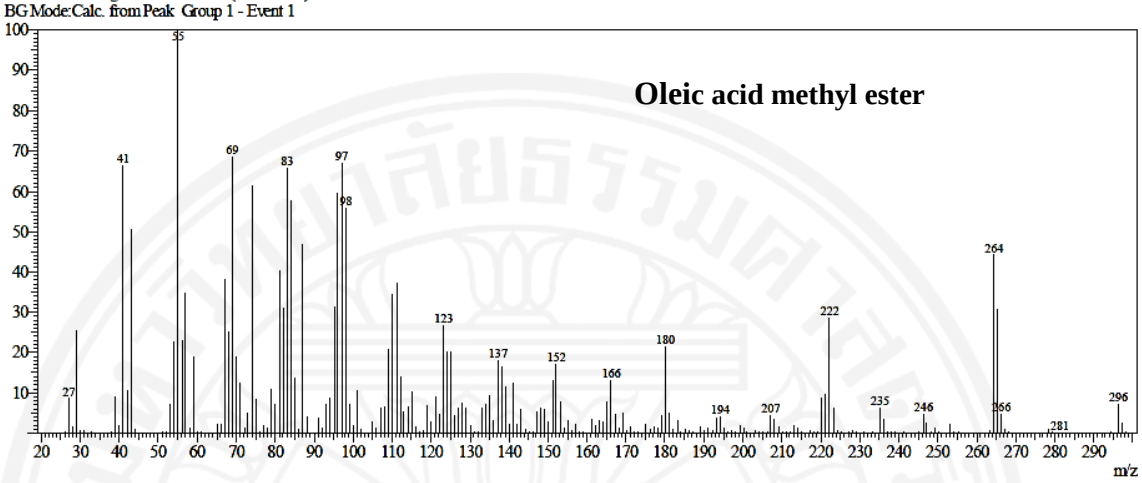
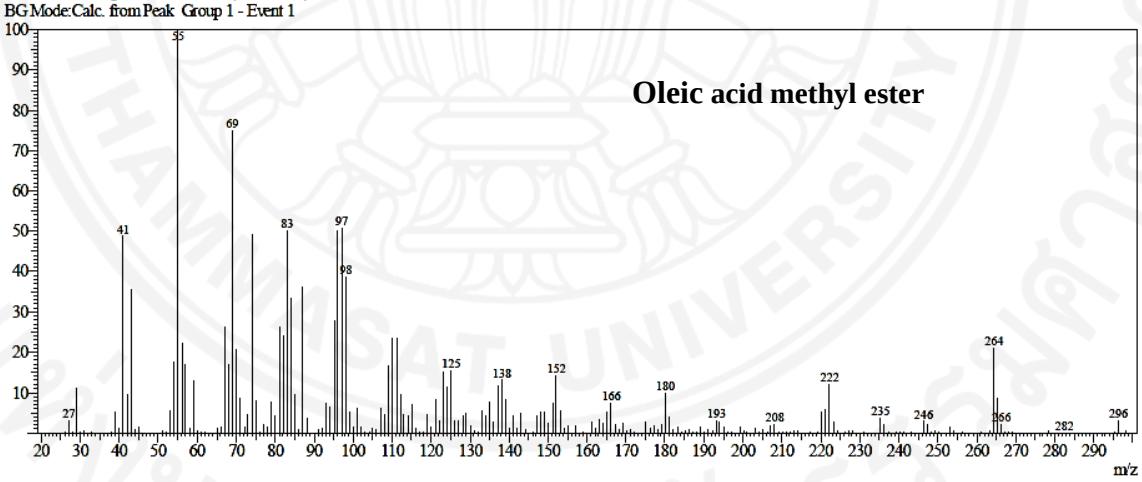
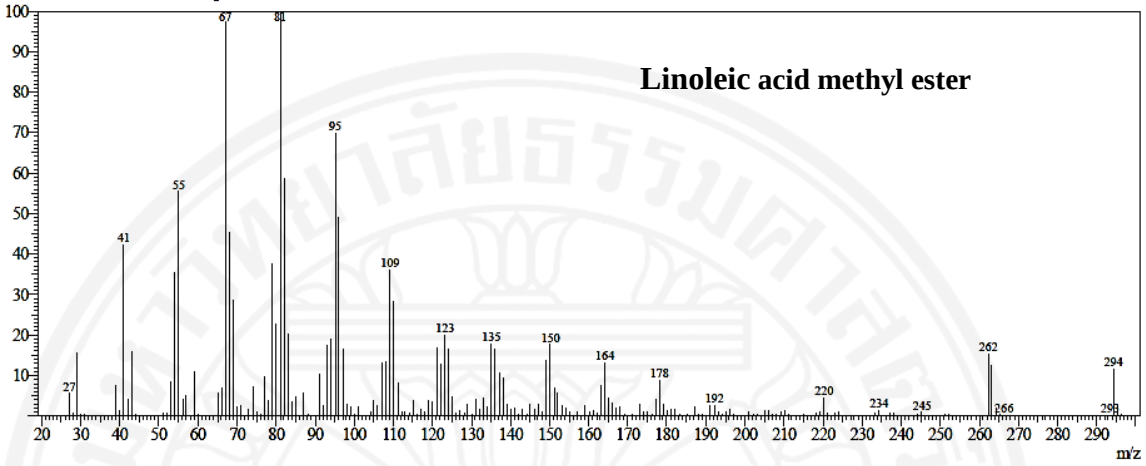
BG Mode:Calc. from Peak Group 1 - Event 1



