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Wax extraction and characterization from full-fat and defatted rice bran

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WAX EXTRACTION AND CHARACTERIZATION FROM FULL-FAT AND DEFATTED RICE BRAN

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PREFACE

The term “Wax” reminds people of white solid fat like candles or bee’s wax, which is neither a correct nor an incorrect concept. Wax is a fat-like material which contains numerous substances and usually has high melting points. The compositions of wax vary from one to another based on its sources. Wax is a ubiquitous material, which exist on the surface of plant material and on animal skins. Wax is highly hydrophobic so it is used as a natural protectant against water and moisture, even against microorganisms. The glassy surfaces of leaves or fruits are due to wax.

Wax is also found in edible oils. Before the oil is consumed, wax is usually separated from the oil by placing the oil at a cold place where the temperature is low enough to make wax crystallize and precipitate at the bottom of the container. This process is called winterization, after which wax is easily removed by filtration or centrifugation. The major component of wax in edible oil is wax esters, which consist of long chain alkyl esters and steryl esters. They are classified as apolar lipid species, whose polarities are lower than triglycerides. Their low polarities and longer chain lengths contribute to crystallization. Not all wax esters are crystallizable, and some wax esters still remain in the oil even after winterization.

Rice bran oil is notable for its high contents of antioxidants and wax. Rice grows in watered paddies and under strong sunshine, which can explain why rice bran, the outer layer of rice grain after the hull is removed by milling processes, contains higher wax and antioxidants than other edible oils. High wax contents in rice bran oil, between 2-4%, has been a major factor that prevented rice bran oil from being marketed as cooking oil.

Rice bran wax has been studied for more than 70 years, and early studies until 1950s focused on rice bran wax separation for industrial use. Compositional analyses of rice bran wax have been conducted since 1980. The wax compositions and wax contents in rice bran oil differ from

one study to another because the researchers used different methods for oil extraction and wax preparation. Some authors did not even make a distinction between wax and wax esters, and no study compared the composition of wax esters from rice bran wax with that from rice bran oil. The difference between crystallizable and noncrystallizable wax esters in rice bran oil has not yet been studied either.

Wax has been considered as a by-product or waste material from oil production and studied mainly for industrial purpose. Its potential nutritional significance was recently recognized after policosanol, which is a mixture of long chain saturated alcohols as well as a component of sugarcane wax, had been intensively studied. Wax esters containing alkyl esters and steryl esters generate fatty acids, fatty alcohols, and sterols after hydrolysis or saponification. Phytosterols are known to improve blood lipid profile. Fatty alcohols like policosanol inhibit cholesterol synthesis in the liver. When absorbed into the body, wax esters are decomposed into those substances, which means that wax esters in wax or oil may have health benefits. However, the exact mechanisms have not been fully studied and there have been only a few studies on the separation or purification processes for those substances from wax, which could be utilized in biological tests.

As mentioned above, rice bran wax and wax esters still have some unknown aspects, which require more thorough studies. These studies should be designed to;

- develop efficient methods for extraction of oil and wax from rice bran,
- establish separation or preparation methods for wax esters from rice bran wax,
- obtain more accurate analytical methods for wax esters using HPLC and GC, and
- test wax components on cells or animals for their possible health-promoting effects.

This dissertation research was designed to identify the composition of rice bran wax esters and

to establish the preparation methods of wax components for biological tests. The approach includes rice bran oil extractions with Soxhlet and Microwave-assisted methods, wax separation from rice bran oil by winterization and solvent fractionation, wax ester separation by HPLC, and compositional analysis of wax esters by GC. The results will give specific information on the compositions of wax esters as well as a possible process for the preparation of biological test samples. In addition, the utility of using commercially defatted rice bran as a source of wax was investigated compared with full-fat rice bran.

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ABSTRACT

Rice bran oil (RBO) contains 3-4% waxes (rice bran wax, RBW), which are composed of wax esters (WE), hydrocarbons, and other minor constituents. Saponified rice bran wax esters (RBWE) generate fatty acids, long chain alcohols, and phytosterols. Phytosterols and long chain fatty alcohols (policosanol) are known to reduce serum cholesterol and inhibit hepatic cholesterol synthesis.

The yields and RBWE contents of RBO and RBW extracted from full-fat RB (FFRB) and defatted RB (DFRB) were determined using 6 different conditions with Soxhlet and Microwave-assisted extraction (MAE). The compositions of WEs from RBO and RBW were also compared. RBW was obtained from RBO by winterization and solvent fractionation. WEs were separated by chromatographic methods, and analyzed as intact WE using a mass analyzer. After saponification of WE, alcohols, sterols, and fatty acids were analyzed by GC.

Crude FFRBO yields were not significantly different among the extraction methods, while MAE (isopropanol, 120°C) showed significantly higher DFRBO yields. DFRBOs had higher concentrations of crude RBW than FFRBOs, and hexane extractions showed higher crude RBW yields. Crude RBW yields from FFRB were higher than from DFRB, while refined RBW yields from FFRB and DFRB were more similar. The refined RBW yields by hexane extractions were much higher than those by isopropanol extractions, and DFRBOs showed higher refined RBW yields than FFRBOs. HPLC results indicated that most WE was contained in RBO raffinate, and around half of the refined RBW consisted of WE. The mass spectra showed that there were more long chain species in WE from RBW. GC results identified C13-C22 fatty acids and the major alcohols in WE from RBW appeared as C32 and C34. Six sterols were identified in WE from RBO.

Results indicate that MAE with hexane is more efficient than Soxhlet for RBWE extraction. DFRB appears to have significant RBW content, which would make it an excellent source for potential commercial exploitation. This study established an efficient procedure for WE analysis as well as for alcohol/sterol separations from RBW for further biological experiments.

CHAPTER 1.
COMPARISON OF OIL AND WAX YIELDS FROM FULL-FAT AND DEFATTED RICE BRAN EXTRACTED USING SOXHLET AND MICROWAVE-ASSISTED EXTRACTIONS

1.1. INTRODUCTION

Harvested rice is in the form of rough rice (paddy) with the edible portion covered with an outer protective layer known as the husk or hull. After being dried, the rice passes though sheller machines to remove the hull material. Shelling produces brown rice, with a thin bran layer surrounding the rice kernel. Abrasive forces in the milling machine remove the outer bran layer on the brown rice and the resultant product is white rice. White rice is consumed after appropriate polishing to further remove any remaining bran layers and to give a desired degree of whiteness and polish. The rice hull and rice bran (RB) are obtained as by-products of the rice milling industry (Juliano, 1985).

Rice bran oil (RBO) has been commercially produced in the US since 1994, and it has a very appealing nut-like flavor. Once extracted, RBO gives very good stability for frying due to its high levels of oryzanol and tocotrienols. It is estimated that current annual world rice bran oil production is less than 800,000 metric tons, or about 1% of all vegetable oils (McCaskill and Zhang, 1999). RB includes the germ and embryo in most commercial milling operations, and represents only about 8% of the paddy weight. However, it contains about three-fourths of the total rice oil. RB itself contains about 15-20% oil, and the typical composition of crude rice bran oil is 68-71% triglycerides, 2-3% diglycerides, 5-6% monoglycerides, 2-3% free fatty acids, 2-3% waxes, 5-7% glycolipids, 3-4% phospholipids, and 4% unsaponifiables (McCaskill and Zhang, 1999; Juliano and Bechtel, 1995; Lu and Luh, 1991). In comparison with other vegetable oils, crude RBO tends to contain higher levels of non-glyceride components including wax esters (WE), most of which are removed during refining processes.

The wax content of RBO can be somewhat variable, depending on cultivar and processing parameters (Belavadi and Bhowmick, 1988). The physical definition of wax is a little confusing because it is usually related with physical properties of wax such as high melting points, and solid conditions at room temperature (Kolattukudy, 1976). Wax can be any hard solid fat at room temperature like Oricuri wax and Japanese wax, but they contain not only wax esters but also a significant amount of glycerides (Bennett, 1975). Waxes in edible oils have been considered as a hazy useless material in vegetable oils and are usually removed by refining processes called winterization before the oils are marketed (Hermann et al, 1999). Wax should be treated as a mixture whose main component is WEs.

The chemical definition of WE is an ester composed of alkyl esters (a long chain fatty acid + a long chain alcohol) or sterol (or steryl) esters (a long chain fatty acid + a sterol). WEs have low solubility in neutral lipids, so at lower temperatures, they crystallize and precipitate as settlings in a crude oil tank (Hermann et al, 1999). According to Ito et al. (1983), RBO comprises 12% of RB, and contains 91% of neutral lipids, 4% of glycolipids, and 5% of phospholipids. Among the neutral lipids, only 9.5% is WE, which are a mixture of 20% of alkyl esters (AE) and 80% of sterol esters (SE). The WE content in RB is only about 1.04%, and the alkyl ester content is only 0.21%. Such a low content of WEs in RB and such complexity in the structures and compositions of RBW make it difficult to analyze RBW and RBWE.

The term “wax” and “wax esters” have been used interchangeably. One of the first-studied forms of wax is Bee’s wax, and later the major component was found to be a wax ester, which caused the misconception of “wax = wax ester”. Thus, the term “wax” is not a single substance but a mixture in which the major component is wax esters, and wax esters are composed of alkyl esters and steryl esters in edible oils. Wax obtained from rice bran is therefore also a mixture,

and it contains numerous substances of which the common property is crystallization at a low temperature. Crude RBO tank settling, which was separated by winterization process (Leibovitz and Ruckenstein, 1984), can be called RBW or crude RBW. This RBW contains not only WEs but also hydrocarbons, acylglycerides, free fatty acids, free alcohols, and many other crystallizable substances. The terms “wax” and “wax esters” are different, and they should be treated in different ways, which requires analysis of the compositional difference between wax and wax esters.

1.2. LITERATURE REVIEW

1.2.1. Studies on the Composition of RBW and RBWEs

There have been only a few studies on rice bran wax (RBW) or rice bran wax esters (RBWE) in rice bran oil (RBO). Cousins et al. (1953) suggested that the tank settlings of crude RBO can be a source of wax. They purified RBW from the tank settlings by dissolving the tank settlings with hexane, acetone, isopropanol, and diethyl ether and filtering the precipitate in 5 different procedures. The wax yield ranged from 8.3 to 13.7%. Even though the wax contents in the study excluded the soft waxes and there was no composition analysis performed, the researchers provided insight into wax purification methods with proper solvents. The composition of waxes from crude RBO tank settlings was studied by Yoon and Rhee (1982) with TLC and GC. They used methyl ethyl ketone to remove the oil and isopropanol to crystallize the wax. Their results showed that there were soft (mp 74 ^{o}C) and hard waxes (mp 79.5 ^{o}C) in the oil according to their melting point, and that the contents of hydrocarbon, fatty alcohol and fatty acid were 5.6%, 3.9% and 0.6% in hard wax, and 1.2%, 4.0%, and 1.0% in soft wax, respectively. The hard wax was mainly composed of saturated fatty alcohols of C24, C26, and C30, saturated fatty acids of C22,

C24, and C26, and n-alkanes of C29 and C30, while the soft wax was mainly composed of saturated fatty alcohols of C24 and C30, saturated fatty acids of C16 and C26, and n-alkanes of C21 and C29. However, their study did not determine alkyl ester composition according to their chain lengths. Both of the studies focused mainly on separation of wax from crude RBO settlings and composition analysis of hydrolyzed wax ester. Belavadi and Bhowmick (1988) investigated crude RBO settlings, and compared the compositions of hydrolyzed and unhydrolyzed wax. They extracted rice bran oil with petroleum ether, separated wax with isopropanol crystallization, compared the composition of the isopropanol-insoluble fraction (IIF, 0.83% of the oil) by TLC, GC, and silica gel column chromatography before and after hydrolysis. They found soft and hard wax fraction by column chromatography of IIF before hydrolysis. The petroleum:benzene (1:1) fraction and ethyl acetate fraction showed melting points of 70.74 ^{o}C (33.9%) and 62.64 ^{o}C (13.3%), respectively, but there were uneluted fractions which did not melt even at 300 ℃ (52.9 %), which the authors identified as alcohols, although they did not provide support for this designation. After alkaline hydrolysis, the composition of IIF showed 40.41% unsaponifable material which was not characterized except for chain lengths, 16.47% ether-insolubles which the author said nothing about, and 25.59% ether-solubles which were identified as fatty acids. The rest of hydrolyzed IIF was not identified. The authors said that there were only 33% of monomeric esters present in IIF and that the IR data indicated the presence of some aromatic moiety such as sterol esters. They used simple solvent systems for column chromatography, and their study did not elucidate the wax ester composition in IIF. The wax ester composition in RBO was thoroughly studied by Ito et al. (1983). Unlike the studies above, they did not use RBO settlings as a starting material. They extracted the total lipid (12% of rice bran) from rice bran with chloroform:methanol (2:1) and water-saturated butanol, and used silica gel column

chromatography to separate neutral lipids (91% of total lipid), glycolipids (4%), and phospholipids (5%). The neutral lipid fraction was rechromatographed on silica gel to isolate the wax fraction (9.5% of neutral lipids) of steryl esters (SE), longer alkyl esters (AE), and shorter alkyl esters (AE), whose ratio was 8:1:1. They used TLC and GC for the composition analysis as well as IR for the identification of wax components, and they identified short chain AEs of C15-C20, and long chain AEs of C38-C58. The main SE was linoleoyl sitosterol. Garcia et al. (1996) used supercritical carbon dioxide (SC-CO_2) for the extraction of RBO and RBW, and compared it with the Soxhlet extraction method. They identified the composition of fatty acids (C14-C34) and fatty alcohols (C22-C34) from the RBOs extracted by each method. SC-CO_2 extraction appeared to extract more long chain fatty acid than hexane extraction. However, they did not separate RBW from the RBO, so it is hard to tell if SC-CO_2 extraction is effective for high yield of wax components. Moreover, the oil yield by SC-CO_2 extraction was unusually low when compared to that of hexane extraction. Vali et al. (2005) studied a process for the preparation of food-grade rice bran wax and determined its composition. They used as starting materials 5 different sediments from crude RBO extracted by a Soxhlet method (hexane), and obtained almost pure WE (>99% purity) after defatting the sediments with hexane and isopropanol, and bleaching them with $NaBH_4$ to remove the resinous matter which is mostly free fatty acids, alcohols, and aldehydes. TLC and GC were used for the WE analysis. The results indicate that rice bran wax is mainly a mixture of saturated AEs of C22 and C24 fatty acids and C24 to C40 aliphatic alcohols, with C24 and C30 being the predominant fatty acid and fatty alcohol, respectively. The alcohol portion of the wax esters also contained small amounts of branched and odd carbon number fatty alcohols. Gunawan et al. (2006) conducted a comprehensive study on the composition of RBWE separated from RBO by acetone winterization, column

chromatography and TLC. They reported that SE and AE accounted for 4.0% of crude RBO, of which 2.8–3.2% and 1.2–1.4% were SE and WE, respectively. By GC–MS, they determined that the major fatty acids in the SE fraction were linoleic acid and oleic acid, and that the sterols were campesterol, stigmasterol, sitosterol, stigmastenol, citrostadienol, cycloartenol, and cycloartanol. AE from RBO consisted of both even and odd carbon numbers ranging from C44 to C64. The major constituents were saturated AEs of C22 and C24 fatty acids and C24 to C40 aliphatic alcohols, with C24 and C30 being the predominant fatty acids and fatty alcohols, respectively.

1.2.2. Studies on the Health Benefits of RBW and RBWEs

RBW suspended in 25% gum arabic solution had an oral LD_{50} of >24 g/kg body weight (bw) in male mice. Hydrogenated RBW (administered as 50% in corn oil) had an oral LD_{50} of >5 g/kg bw in white rats, which were necropsied 14 days after dosing; one male rat had a dilated right kidney. Ten albino rats (5 male and 5 female) that orally received RBW (a 12.5% suspension heated and cooled in corn oil) at a dose of 5 g/kg bw, were observed for 14 days, and dissected. No gross changes were observed in nine, but one showed two red nodules (3mm i.d.) attached to fat adjacent to the bladder. The LD_{50} was >5 g/kg bw (No author listed, 2006).

Hansen and Mead (1965) studied the effect of waxes on rat growth by feeding diets with a defined wax such as oleyl palmitate at either 4 or 15 g/100 g diet for 2–4 weeks, in which absorption of the wax was about 50%, and the animals fed at this level developed steatorrhea. This indicates that intact wax esters are not absorbable, and that for uptake to occur, the esterified fatty alcohol must be released by a lipase or other carboxyl esterase.

Efficiency of long chain species uptake decreases as chain length and hydrophobicity increase, and depends on the secretion of bile acids, colipases and a carboxyl esterase and the existence of

competing substrates for those enzymes. Pancreatic lipase hydrolyzes triacylglycerol at approximately 10 times the rate of waxes, so the presence of dietary fats may stimulate secretion of bile and pancreatic enzymes yet inhibit wax hydrolysis. Hydrolysis of WEs releases fatty acids and fatty alcohols, both of which are readily absorbed by the intestinal epithelium (Hargrove et al. 2004). Based on the studies in the previous section (Sec. 1.1.1.1), hydrolysis of WEs also releases sterols.

Long chain fully saturated aliphatic alcohols (C24-C34), known as policosanol, especially when extracted from sugar cane wax, have been widely studied mainly by Cuban scientists (Pepping, 2003; no author listed, 2004). Octacosanol (CH_3-$(CH2)_{26}$-CH_2-OH, C28) is the predominant moiety, comprising approximately 63% of the mixture (Granja et al., 1997). Policosanol is a drug currently in use in combination with dietary therapy in patients with hypercholesterolemia (Gouni-Berthold, 2002). The health-promoting effects of policoanol has been well reviewed (Janikula, 2002; Gouni-Berthold and Berthold, 2002;; McCarty, 2002, 2005; Pepping, 2003; Taylor et al. 2003; Jacoby and Mohler, 2004; No author listed, 2004). Policosanol have been found to show little or no toxicity or harmful side effects in various animals with the concentration range of 0.25-5000 mg/kg bw for 3 weeks to 18 months. Policosanol has significant anti-platelet effects or anti-coagulation effects in blood in both humans and animal models. Policosanol prevents the development of atherosclerosis by inhibiting LDL oxidation as well as neointimal formation and by accelerating LDL metabolism. Policosanol appears to decrease synthesis and increase degradation of HMG-CoA, the rate-limiting step in cholesterol synthesis. It is thought to interfere with the synthesis and degradation of the enzyme. Singh et al. (2006) found that policosanol inhibits cholesterol synthesis in hepatoma cells by AMP-kinase activation, which indirectly down-regulates HMG-CoA

reductase, and that triacontanol (C30) is more effective than octacosanol (C28). However, several other studies recently reported that sugar cane policosanol has no or little direct effects on hypercholesterolemia in human and animal subjects, and that policosanol does not alter the serum lipid profile over an 8-wk period in adults with mild hypercholesterolemia (Kassis and Jones, 2006; Dulin et al., 2006; Kassis et al., 2007; Francini-Pesenti et al., 2008). Rice policosanol has also been tested in human and animal subjects. Rice policosanol treatment did not change significantly neither fibrinogen nor coagulation factors VII, VIII, XII and XIII (Reiner and Tedeschi-Reiner, 2007). Rice policosanol significantly reduced plasma total cholesterol and increased Apo AI but did not change plasma triglycerides, HDL, HDL2, HDL3 and LDL cholesterol, ox-LDL, Lp(a), Apo B, fibrinogen, homocysteine or CRP levels (Reiner et al., 2005). Rice bran policosanols have no significant favorable effect in changing lipid levels in hamsters (Wang et al., 2003). All the studies indicate that policosanol does not improve blood lipid profile but inhibits cholesterol synthesis.

RBW contains a significant amount of steryl esters, which can produce phytosterols on saponification. Rice bran wax has high levels of phytosterols when it is properly prepared (Norton, 1995). Phytosterols reduce total cholesterol and HDL-C levels (Wang et al., 2003). These phytosterols from edible oils have been found to have blood cholesterol-lowering effects on human subjects (Moruisi et al., 2006; Wang et al., 2005; Kerckhoffs et al. 2002). Vissers et al. (2000) found that plant sterols (2.1g/day for 3 weeks) from rice bran oil lowered serum total cholesterol by 5% and LDL cholesterol by 9% in normolipemic humans. The effect of rice bran oil sterols is probably due to ss-sitosterol and other 4-desmethylsterols and not to 4,4'-dimethylsterols. Meijer et al. (2003) investigated the effect of three types of plant sterols, free, esterified with FA, or with phenolic acids, on cholesterol absorption. Rice bran sterols

containing 70% 4,4'-dimethylsterols tended to lower cholesterol absorption efficiency by 7% and plasma total cholesterol by 5%. Trautwein et al. (2002) found that not only sterols but also esterified sterol (steryl esters) were equally effective in lowering plasma cholesterol and LDL-cholesterol, and that sterols achieved their cholesterol-lowering effect by stimulating fecal cholesterol excretion through inhibiting intestinal cholesterol absorption.

1.2.3. RBO Extraction and Winterization

RBO has been prepared by various extraction methods in the laboratory. Ito et al., (1983) used solvent extractions with methanol:chloroform (2:1), and the RBO yield was 12%. Soxhlet extraction was used with hexane or petroleum ether (Belavadi & Bhowmick, 1988; Garcia et al.,1996; Vali et al., 2005), or supercritical carbon dioxide extraction (SC-CO_2) (Zhao et al., 1987; Saito et al., 1993; Shen et al., 1997; Xu & Godber , 2000) was also used. The RBO yields reached 14-21% with Soxhlet and 13-22% with SC-CO_2. Zigoneanu et al. (2007) determined the levels of antioxidant components in rice bran oil extracted by MAE. They obtained RBO yields of 10-15% by MAE with isopropanol and 12-14% by MAE with hexane. The RBO yields varied from 12% to 20% in the studies mentioned above, but they did not specify which oil extraction method would be most suitable for RBW preparation.

Waxes have low solubility in oil at low temperatures, and cause turbidity and crystallization, resulting in sediment formation in crude oil settling tanks. Tank settlings are removed during a refining process called winterization to obtain clearer RBO (Krishna, 1993; Ramakrishna et al., 1987). There are 3 winterization methods; conventional winterization (dry winterization), crystallization with water and emulsifying agents (wet winterization), and solvent winterization (Hermann et al, 1999). Sah et al. (1983), De and Bhattacharyya (1998), Rajam et al. (2005), and

Ghosh and Bandyopadhyay (2005) have studied the winterization of crude RBO, but there studies focused not on the separation of RBW from RBO but the acquisition of clearer RBO. The wax separated by winterization contains not only wax esters but also other lipid species, so to obtain purer forms of wax esters, defatting or washing with organic solvents is necessary, which is called solvent fractionation (Cousins et al., 1953; Vali et al., 2005). Yoon and Rhee (1982) used methyl ethyl ketone to remove the oil and isopropanol in order to crystallize the wax. Vali et al. (2005) used hexane and isopropanol to separate polar lipids from crude RBO. Gunawan et al. (2006) performed acetone fractionation to separate wax esters from other nonpolar lipids. Several studies have been published on RBW separation by winterization, but there is still a lack of information on the relationship between RBO extraction methods and RBW yields by winterization followed by solvent fractionation.