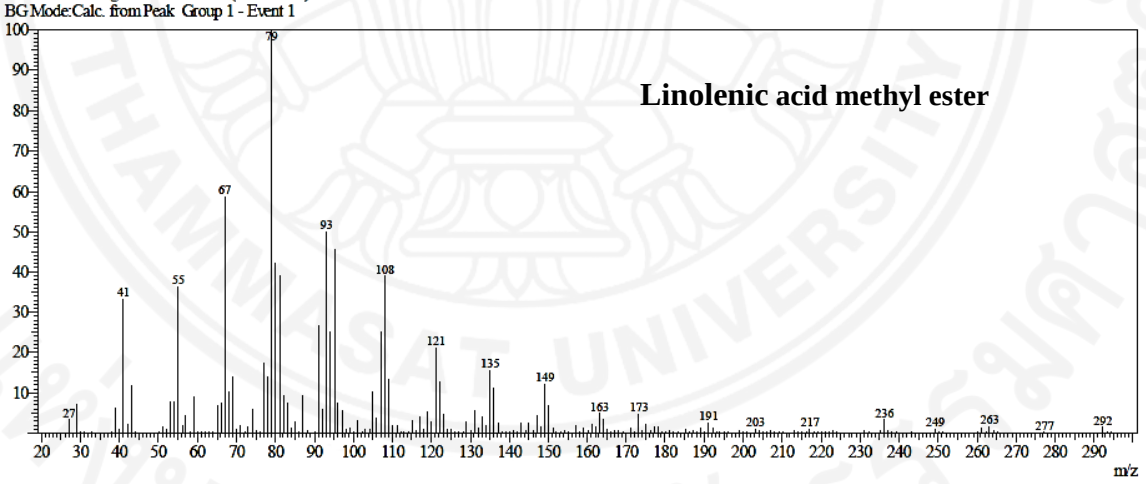
Peak#:9 R.Time:18.797(Scan#:3760)
MassPeaks:334
RawMode:Averaged 18.790-18.800(3759-3761)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:10 R.Time:19.886(Scan#:3978)
MassPeaks:332
RawMode:Averaged 19.880-19.890(3977-3979)
BG Mode:Calc. from Peak Group 1 - Event 1



Peak#:11 R.Time:21.565(Scan#:4314)
MassPeaks:351
RawMode:Averaged 21.560-21.570(4313-4315)
BG Mode:Calc. from Peak Group 1 - Event 1



APPENDIX D

Fatty acid composition

Table 1D Fatty acid composition of L-RBO over one-year period by ^{1}H NMR technique.