HPLC methods for WE separation will be reviewed in detail in Chapter 2.

1.3. OBJECTIVES OF STUDY

The studies mentioned in Sec.1.1.1.1 provided general aspects of the composition of RBW. Cousins et al. (1953), Belavadi and Bhowmick (1988), and Vali et al. (2005) used RBO tank settling as starting material. Although the yields of those settlings are not specified in the original papers, they all dissolved the tank settlings in organic solvents at an elevated temperature, cooled the solution to a low temperature (winterization), and separated the precipitate (filtration). They used solvent fractionation (Kreulen, 1976), in order to obtain purer forms of WEs. Ito et al. (1983), Garcia et al. (1996) and Gunawan et al. (2006) extracted RBO to separate RBWE. Although the RBO extraction methods and the RBO yields were different, they separated RBWE with column chromatography or thin layer chromatography. Gunawan et al. (2006) also used

acetone fractionation before WE separation after RBO extraction.

These authors indicated that they had analyzed RBW but actually what they analyzed was WEs in RBW or in RBO. Some researchers did not distinguish the difference between "wax and wax esters" or between "WE from RBO and WE from RBW". None of the studies cited above separated WEs from RBO and RBW that were derived from the same RBO, and analyzed. This makes it very difficult to combine the results of the studies in order to obtain proximate composition of RBWE or to set up an experimental plan for RBW analysis. Moreover, there are considerable compositional differences between WEs from RBW (or tank settlings) and from RBO itself, which requires further studies.

The ultimate goal of this research was to establish a process to recover wax components from rice bran for further testing of potential health benefits. The specific objectives of this study were 1) to find efficient methods for RBO and RBW extraction, 2) to compare the RBW contents of FF RB and DF RB, 3) to establish the amounts of WE in RBO and RBW, and 4) to suggest efficient preparation methods for RBW analysis.

1.4. METHODS AND MATERIALS

1.4.1. Sample Preparation

Oil was extracted from two kinds of rice bran (RB), full-fat rice bran (FF RB) and defatted rice bran (DF RB). Both were provided by Riceland Foods (Stuttgart, Arkansas). FF RB was provided as extruded collets with 1-cm i.d., which was ground with a food grinder. DF RB was provided as a powder. The moisture contents of the RBs were determined to be 8.31% and 9.37% in FF RB and DF RB, respectively, after they were dried at 60 °C for 24 hrs and 80 °C for 24 hrs. The RB was filtered through a 20-mesh sieve, and the filtered RB was stored at –20 °C.

1.4.2. Oil Extraction

Two extraction methods were used; Soxhlet extraction and Microwave-assisted extraction (MAE) (Zigoneanu et al. 2007). The oil extraction and crude RBO recovery procedure is depicted in fig. 1.1. For Soxhlet extraction, 20 g of RB was weighed into a cellulose thimble (30 mm x 77 mm, Whatmann, Maidstone, UK), and the thimble was placed in a Soxhlet device. Two hundred milliliters of hexane was used as the extraction solvent and the extraction was performed for 7 hrs. The temperature in the extraction chamber was approximately 62 to 63 °C. After the solvent was removed with a rotary evaporator, the weight of crude RBO was measured. For MAE method, an Ethos E apparatus (Milestone, Monroe, CT) was used. Forty grams of RB was placed in an extraction vessel, and 150 ml of hexane or isopropanol was added. Three extraction chambers were placed in the apparatus for 1 extraction. The extraction temperature was set to 80 °C for hexane extraction and 80 °C or 120 °C for isopropanol extraction. The extraction was continued for 30 min at the desired temperature. After extraction, the mixture of the solvent and the RB was filtered through a Buchner funnel (pore size 15 – 20 μm). During the filtration, the RB was washed with hot hexane or isopropanol. The filtered solution was removed into a 500-ml round-bottom flask, and the extract was recovered after solvent evaporation by a rotary evaporator. The isopropanol extract contained a small amount of material that was not soluble at 80 °C, so two different procedures were adopted to separate the crude RBO; hexane separation (HS) and hexane-water separation (HWS). For hexane separation, 100 ml of hexane was added to the extract and heated at 60 °C until the solution became clear. It was filtered when still hot, and the crude RBO was obtained after hexane evaporation. For hexane-water separation, 100 ml of hexane and 20 ml of water was added to the extract, and heated at 60 °C until the solution became clear. It was removed into a separation funnel, and the hexane layer and the

water layer was separated. The hexane layer was further dehydrated by mixing with 1g of sodium sulfate. The crude RBO was obtained after the hexane was evaporated. After the extracted crude rice bran oil (RBO) was weighed and the yield was calculated, the RBO samples were stored at –20°C.

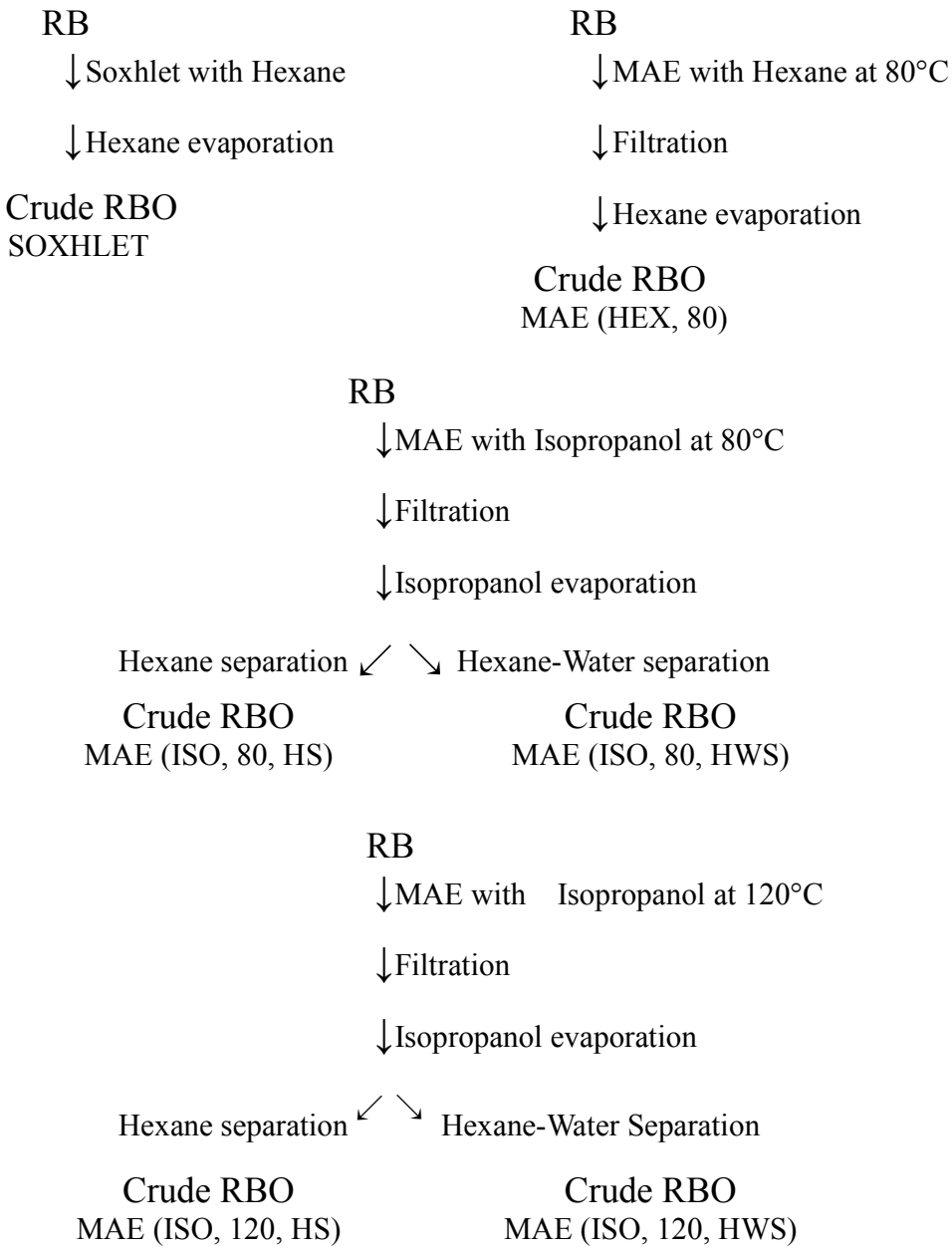


Figure 1.1. RBO Extraction and Recovery Procedure

1.4.3. Refinement of Crude RBO

The refining process of crude RBO was modified from the method of De and Bhattacharyya (1998), and the processes are illustrated in fig.1.2. Crude RBO (about 2 ml) was weighed into a clean test tube, heated to 80 °C, and then 1 ml of distilled water was added to the oil. It was heated until the oil became clear, vortexed, and centrifuged at 80 °C and 1500 g for 20 min (Vacuum centrifuge evaporator, CentriVap Console Labconco, Kansas City, Missouri). The washed oil was carefully transferred to another test tube. The same procedure was used with 1 ml of 0.2% (w/w) aqueous phosphoric acid, and then 1 ml of hot water containing 50 ul of 10 % CaO (Mallinckrodt Baker, Phillipsburg, NJ). The oil was washed with water again. After degumming, the oil was transferred to a centrifuge tube with a conical bottom, and bleached with 100 mg of activated carbon (100-200 mesh, Eastman organic chemicals, Rochester, NY) and 100 mg of Fuller's earth (100 – 200 mesh, Sigma, St. Louis, MO). The mixture was centrifuged at 80 °C and 1500 g for 20 min. After bleaching, the oil was transferred to a centrifuge tube, dehydrated with 100 mg of sodium sulfate, and centrifuged at 80 °C and 1500 g for 20 min. The dehydrated oil was transferred to a clean test tube and dissolved in 4 ml of hexane. The hexane solution was heated and filtered through a syringe filter (0.2 μm, 25-mm PTFE, Whatman, Madstone, UK). The processed RBO was recovered after hexane evaporation, weighed and stored at -20 °C.

1.4.4. Winterization

One gram of the processed RBO was mixed with the same volume of hexane, and the solution was heated at 60 °C until the solution became transparent. It was cooled to room temperature, and then placed in an incubator at 20 °C for 1 hr. The temperature of the incubator was further

reduced to 18, 16, 14, and 12 °C, and maintained at each temperature for 1 hr. Finally, the solution was cooled at 10 °C, and left overnight for the completion of wax crystallization. After the winterization was finished, the yellowish crude rice bran wax (RBW) was recovered after the solution was centrifuged (10,000g) at 10 °C for 20 min. After the supernatant (dewaxed RBO) was carefully removed, the precipitate (Crude RBW) was dried in a centrifugal evaporator. The weight of the crude RBW was measured, and the yield from the processed RBO was calculated. The winterization steps were shown in fig.1.3.

Crude RBO (2 ml)
↓ Distilled water
↓ Phosphoric acid
↓ CaO
Degummed RBO
↓ Activated carbon and Fuller's earth
Bleached RBO
↓ Sodium Sulfate
Dehydrated RBO
↓ Dissolved in hexane
↓ Filtered (0.2 um)
↓ Solvent evaporation
Processed RBO
↓ Weight measured

Figure 1.2. Processes of Crude RBO refinement.

1.4.5. Solvent Fractionation

The solvent fractionation method was modified from Vali et al. (2005), and depicted in fig. 1.3. It started with the addition of the same volume of hexane into the crude RBW. The mixture was heated at 60 °C until the solution became clear. The same winterization procedure as above was followed, and the supernatant was added to the dewaxed RBO (RBO raffinate). To the hexane-

washed RBW, the same volume of isopropanol was added and heated at 80 °C. After winterization and centrifugation, the supernatant (Isopropanol-soluble fraction) was set aside. The isopropanol-washed RBW was distilled in the same volume of isopropanol containing a droplet of 10% aqueous sodium borohydride (Sigma, St. Louis, MO) and filtered through a 0.2-μm membrane filter while the solution was still hot. The filtered particles were added to the isopropanol-soluble fraction. The filtered solution was placed in the centrifugal evaporator, and after solvent evaporation, the remaining material was washed with 1 ml of distilled water. The precipitate (Refined RBW) was dried at 80 °C, and the yield was calculated. The water used for the washing was added to the isopropanol-soluble fraction. After the washed-out fraction was dried in the centrifugal evaporator, 2 ml of hexane was added to the dried matter and the mixture was heated at 60 °C with vigorous agitation. The hexane layer was removed into a clean test tube. This lipid extraction was done twice. After hexane evaporation, the extracted lipid (isopropanol-soluble RBW, IS RBW) was weighed. All the final products, RBO raffinate, Refined RBW, and IS RBW were stored at -20 °C for HPLC analysis.

1.4.6. WE Preparation and Determination of WE Contents by HPLC

HPLC analysis of RBO samples was performed with a Waters Delta Prep 4000 preparative chromatography system (Waters Corporation, Milford, Massachusetts, USA), and an ELSD detector (Shimadzu low temperature evaporative light scattering detector ELSD-LT2, Shimadzu, Kyoto, Japan). A 2-ml sample loop was connected with the preparative HPLC system. The data was processed with a Shimadzu GC solution program (Shimadzu Corporation, Kyoto, Japan). Only HPLC-grade solvents were used for all analysis. The standard materials for HPLC analysis, octacosane (C28), stearyl stearate (SS), stearate methyl ester (SME), behenate methyl ester

(BME), cholesteryl stearate (CS), and tristearine (TS) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

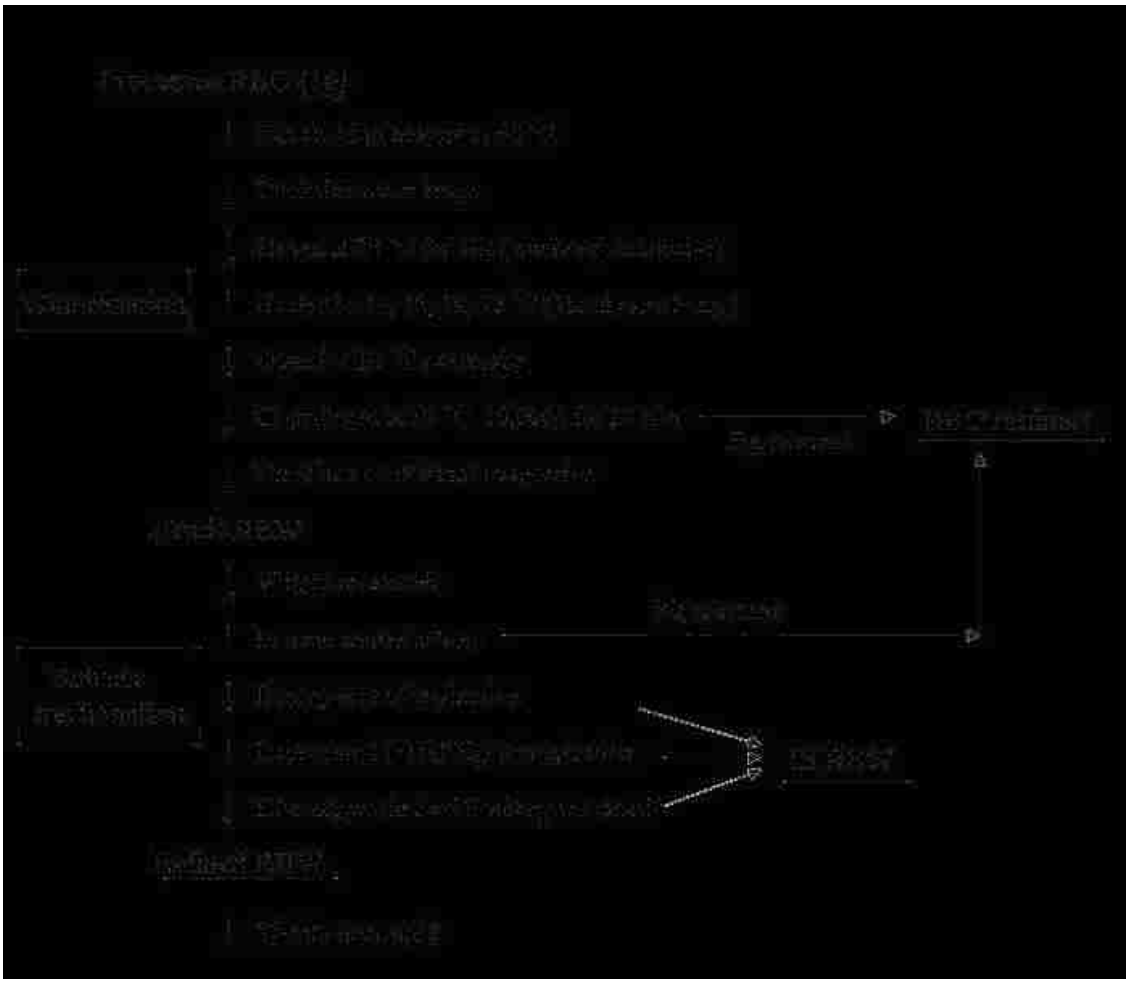


Figure 1.3. Winterization and Solvent fractionation. The underlined items were the final products of all the processes.

RBWE was separated from the processed RBO, RBO raffinate, IS RBW, and Refined RBW by low pressure column chromatography and preparative HPLC. For low pressure column chromatography, 30 g of silica gel (60 – 200 mesh, EM Science, Gibbstown, NJ) was baked at 150 °C overnight and hydrated with 1.0 ml of distilled water. The silica gel was mixed with 200 ml of hexane, and the hexane slurry was packed into a glass column with a 300-ml solvent

reservoir (2 cm i.d x 25 cm length). Glass balls (3 mm i.d.) were placed just above the silica gel surface to prevent surface fluctuation. Five hundred milligrams of each of the processed RBO, RBO raffinate, IS RBW, or refined RBW from FF RBO or DF RBO extracted by the Soxhlet method was mixed with 10 ml of hexane, and heated until it became transparent. The hexane solution was loaded onto the glass column. The portion of the column packed with glass balls, just above the silica gel surface, was wrapped with heating tape to prevent crystallization. Slight air pressure was applied to increase the flow rate. The WE-rich fraction was eluted with hexane:diethyl ether (95:5, v/v), and the silica gel was washed with 200 ml of acetone and methanol. The solvent was heated at 50 °C before use. After solvent evaporation, the WE-rich fraction was weighed, and mixed with 4 volumes hexane. One milliliter of the hexane solution containing about 250 μg of the WE-rich fraction was injected into the preparative HPLC and loaded onto a preparative column. For this preparative HPLC, 220 g of TLC-grade silica gel (SilicAR TLC-7, 35 – 60 μm, Mallinckrodt, Phillipsburg, NJ, USA) was packed into a stainless steel column (5 cm i.d., Botanicals, Montgomeryville, PA) and compressed to the length of 20 cm. The evaporation temperature for ELSD was set to 50°C, and nitrogen was used for solvent evaporation at the pressure of 30 psi. The sensitivity of ELSD was set to 1 (lowest) and ELSD data acquisition started 5 min after injection. The column, the stainless steel tubing and the sample loop were wrapped with heating tapes to maintain the temperature of the solvent and the column at 40 °C. The injected lipid was eluted with hexane (Solvent A), hexane:diethyl ether (10:1, v/v, Solvent B), and chloroform (Solvent C); Solvent A100% for the first 5 min, A50%B50% for the next 15 min, and C100% for the rest of the time. The flow rate was fixed at 40 ml/min, and the eluent was distributed to the ELSD and the fraction collector at the ratio of 2.5:37.5. The WE fraction was collected and weighed after solvent evaporation. The WE was

used as the weight standard for analytical HPLC.

For analytical HPLC, the processed RBO, RBO raffinate, IS RBW, or refined RBW was made into 10 mg/ml solutions with hexane, and 10 μl of each sample was injected. Two analytical silica (2SI) columns (Supelcosil LC-SI column, 250 mm x 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA) connected consecutively with a 5-cm stainless steel tubing and coupled with a guard column (50 mm x 2 mm), which was manually packed with TLC-grade silica gel (SilicAR TLC-7, 35 – 60 μm, Mallinckrodt, Phillipsburg, NJ, USA), were used for the analysis. The column, the stainless steel tubing and the sample loop were wrapped with heating tapes to maintain the temperature of the solvent and the column at 50 °C. The ELSD sensitivity was set to 3 (middle), and the ELSD data acquisition started 2 min after injection. The standard substances were prepared in hexane at 1 mg/ml, and 1 to 25 μg (1 to 25 μl) was injected. The injected lipids were eluted with Hexane (Solvent A) for the first 5 min, and for the next 10 min, hexane:diethyl ether (20:1, v/v, Solvent B) was used. The columns were washed with methanol:acetone (50:50, v/v, Solvent C) for 10 min. During operation, the flow rate was fixed at 2.5 ml/min. The WE obtained from preparative HPLC was prepared in hexane at the concentration of 1.0 mg/ml, and 1, 2, 3, 4, 5, 10, 15, 20, 25, and 50 μg was injected. The standard curves appear in fig.1.4. The amounts of WE in the RBO and RBW samples were calculated by the regression equations.

1.4.7. Data Analysis

For statistical data analysis, 4 sets of RBO samples were prepared in each of 6 extraction conditions for FF RB and DF RB. The data were analyzed with SAS One-way ANOVA program ($p<0.05$), and Tukey's studentized range test was used for the comparison of any significant differences ($p<0.05$).