	Oct-13	Nov-13	Jan-14	Apr-14	May-14	Jun-14	Jul-14	Aug-14	Sep-14	Oct-14	Nov-14
Method I											
%S	24.44±0.69	23.79±0.09	26.56±0.07	24.35±1.44	29.88±0.47	26.90±0.32	28.90±0.41	25.90±0.11	24.29±0.02	24.73±0.78	26.67±0.07
%O	49.94±0.48	48.67±0.18	47.60±0.11	39.23±0.52	47.13±0.10	48.88±0.15	47.45±0.12	49.86±0.51	50.84±0.70	55.29±0.94	49.03±0.17
%L	22.70±0.28	25.07±0.56	23.58±0.13	32.49±0.92	20.10±0.75	20.99±0.52	20.37±0.25	20.49±0.90	21.60±1.28	14.42±0.47	21.08±0.23
%Ln	2.91±0.05	2.46±0.29	2.26±0.06	3.91±0.15	2.88±0.36	3.23±0.18	3.35±0.07	3.74±0.39	3.27±0.56	5.57±0.49	3.23±0.11
Method II											
%S	26.80±0.10	39.24±0.87	22.81±0.11	36.03±1.63	39.24±0.87	20.42±0.23	14.65±0.47	40.24±0.41	34.39±0.94	33.82±0.68	42.35±0.17
%O	47.66±0.30	33.60±0.81	51.27±0.07	27.92±1.14	37.64±1.22	55.16±0.45	61.22±0.69	36.07±0.30	41.07±0.34	46.70±0.61	33.85±0.17
%L	22.56±0.25	24.31±0.66	23.75±0.13	31.57±0.85	20.37±0.68	21.41±0.48	21.25±0.27	19.42±1.03	20.94±1.44	13.40±0.56	20.07±0.26
%Ln	2.98±0.05	2.84±0.34	2.17±0.06	4.47±0.25	2.75±0.32	3.02±0.15	2.87±0.07	4.28±0.46	3.61±0.64	6.08±0.52	3.74±0.13
Method III											
%S	18.55±0.55	19.64±0.30	23.66±0.50	20.71±0.14	21.31±0.19	21.07±0.04	22.52±0.23	17.26±0.17	16.95±0.39	16.80±1.70	19.11±0.29
%O	35.33±0.32	32.91±0.24	32.54±0.26	15.88±0.75	35.13±0.29	34.49±0.21	33.31±0.28	35.88±0.33	29.50±0.53	40.43±2.26	35.33±0.31
%L	30.75±0.16	31.63±0.04	29.20±0.16	42.27±0.50	29.03±0.12	29.63±0.12	29.39±0.07	31.24±0.18	30.88±0.16	28.25±0.38	30.38±0.08
%Ln	15.37±0.08	15.82±0.02	14.60±0.08	21.14±0.25	14.52±0.06	14.81±0.06	14.69±0.04	15.62±0.09	15.44±0.08	14.13±0.19	15.19±0.04
Method IV											
%S	24.44±0.69	23.79±0.09	26.56±0.07	24.35±1.44	29.88±0.47	26.90±0.32	28.90±0.41	25.90±0.11	24.29±0.02	24.73±0.78	26.67±0.07
%O	32.77±0.38	31.21±0.13	31.30±0.07	15.14±0.51	31.31±0.41	31.94±0.20	30.60±0.05	32.14±0.30	33.57±0.15	36.93±1.68	32.03±0.17
%L	28.52±0.22	30.00±0.07	28.09±0.02	40.34±1.19	25.87±0.05	27.44±0.19	27.15±0.29	27.97±0.15	28.15±0.19	25.56±0.60	27.54±0.14
%Ln	14.26±0.11	15.00±0.03	14.05±0.01	20.17±0.59	12.94±0.02	13.72±0.09	13.50±0.14	13.99±0.08	14.08±0.09	12.78±0.30	13.77±0.07

Table 2D Fatty acid composition of Y-RBO over one-year period by ^{1}H NMR technique.