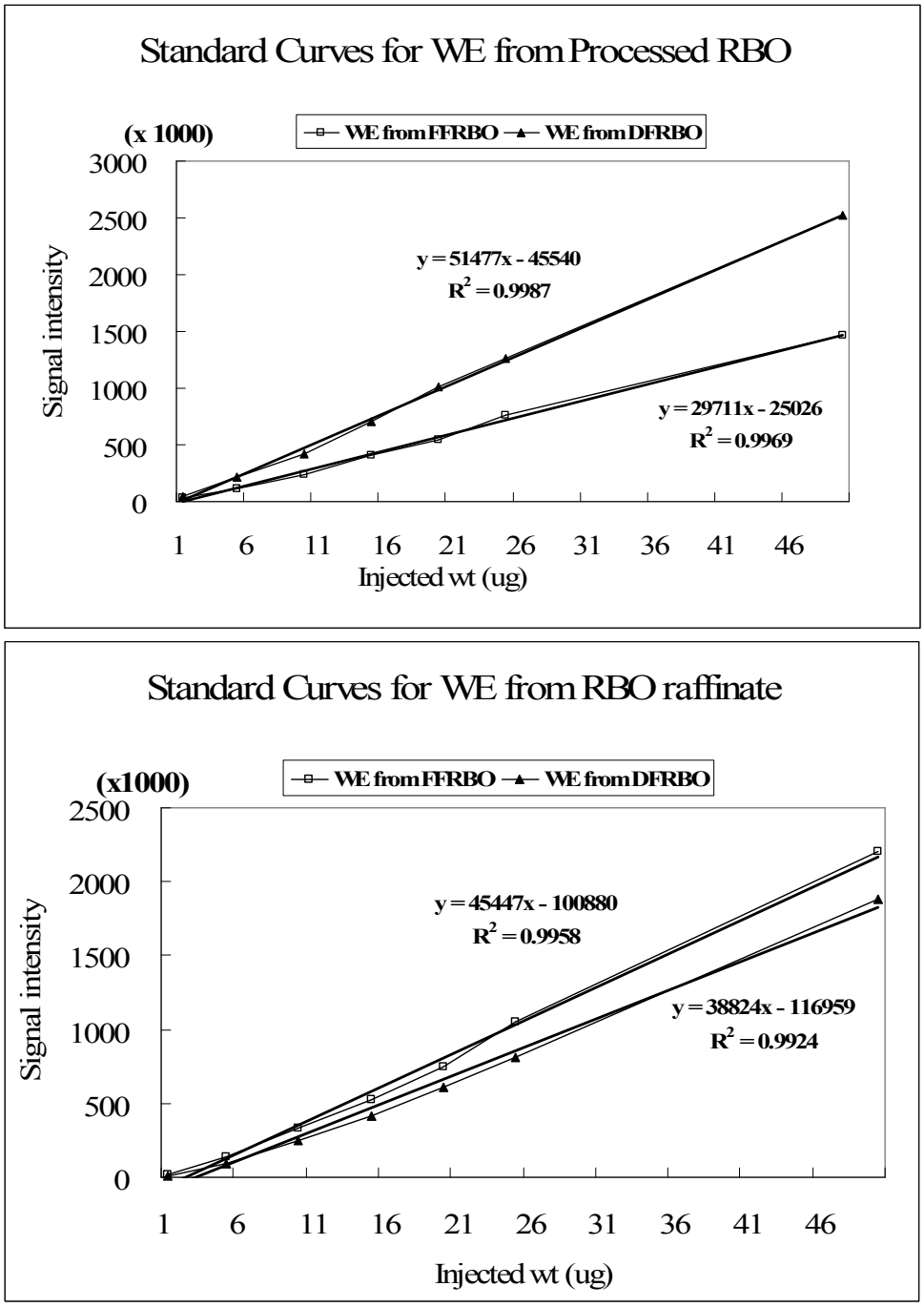
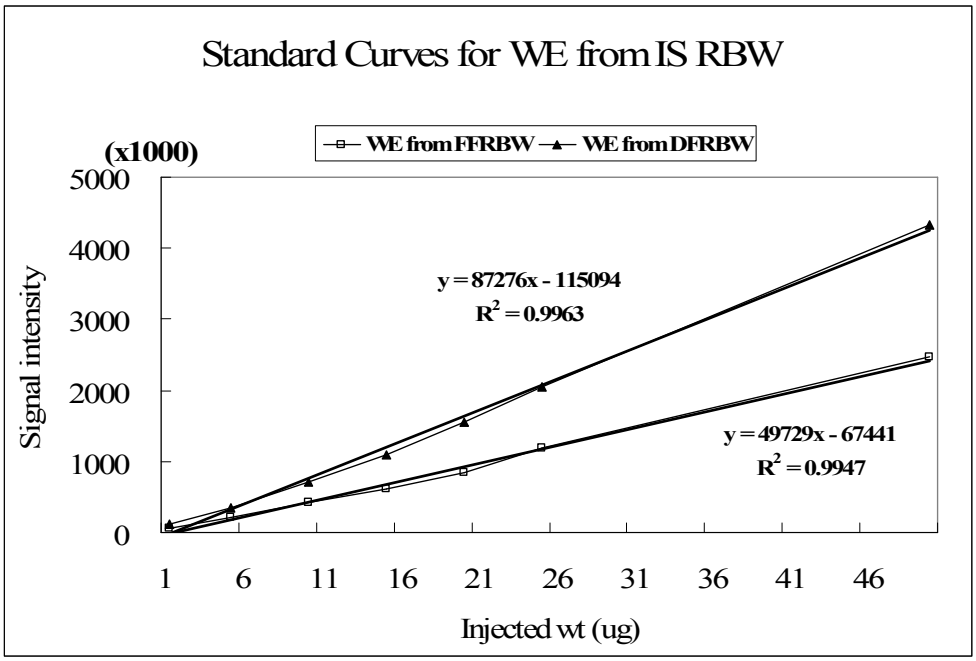


Figure 1.4. Standard curves and Regression equations for WE.



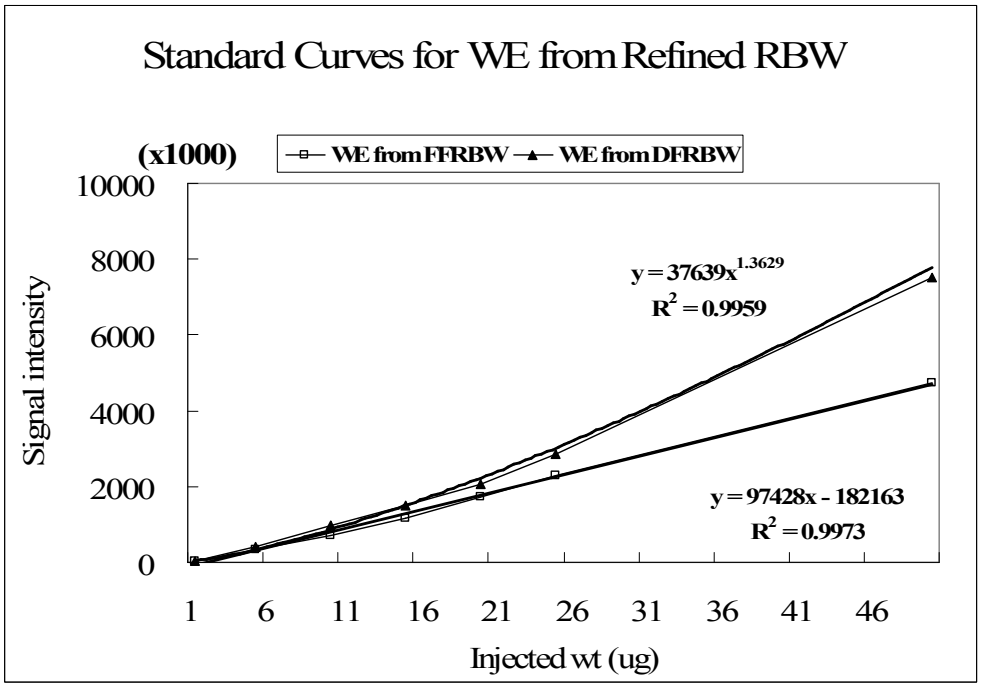


Figure 1.4. (Continued)

1.5. RESULTS

The crude RBO yields from FF RB were almost 5 or 6 times larger than those from DF RB (table 1.1). There was no significant difference between the oil yields (~20%) from FF RB due to the extraction methods. On the other hand, for DF RB, MAE with isopropanol at 120°C showed significantly higher crude oil yields (4.39 - 4.67%) than the other methods (3.14 - 3.81%). Isopropanol extractions produced slightly higher oil yields from DF RB than hexane extractions. The hexane separation (HS) method for crude RBO recovery showed slightly higher yields than the hexane-water separation (HWS) in MAE at 80 ^{o}C from DF RB, but the difference was not statistically significant in other MAE methods ($p=0.63$). Table 1.1 also shows the yields of the processed RBO from the crude RBO. The crude FF RBO samples were found to contain less material (i.e. higher yields) which could be removed during oil processing. The crude RBO prepared by MAE with isopropanol at 80 °C presented the lowest yields of the processed FF RBO (67.18 – 68.69%) and DF RBO (31.37 – 36.00 %), and the other methods showed higher yields with FF RB (72.36 – 76.30%) and DF RB (53.36 – 60.42%), which means that higher extraction temperature with isopropanol significantly contributed to the processed RBO yields. The processed RBO yields from DF RB with HS and HWS were significantly higher ($p<0.05$) than with HS, but those from FF RB were not significantly different ($p>0.05$).

Crude RBW yields (4.81 – 17.57 mg/g RB) from FF RB were higher than those from DF RB (1.12 – 7.60 mg/g RB) (table 1.2). Hexane extracted 2 or 3 times more crude RBW than isopropanol from RB. Soxhlet and MAE with hexane extracted more crude RBW from FF RB and from DF RB, respectively. HS and HWS for crude RBO recovery showed a significant difference in the crude RBW yield from FF RB, but not from DF RB. The elevated extraction temperature yielded more crude RBW from DF RB, but not from FF RB. The processed DF

RBO had a higher concentration of crude RBW (9.40 – 39.44 %) than that from FF RBO (3.67 – 11.24%), and the hexane-extracted RBOs showed higher crude RBW yields than the isopropanol-extracted RBOs (table 1.2). The RBO raffinate yields, after hexane winterizations, were higher in the isopropanol-extracted RBO than the hexane-extracts, and the differences were smaller in the FF RBO raffinate.

Table 1.1. Stepwise yields[a] of RBO

Extraction Methods	RB	Crude RBO	Processed RBO
		wt% from RB[b]	wt% from Crude RBO[b]
SOXHLET	FF RB	20.49 ± 0.08^{c}	76.30 ± 1.91^{c}
MAE (HEX, 80)	FF RB	20.25 ± 0.36^{c}	74.64 ± 1.87^{c}
MAE (ISO, 80, HS)	FF RB	20.91 ± 0.15^{c}	67.18 ± 1.68^{d}
MAE (ISO, 80, HWS)	FF RB	20.45 ± 0.43^{c}	68.69 ± 1.72^{d}
MAE (ISO, 120, HS)	FF RB	20.94 ± 0.47^{c}	72.36 ± 1.81^{c}
MAE (ISO, 120, HWS)	FF RB	19.95 ± 0.44^{c}	75.95 ± 1.90^{c}
SOXHLET	DF RB	3.14 ± 0.11^{c}	54.94 ± 1.12^{c}
MAE (HEX, 80)	DF RB	3.19 ± 0.07^{c}	60.42 ± 1.51^{d}
MAE (ISO, 80, HS)	DF RB	3.81 ± 0.13^{d}	31.37 ± 0.78^{e}
MAE (ISO, 80, HWS)	DF RB	3.32 ± 0.14^{c}	36.00 ± 0.90^{f}
MAE (ISO, 120, HS)	DF RB	4.67 ± 0.21^{e}	53.36 ± 1.33^{c}
MAE (ISO, 120, HWS)	DF RB	4.39 ± 0.13^{e}	56.77 ± 1.42^{g}

a: n=4, and all values are expressed as mean value ± SD. b: All yields are significantly higher with FF RB in all extraction methods ($p<0.05$). c-g: Values with different superscripts are significantly different within the subgroup (with the same RB in the same column) ($p<0.05$). The graphical illustration of table1.1 appears in Appendix 1.