	Oct-13	Nov-13	Apr-14	May-14	Jun-14	Jul-14	Aug-14	Sep-14	Oct-14	Nov-14
Method I										
%S	24.99±0.10	25.38±0.27	22.37±2.41	28.00±0.14	26.50±0.24	25.59±0.15	26.63±0.13	25.02±0.02	28.05±0.18	26.11±0.16
%O	48.08±0.60	48.44±0.17	36.40±0.80	47.71±2.15	48.20±0.15	48.77±±0.20	50.46±0.15	50.07±0.06	49.25±0.28	48.26±0.71
%L	24.36±1.05	23.82±0.09	38.73±1.70	19.40±1.38	22.30±0.43	22.34±0.07	18.74±0.18	21.10±0.13	18.87±0.42	22.68±1.44
%Ln	2.58±0.47	2.37±0.04	2.50±0.09	3.57±0.68	3.00±0.15	3.30±0.02	4.18±0.17	3.81±0.07	3.82±0.35	2.94±0.70
Method II										
%S	30.28±12.75	26.39±0.10	28.09±1.40	26.28±0.17	23.16±0.43	38.94±0.74	25.97±1.09	39.21±3.55	41.59±1.37	45.98±1.58
%O	43.78±13.80	47.45±0.09	30.82±2.71	50.68±0.87	51.44±0.24	35.86±0.78	51.09±1.01	36.41±3.48	36.24±1.30	28.98±0.88
%L	23.26±1.18	23.77±0.08	38.44±1.54	19.53±1.34	22.50±0.43	21.46±0.04	18.79±0.26	20.02±0.14	17.83±0.62	21.50±1.81
%Ln	2.68±0.27	2.39±0.04	2.65±0.17	3.51±0.66	2.90±0.15	3.74±0.01	4.16±0.20	4.35±0.07	4.34±0.46	3.53±0.88
Method III										
%S	19.45±0.40	20.32±0.44	19.90±0.20	19.31±0.07	19.86±0.20	18.70±0.19	19.48±0.07	17.35±0.08	19.55±0.93	19.10±0.14
%O	33.01±0.40	33.94±0.04	12.42±1.02	36.08±0.32	33.85±0.24	33.87±0.23	35.91±0.35	35.16±0.06	35.98±0.92	33.99±0.11
%L	31.69±0.0	30.49±0.27	45.12±0.56	29.74±0.24	30.86±0.03	31.62±0.04	29.74±0.19	31.66±0.02	29.65±0.05	31.28±0.02
%Ln	15.85±0.0	15.25±0.13	22.56±0.28	14.87±0.12	15.43±0.01	15.81±0.02	14.87±0.10	15.83±0.01	14.82±0.02	15.64±0.01
Method IV										
%S	24.99±0.10	25.38±0.27	22.37±2.41	28.00±0.14	26.50±0.24	25.59±0.15	26.63±0.13	25.02±0.02	28.05±0.18	26.11±0.16
%O	30.74±0.25	31.39±0.15	12.02±0.73	32.19±0.24	31.05±0.04	31.00±0.20	32.72±0.34	31.89±0.02	32.17±0.39	31.04±0.06
%L	29.51±0.12	28.56±0.36	43.74±1.87	26.54±0.25	28.30±0.18	28.94±0.03	27.10±0.16	28.72±0.01	26.52±0.36	28.53±0.09
%Ln	14.76±0.06	14.28±0.08	21.87±0.94	13.27±0.12	14.15±0.09	14.47±0.02	13.55±0.08	14.36±0.01	13.26±0.18	14.28±0.04

Table 3D Fatty acid composition of L-RBO (left) and Y-RBO (right) in different storage conditions by ^{1}H NMR technique.

	Method I	Method II	Method III	Method IV		Method I	Method II	Method III	Method IV
LRBO-1					**YRBO-1**				
%S	24.44±0.69	26.80±0.10	18.55±0.55	24.44±0.69	%S	24.99±0.10	30.28±12.75	19.45±0.40	24.99±0.10
%O	49.94±0.48	47.66±0.30	35.33±0.32	32.77±0.38	%O	48.08±0.60	43.78±13.80	33.01±0.40	30.74±0.25
%L	22.70±0.28	22.56±0.25	30.75±0.16	28.52±0.22	%L	24.36±1.05	23.26±1.18	31.69±0.0	29.51±0.12
%Ln	2.91±0.05	2.98±0.05	15.37±0.08	14.26±0.11	%Ln	2.58±0.47	2.68±0.27	15.85±0.0	14.76±0.06
LRBO-2					**YRBO-2**				
%S	24.73±0.78	33.82±0.68	16.80±1.70	24.73±0.78	%S	28.05±0.18	41.59±1.37	19.55±0.93	28.05±0.18
%O	55.29±0.94	46.70±0.61	40.83±2.26	36.93±1.68	%O	49.25±0.28	36.24±1.30	35.98±0.92	32.17±0.39
%L	14.42±0.47	13.40±0.56	28.25±0.38	25.56±0.60	%L	18.87±0.42	17.83±0.62	29.65±0.05	26.52±0.36
%Ln	5.57±0.49	6.08±0.52	14.13±0.19	12.78±0.30	%Ln	3.82±0.35	4.34±0.46	14.82±0.02	13.26±0.18
LRBO-3					**YRBO-3**				
%S	26.68±0.25	32.79±0.53	18..50±0.48	26.68±0.25	%S	25.48±0.72	35.65±0.30	17.80±0.64	25.48±0.72
%O	48.05±0.69	42.18±0.80	33.00±0.47	29.68±0.34	%O	49.90±0.35	40.24±0.65	33.13±0.38	30.03±0.04
%L	21.44±0.84	20.98±0.89	32.34±0.04	29.09±0.10	%L	19.58±0.34	18.55±0.25	32.71±0.37	29.66±0.49
%Ln	3.82±0.41	4.06±0.43	16.17±0.02	14.55±0.05	%Ln	5.04±0.13	5.55±0.18	16.36±0.18	14.83±0.25
LRBO-4					**YRBO-4**				
%S	27.28±0.20	32.48±0.27	19.38±0.30	27.28±0.20	%S	27.72±0.03	42.99±0.06	22.65±0.37	27.72±0.03
%O	48.70±0.38	43.75±0.44	32.60±0.53	29.41±0.45	%O	48.21±0.18	33.39±0.27	33.77±0.08	31.56±0.16
%L	19.17±0.21	18.67±0.21	32.10±0.18	28.88±0.18	%L	21.02±0.29	20.09±0.31	29.05±0.25	27.15±0.13
%Ln	4.85±0.02	5.10±0.02	16.01±0.09	14.44±0.09	%Ln	3.06±0.09	3.53±0.11	14.53±0.13	13.58±0.06