The refined RBW yields from crude RBW indicates that the solvent fractionation removed a large part of crude RBW and left a relatively small amount of refined RBW (table 1.3). The refined wax yields from FF RB were similar to those from DF RB. The HS and HWS methods for crude RBO recovery did not have any significant effects on the refined RBW yields from FF RB, but did on those from DF RB. The refined RBW yields from the processed RBO indicated

that hexane is more efficient for wax extraction than isopropanol. Soxhlet extraction and MAE with hexane yielded 1.68% and 1.54% from the processed FF RBO and 9.45% and 11.78% from the processed DF RBO, respectively, while the MAE with isopopanol achieved less than 0.15% from the processed FF RBO and less than 1.52% from the processed DF RBO. The yields from crude RBW were 3 or 4 times higher with hexane extraction than isopropanol extraction from both of the RBs. The extraction temperature with MAE appeared to affect the refined RBW yields, but the difference was very small, compared to the difference from hexane extractions (table 1.3).

The weight percentages of IS RBW as well as red precipitates from the crude RBW also appear in table 1.4, which shows that the solvent fractionation process removed a great portion of the crude RBW. Hexane-extracted RBO yielded relatively smaller percentages of IS RBW.

The chromatograms from preparative HPLC are shown in fig.1.5 when the WE-rich fraction from the processed FF RBO or DF RBO was injected. The purpose of this prep HPLC was to prepare the weight standards for analytical HPLC. The chromatograms of WE-rich fractions from the RBO raffinates, the IS RBWs, and the refined RBWs appeared with similar peak patterns (chromatograms not shown).

The chromatograms of the analytical method are shown in fig.1.6. The WE appeared as a sharp peak near 6 min. The WE standards were detected at the same positions. The hydrocarbons appeared between 1 and 2 min, and the triglycerides at 16 min. Figure 1.7 shows the chromatograms with injections of refined FF RBO, FF RBO raffinate, and refined FF RBW. The chromatograms of analytical HPLC with injections of refined DF RBO, DF RBO raffinate, and refined DF RBW appear in fig 1.8. In both figures, refined RBW represented stronger signals than RBO samples, and the refined DF RBW yielded the highest peak among all other samples.

Table 1.2. Stepwise yields[a] of Crude RBW

Extraction Methods	RB	Crude RBW			RBO raffinate
		from RB (mg/g)[b]	wt% from Crude RBO[c]	wt% from Processed RBO[c]	wt% from Processed RBO[b]
SOXHLET	FF RB	17.57±0.34[d]	8.57±0.17[d]	11.24±0.22[d]	88.76±4.22[d]
MAE (HEX, 80)	FF RB	13.69±0.29[e]	6.76±0.09[e]	9.06±0.42[e]	90.94±3.52[d]
MAE (ISO, 80, HS)	FF RB	6.18±0.26[f]	2.95±0.11[f]	4.40±0.21[f]	95.60±4.61[e]
MAE (ISO, 80, HWS)	FF RB	5.63±0.07[g]	2.75±0.12[g]	4.30±0.20[f]	96.59±4.04[e]
MAE (ISO, 120, HS)	FF RB	6.42±0.28[f]	3.14±0.17[f]	4.47±0.14[f]	95.65±4.23[e]
MAE (ISO, 120, HWS)	FF RB	4.81±0.26[h]	2.41±0.18[g]	3.67±0.14[g]	96.83±4.37[e]
SOXHLET	DF RB	6.30±0.28[d]	20.01±0.66[d]	35.87±1.14[d]	67.63±2.81[d]
MAE (HEX, 80)	DF RB	7.60±0.12[e]	23.83±1.07[e]	39.44±1.61[e]	60.56±2.61[e]
MAE (ISO, 80, HS)	DF RB	1.14±0.07[f]	3.00±0.17[f]	9.56±0.45[f]	90.44±3.55[f]
MAE (ISO, 80, HWS)	DF RB	1.12±0.09[f]	3.38±0.18[f]	9.40±0.48[f]	90.60±3.78[f]
MAE (ISO, 120, HS)	DF RB	2.48±0.17[g]	5.30±0.19[g]	9.94±0.29[f]	90.06±3.62[f]
MAE (ISO, 120, HWS)	DF RB	2.45±0.11[g]	5.59±0.21[g]	9.85±0.25[f]	90.15±4.25[f]

a: n=4, and all values are expressed as mean value ± SD. b: Values with FF RB are significantly higher than those with DF RB in all extraction methods ($p<0.05$). c: Values with DF RB are significantly higher than those with FF RB in all extraction methods ($p<0.05$). d-h: Values with different superscripts are significantly different within the same subgroup (with the same RB in the same column) ($p<0.05$). The graphical illustration of table1.2 appears in Appendix 2.

Table 1.3. Stepwise yields[a] of Refined RBW.

Extraction Methods	RB	from RB (mg/g)	wt% from Crude RBO[b]	wt% from Processed RBO[b]	wt% from Crude RBW[b]
SOXHLET	FF RB	2.63±0.13[c]	1.28±0.06[c]	1.68±0.14[c]	14.97±0.29[c]
MAE (HEX, 80)	FF RB	2.33±0.12[d]	1.15±0.06[c]	1.54±0.08[c]	16.59±0.32[d]
MAE (ISO, 80, HS)	FF RB	0.21±0.01[e]	0.10±0.01[d]	0.15±0.01[d]	4.63±0.49[e]
MAE (ISO, 80, HWS)	FF RB	0.20±0.01[e]	0.10±0.01[d]	0.14±0.01[d]	4.12±0.07[e]
MAE (ISO, 120, HS)	FF RB	0.13±0.01[f]	0.06±0.01[e]	0.10±0.01[e]	3.18±0.18[f]
MAE (ISO, 120, HWS)	FF RB	0.14±0.01[f]	0.07±0.01[e]	0.11±0.01[e]	3.64±0.23[f]
SOXHLET	DF RB	2.33±0.12[c]	5.29±0.26[c]	9.45±0.47[c]	24.64±0.51[c]
MAE (HEX, 80)	DF RB	2.27±0.11[c]	7.12±0.36[d]	11.78±0.73[d]	25.74±0.58[c]
MAE (ISO, 80, HS)	DF RB	0.17±0.01[d]	0.44±0.02[e]	1.40±0.14[e]	6.80±0.22[d]
MAE (ISO, 80, HWS)	DF RB	0.21±0.01[e]	0.64±0.03[f]	1.52±0.03[f]	6.73±0.33[d]
MAE (ISO, 120, HS)	DF RB	0.33±0.02[f]	0.50±0.03[e]	1.32±0.07[e]	7.58±0.15[e]
MAE (ISO, 120, HWS)	DF RB	0.35±0.02[f]	0.61±0.03[f]	1.41±0.07[e]	7.01±0.39[d]

a: n=4, and all values are expressed as mean value ± SD. b: Values with DF RB are significantly higher than those with FF RB in all extraction methods ($p<0.05$). c-f: Values with different superscripts are significantly different within the same subgroup (with the same RB in the same column) ($p<0.05$). The graphical illustration of table1.3 appears in Appendix 3.

Table 1.4. Yields (wt%)[a] of IS RBW and other impurities from crude RBW

Extraction Methods	RB	IS RBW	Red precipitate and other impurity
SOXHLET	FF RB	22.34±1.25[b]	62.04±3.10[b]
MAE (HEX, 80)	FF RB	25.14±1.17[c]	61.30±3.06[b]
MAE (ISO, 80, HS)	FF RB	52.18±2.77[d]	41.61±2.08[c]
MAE (ISO, 80, HWS)	FF RB	56.67±2.14[e]	39.17±1.96[c]
MAE (ISO, 120, HS)	FF RB	50.64±2.83[d]	51.17±2.56[d]
MAE (ISO, 120, HWS)	FF RB	59.09±2.95[e]	37.27±1.86[c]
SOXHLET	DF RB	16.53±0.83[b]	57.06±2.85[b]
MAE (HEX, 80)	DF RB	17.05±0.85[b]	53.85±2.69[c]
MAE (ISO, 80, HS)	DF RB	42.52±1.66[c]	50.18±2.51[d]
MAE (ISO, 80, HWS)	DF RB	43.29±1.76[c]	51.87±2.59[c]
MAE (ISO, 120, HS)	DF RB	50.00±1.50[d]	48.18±2.41[d]
MAE (ISO, 120, HWS)	DF RB	53.85±2.69[d]	39.42±1.97[e]

a: n=4, and all values are expressed as mean value ± SD. b-e: Values with different superscripts are significantly different within the same subgroup (with the same RB in the same column) ($p<0.05$). The graphical illustration of table1.4 appears in Appendix 4.

The WE separated from the prep method was injected into the analytical columns, and the weight standard curves were drawn (fig. 1.4). The standard curves for WEs from DF samples showed stronger signals than those from FF samples, except the curves for WE from the RBO raffinate. From each curve, its own trend line and the regression equation were found, and based on those equations, the amount of WE in the injected sample was calculated.

The WE contents in the processed RBO and the final products are shown in the table 5. The WE contents in the hexane-extracted processed FF RBOs and DF RBOs were almost 2 and 3 times larger, respectively, than the isopropanol-extracted processed RBOs. This trend was also shown in the WE contents of the other final products. Unlike RBW, MAE with hexane appeared more efficient in RBWE extraction than Soxhlet. The WE contents in the RBO raffinate samples reached 10 % and 11 % in hexane-extracted FF RBOs and DF RBOs, respectively. Comparing the WE content in the processed RBO and the RBO raffinate extracted with hexane, only about 10% of WE was removed by winterization and solvent fractionation from the processed FF RBO, and 34-37% of WE from the processed DF RBO.

In the case of MAE-extracted RBO, about 3 and 15% of WE was removed from the processed FF RBO and DF RBO. IS RBWs also had significant WE contents, which were higher in MAE with hexane. The crude RBO recovery methods, HS and HWS, did not show any significant difference. Nearly half of the refined RBW was found to be WE, and the WE contents in the refined RBW samples from FF RB and DF RB were not significantly different ($p=0.12$).

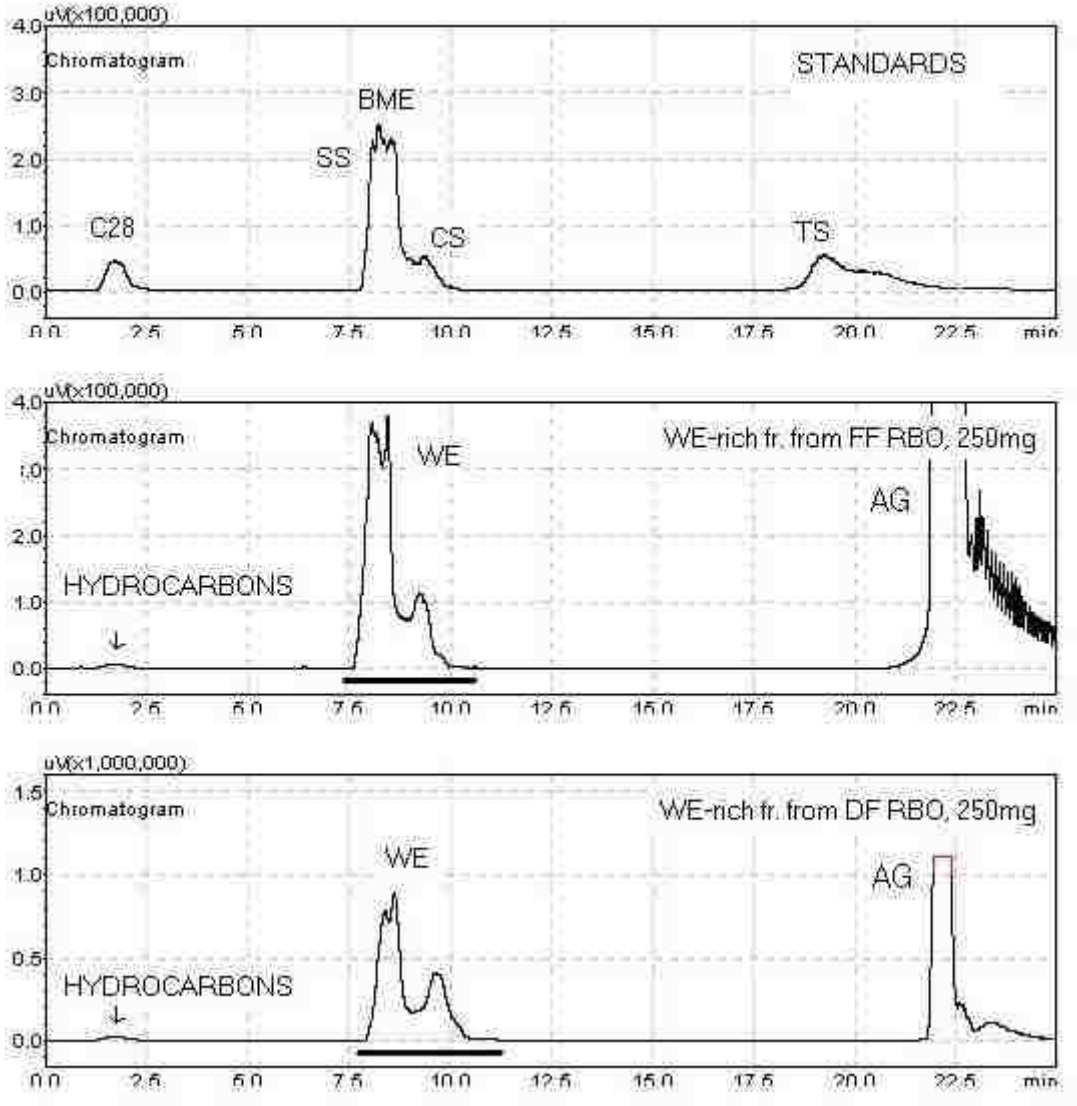


Figure 1.5. Chromatograms of preparative HPLC. Standard mixture containing 100 μg of each (upper), WE-rich fraction from FF RBO (middle) and DF RBO (bottom). C28: octacosane, SS: stearyl stearate, BME: behenate methyl ester, CS: cholesteryl stearate, TS: tristearine, WE: wax esters, AG: acylglycerides.

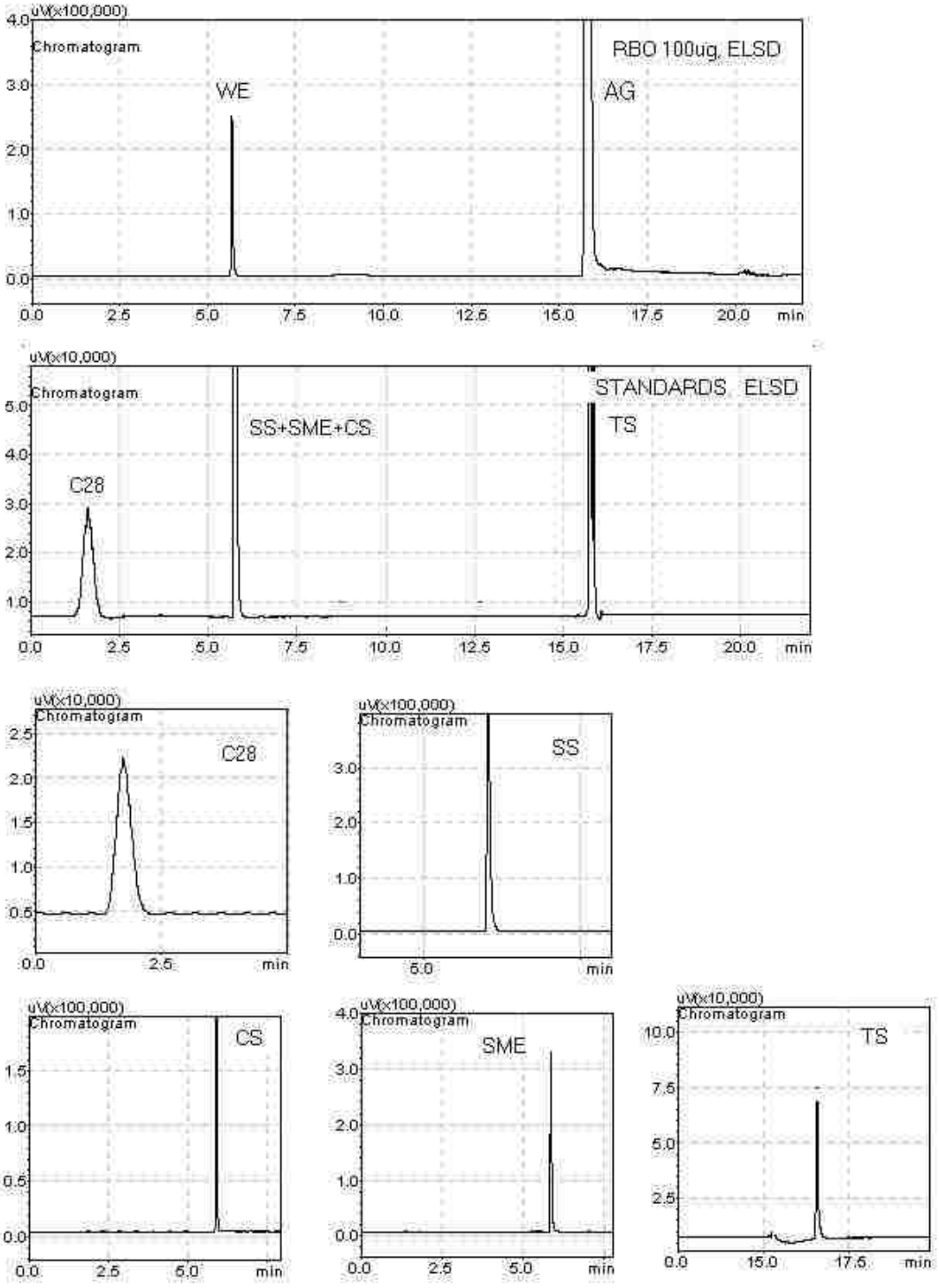


Figure 1.6. Chromatograms of analytical HPLC. FF RBO (upper), the standard mixture (middle), and 5 standards (bottom). C28: octacosane, SS: stearyl stearate, SME: stearate methyl ester, CS: cholesteryl stearate, TS: tristearine, WE: wax esters, AG: acylglycerides.

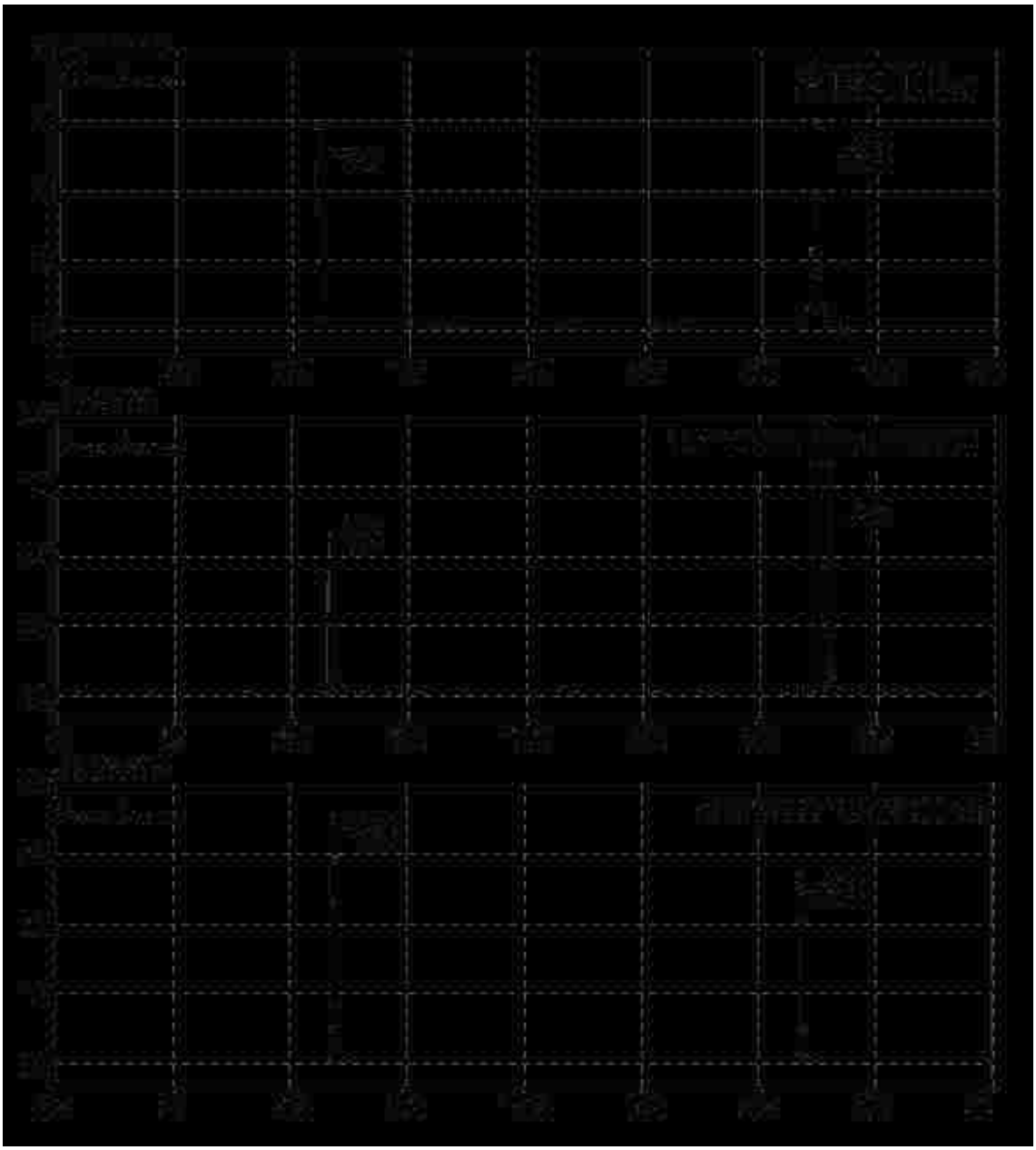


Figure 1.7. Chromatograms of analytical HPLC with injections of refined FF RBO (upper), FF RBO raffinate (middle), and refined FF RBW (bottom). WE: wax esters, AG: acylglycerides.

The WE distributions among the final products are shown in table 6. The amount of WE in the processed RBO was considered as 100 %. More than 80 % of the total WE was contained in the RBO raffinate, except for the DF RBO raffinates that had less than 65 % of WE contents. IS RBWs contained less than 5% of the total WE. The refined DF RBWs were found to have significantly larger contents of WE than the refined FF RBWs, and especially

the refined DF RBW from hexane-extraction methods contained more than 30 % of total WE, which were 4 times larger than those of the refined FF RBW from the same extraction methods. The WE contents, less than 1.45 % in the refined FF RBW and less than 10.42 % in the refined DF RBW from isopropanol-extraction methods, were much lower than those in the refined RBW from hexane-extraction methods. The crude RBO recovery methods, HS and HWS, showed similar values. The WE contents in the final products from MAE with hexane and Soxhlet extraction did not show significant differences except those in IS RBW.