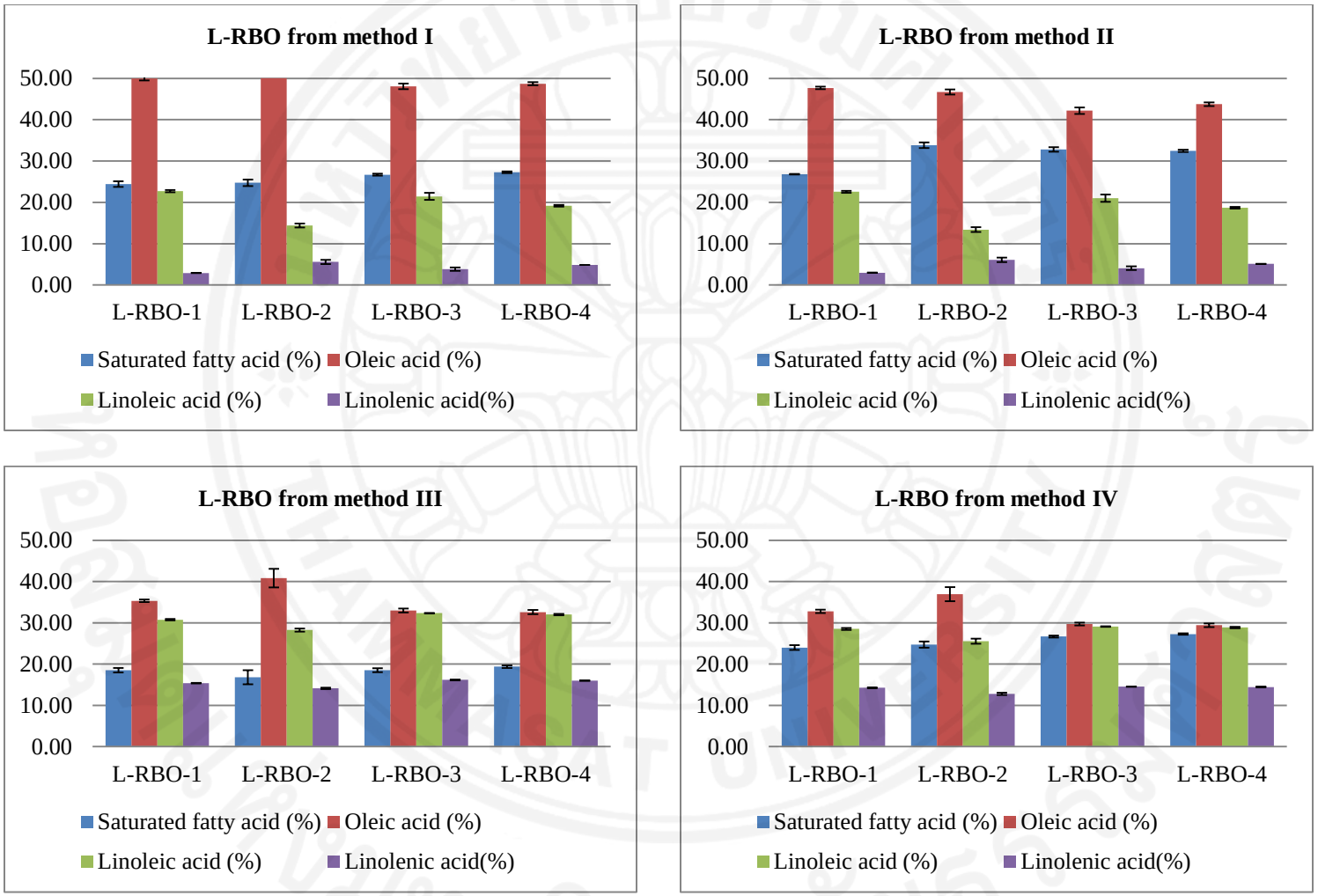


Figure 1D Fatty acid composition of L-RBO in different storage conditions by ^{1}H NMR technique (method I-IV).

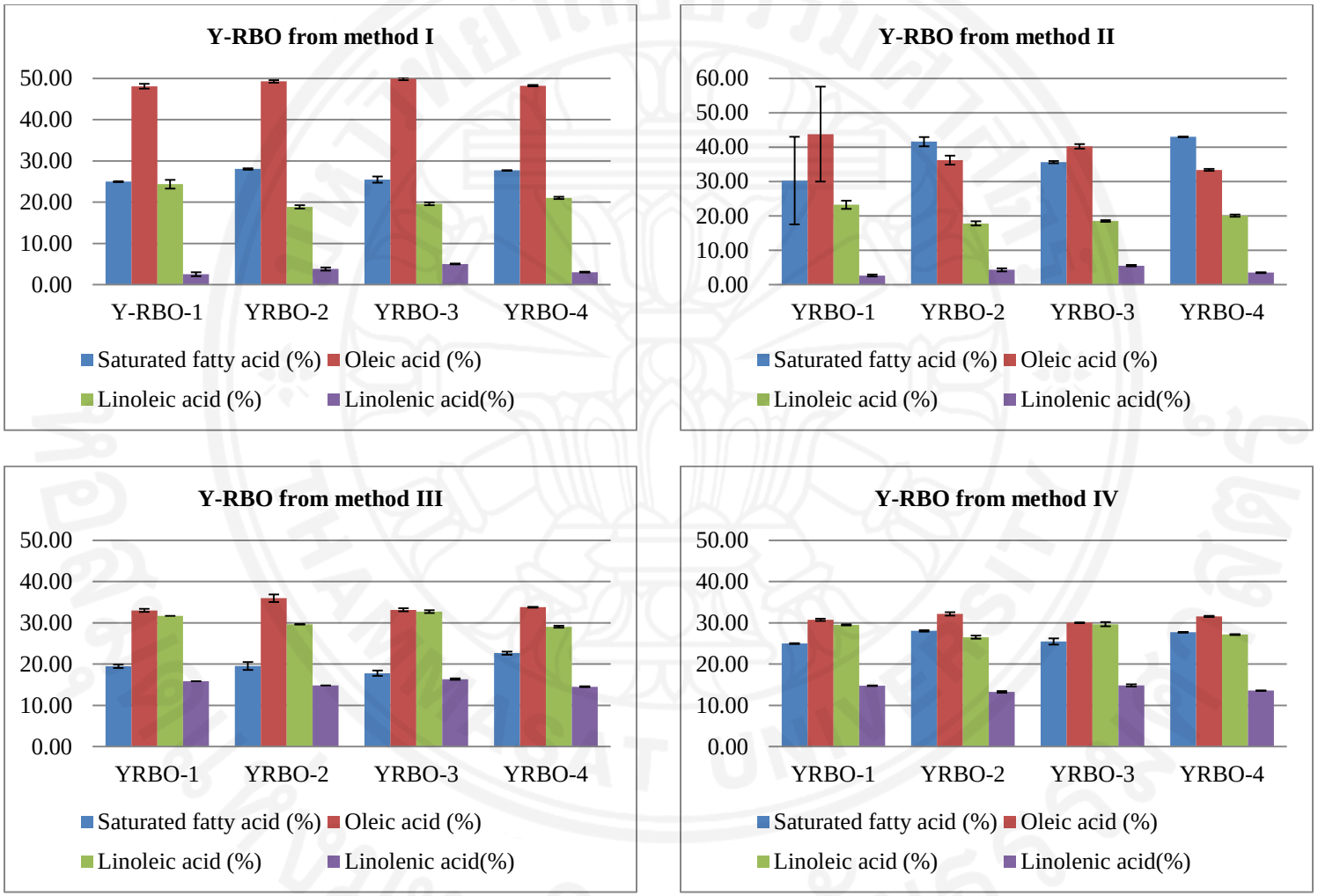


Figure 2D Fatty acid composition of -RBO in different storage conditions by ^{1}H NMR technique (method I-IV).

APPENDIX F

Statistical analysis

t-Test: Two-Sample Assuming Equal Variances

AV			t Stat	t Critical
L-RBO-1	and	L-RBO-2	1.518	4.303
L-RBO-1	and	L-RBO-3	1.213	2.776
L-RBO-1	and	L-RBO-4	4.131	4.303
L-RBO-3	and	L-RBO-4	13.771	2.776

AV			t Stat	t Critical
Y-RBO-1	and	Y-RBO-2	-5.014	2.776
Y-RBO-1	and	Y-RBO-3	-2.204	2.776
Y-RBO-1	and	Y-RBO-4	8.864	2.776
Y-RBO-3	and	Y-RBO-4	43.959	2.776

IV			t Stat	t Critical
L-RBO-1	and	L-RBO-2	16.630	4.303
L-RBO-1	and	L-RBO-3	37.108	2.776
L-RBO-1	and	L-RBO-4	13.795	4.303
L-RBO-3	and	L-RBO-4	-3.828	2.776

IV			t Stat	t Critical
Y-RBO-1	and	Y-RBO-2	15.668	2.776
Y-RBO-1	and	Y-RBO-3	-21.916	2.776
Y-RBO-1	and	Y-RBO-4	10.744	2.776
Y-RBO-3	and	Y-RBO-4	0.028	2.776

PV			t Stat	t Critical
L-RBO-1	and	L-RBO-2	89.583	2.776
L-RBO-1	and	L-RBO-3	-12.523	2.776
L-RBO-1	and	L-RBO-4	3.638	2.776
L-RBO-3	and	L-RBO-4	11.384	2.776

PV			t Stat	t Critical
Y-RBO-1	and	Y-RBO-2	17.852	2.776
Y-RBO-1	and	Y-RBO-3	15.724	2.776
Y-RBO-1	and	Y-RBO-4	2.516	2.776
Y-RBO-3	and	Y-RBO-4	-20.053	2.776

t-Test: Paired Two Sample for Means (p=0.05)

	t Stat	t Critical
GC-MS and method I		
L-RBO-4	-1.1168E-16	3.182
Y-RBO-4	0.0327	3.182
GC-MS and method II		
L-RBO-4	-0.00058	3.182
Y-RBO-4	-0.027	3.182
GC-MS and method III		
L-RBO-4	-0.00051	3.182
Y-RBO-4	-0.015	3.182
GC-MS and method IV		
L-RBO-4	2.01E-16	3.182
Y-RBO-4	-0.017	3.182

t-Test: Two-Sample Assuming Equal Variances (p=0.05)

Saturated fatty acid from method I	t Stat	t Critical
L-RBO-1 and L-RBO-2	1.320	2.776
L-RBO-1 and L-RBO-3	-7.504	2.776
L-RBO-1 and L-RBO-4	-9.483	2.776
L-RBO-3 and L-RBO-4	-3.217	2.776

Saturated fatty acid from method I	t Stat	t Critical
Y-RBO-1 and Y-RBO-2	25.188	2.776
Y-RBO-1 and Y-RBO-3	1.165	4.303
Y-RBO-1 and Y-RBO-4	-43.533	2.776
Y-RBO-3 and Y-RBO-4	-5.350	4.303

BIOGRAPHY

Name	Miss Chutima Ponchurdchai
Date of Birth	July 20, 1990
Educational Attainment	2012: Bachelor degree of Science in Chemistry, Thammasat University
Position	Graduate student
Work experience	Teaching assistant at Faculty of Science and Technology, Thammasat University

Publications

Chutima Ponchurdchai and Jirada Singkhonrat. Preparative separation of sweeteners (Stevioside and Rebaudioside A) from *Stevia rebaudiana* Bertoni crude extracts by macroporous resins. The 39th Congress on Science and Technology of Thailand (STT 39), October 21-23, 2013, Bangkok International Trade & Exhibition Centre (BITEC), Bangkok, Thailand (poster presentation).

Chutima Ponchurdchai and Jirada Singkhonrat. ^{1}H-NMR fingerprinting of cold-pressed rice bran oil. Pure and Applied Chemistry International Conference (PACCON 2014), January 8-10, 2014, Centara Hotel and Convention Centre Khon Kaen, Thailand (poster presentation).

Chutima Ponchurdchai and Jirada Singkhonrat. Determination of saturated and unsaturated fatty acids in cold-pressed rice bran oils by ^{1}H-NMR. 3rd Rice National Conference 2014, Miracle Grand Convention Hotel, Bangkok, Thailand (poster presentation).

Chutima Ponchurdchai and Jirada Singkhonrat. Monitoring of stability evaluation of unrefined organic rice bran oil using ^{13}C-NMR spectroscopic. Pure and Applied

Chemistry International Conference (PACCON 2015), January 21-23, 2015, Amari Watergate Hotel, Bangkok, Thailand (poster presentation).

Chutima Ponchurdchai and Jirada Singkhonrat. Monitoring the effect of different storage conditions of cold-pressed rice bran oil. 2015 6th International Conference on Chemistry and Chemical Engineering (ICCCE 2015), August 5-6, 2015, Hotel Mercure Paris Vélizy, France (oral presentation).