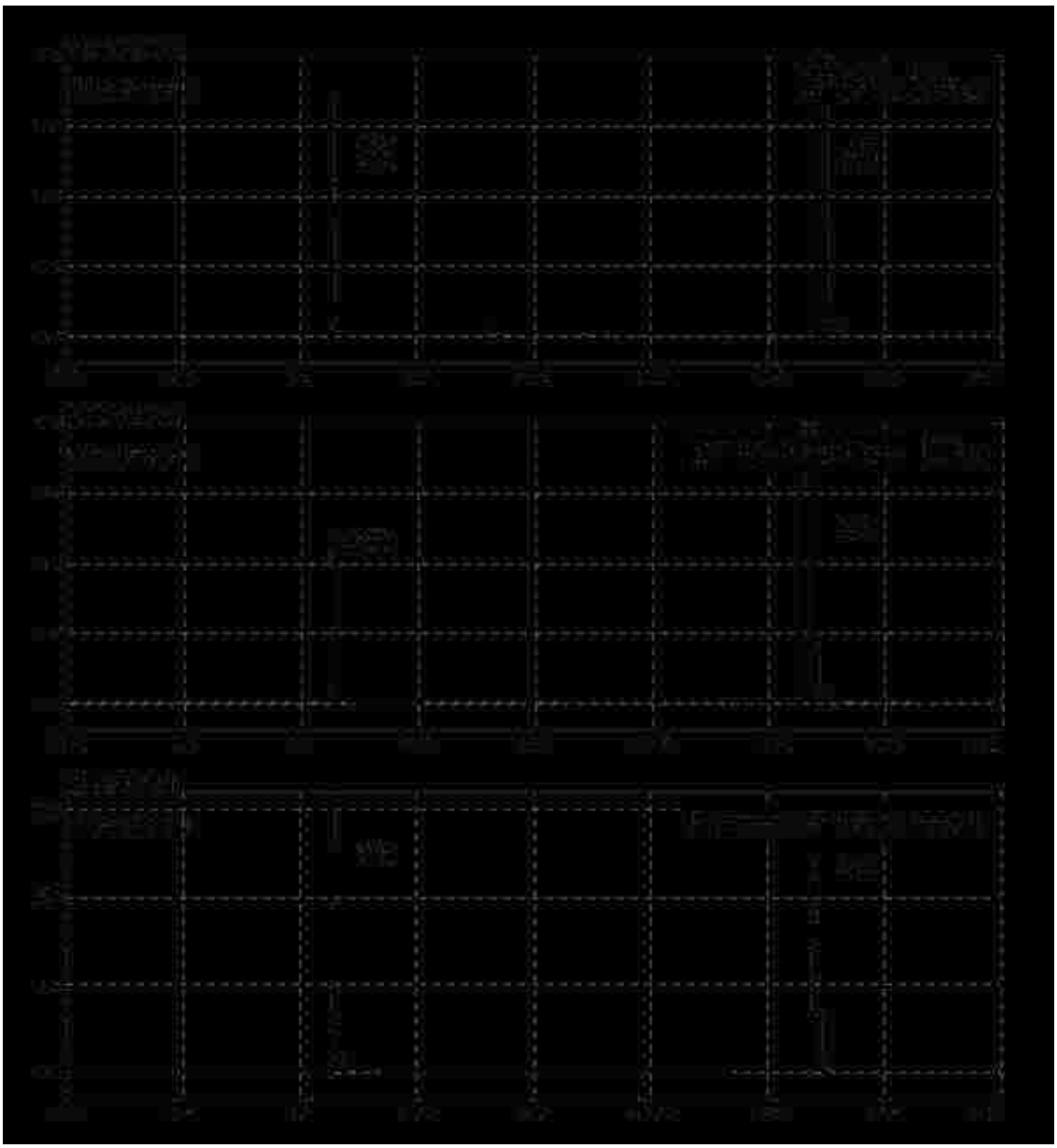


Figure 1.8. Chromatograms of analytical HPLC with injections of refined DF RBO (upper), DF RBO raffinate (middle), and refined DF RBW (bottom). WE: wax esters, AG: acylglycerides.

Table 1.5. WE contents[a] (%) in the processed RBO and final products

Extraction Methods	RB	Processed RBO	RBO raffinate	IS RBW	Refined RBW
SOXHLET	FF RB	10.32±0.52^{b,g}	9.34±0.28^{b,g}	7.29±0.36^{b}	47.45±1.48^{b}
MAE (HEX, 80)	FF RB	11.18±0.55^{c,g}	10.04±0.27^{c,g}	12.58±0.27^{c}	55.97±2.80^{c}
MAE (ISO, 80, HS)	FF RB	5.69±0.07^{d}	5.54±0.26^{d}	4.19±0.21^{d}	54.89±2.54^{c}
MAE (ISO, 80, HWS)	FF RB	6.47±0.27^{e}	6.29±0.24^{e}	5.82±0.07^{e}	52.94±2.65^{c}
MAE (ISO, 120, HS)	FF RB	5.71±0.27^{d}	5.59±0.24^{d}	4.96±0.25^{f}	43.65±2.67^{d}
MAE (ISO, 120, HWS)	FF RB	5.23±0.16^{f}	5.08±0.22^{d}	5.62±0.09^{e}	49.40±1.47^{b}
SOXHLET	DF RB	15.86±0.56^{b,g}	10.44±0.34^{b,g}	8.20±0.31^{b}	52.21±1.95^{b}
MAE (HEX, 80)	DF RB	18.31±0.85^{c,g}	11.54±0.29^{c,g}	12.15±0.51^{c}	50.44±1.52^{b}
MAE (ISO, 80, HS)	DF RB	5.46±0.25^{d}	4.69±0.22^{d}	4.66±0.09^{d}	40.54±1.88^{c}
MAE (ISO, 80, HWS)	DF RB	5.99±0.47^{e}	5.12±0.19^{e}	4.36±0.22^{d}	44.05±2.20^{d}
MAE (ISO, 120, HS)	DF RB	6.02±0.27^{e}	5.12±0.27^{e}	5.68±0.24^{e}	47.03±1.35^{d}
MAE (ISO, 120, HWS)	DF RB	6.43±0.19^{e}	5.51±0.11^{f}	4.81±0.24^{d}	45.12±2.26^{d}

a: n=4, and all values are expressed as mean value ± SD. b-f: Values with different superscripts are significantly different within the same subgroup (with the same RB in the same column) ($p<0.05$). g: Values with DF RB are significantly higher than those with FF RB ($p<0.05$). The graphical illustration of table1.5 appears in Appendix 5.

Table 1.6. WE distribution data[a] (%) in the final products. The amount of WE in the processed RBO was considered as 100%.

Extraction Methods	RB	Processed RBO (total %)	RBO raffinate[b]	IS RBW[b]	Refined RBW[b]
SOXHLET	FF RB	100	90.47±2.68[c]	1.80±0.09[c]	7.73±0.62[c]
MAE (HEX, 80)	FF RB	100	89.82±2.40[c]	2.46±0.12[d]	7.73±0.39[c]
MAE (ISO, 80, HS)	FF RB	100	97.27±4.55[d]	1.28±0.06[e]	1.45±0.14[d]
MAE (ISO, 80, HWS)	FF RB	100	97.13±3.74[d]	1.72±0.09[c]	1.15±0.01[e]
MAE (ISO, 120, HS)	FF RB	100	97.92±4.22[d]	1.31±0.07[e]	1.07±0.04[e]
MAE (ISO, 120, HWS)	FF RB	100	97.15±4.21[d]	1.85±0.09[c]	1.00±0.09[e]
SOXHLET	DF RB	100	65.83±2.15[c]	3.06±0.10[c]	31.11±1.56[c]
MAE (HEX, 80)	DF RB	100	63.03±1.60[c]	4.51±0.16[d]	32.46±2.00[c]
MAE (ISO, 80, HS)	DF RB	100	86.00±4.03[d]	3.58±0.18[e]	10.42±1.05[d]
MAE (ISO, 80, HWS)	DF RB	100	85.42±3.17[d]	3.41±0.17[e]	11.18±0.21[d]
MAE (ISO, 120, HS)	DF RB	100	84.98±4.49[d]	4.75±0.24[d]	10.28±0.52[d]
MAE (ISO, 120, HWS)	DF RB	100	85.76±1.71[d]	4.33±0.22[d]	9.91±0.49[d]

a: n=4, and all values are expressed as mean value ± SD. b: Values with FF RB are significantly different from those with DF RB.
c-e: Values with different superscripts are significantly different within the same subgroup (with the same RB in the same column) ($p<0.05$).
The graphical illustration of table1.6 appears in Appendix 6.

1.6. DISCUSSION

The goal of this research was to characterize the relative distribution of "wax" components that could be recovered from either FF or DF RB for further evaluation of potential health promoting potentials. The long term utility of this research would be to develop processing procedures for the recovery of the most promising fractions from either type of RB. The crude RBO yields from FF RB and DF RB were about 20% and 3.5%, respectively. There is no published data comparable for yield from DF RB, and that from FF RB is at the upper level of the range found in other studies (Zhao et al., 1987; Saito et al., 1993; Shen et al., 1997; Xu & Godber, 2000). The extraction conditions of MAE were chosen on the basis of Zigoneanu et al. (2007). They found that the RBO yield from FF RB by MAE with isopropanol for 15 min reached a maximum level (15%) at 120 °C, and that the RBO yield (14%) by MAE with hexane for 15 min was not significantly dependent on temperature. Their yields were much lower than those (20%) found in this study, and the yield difference probably resulted from the difference in extraction times. The extracted RBO was solid at room temperature, so when it was removed into another vessel or was mixed with other reagents, it was heated at 80 °C. This high melting point could cause errors in the experiment unless glass Pasteur pipettes were heated by a flame and washed with hot solvent to prevent the oil from sticking onto the inside-pipette wall, whenever the oil was transferred. Crystallizable lipid species are known to cause problems in experimental procedures such as filtration and HPLC analysis because they frequently clog filters, columns, or parts of the HPLC. In this study, it was found that an elevated temperature was necessary throughout all experimental procedures.

During the degumming process, the RBO was mixed with aqueous solution, vortexed, and centrifuged at high temperature. This vortexing step produced a stable emulsion layer between

water and oil layers that remained even after centrifugation. This emulsion probably contained a significant portion of the RBO sample, but it could not be incorporated into the winterization process. The centrifugation step was done using a vacuum centrifugal evaporator (CentriVap Console Labconco, Kansas City, MO) because it could be maintained at an elevated temperature necessary to prevent crystallization of wax. However, its maximum centrifugal force was not sufficient to completely separate the aqueous and nonaqueous layers, which affected the true RBO yield calculation. In spite of the difficulties encountered in the degumming process of RBO, it seemed necessary to remove the phospholipids because they can significantly reduce wax crystal size, possibly due to their emulsifying ability (Morrison and Thomas, 1976), resulting in diminished filtration rates (Leibovitz and Ruckenstein, 1984). The yields of the processed RBO from the crude RBO seems to be dependent on degumming methods. The yields may vary according to the degumming methods, the experimental conditions, and the laboratory workers. However, it became clear that the temperature should be maintained high enough for the RBO to be liquid during the RBO refining process.

At first it appears that isopropanol extracted more RBO than hexane. However, a yellowish particulate material was evident in the isopropanol extracts. It is possible that because isopropanol is a relatively more polar solvent than hexane, it could extract more polar substances from RB than hexane. The particulate matter could be dissolved in water but not in hexane. The amount of this material varied from one RBO sample to another and was not consistent from one extraction to another. Hexane appeared to be more appropriate for wax extraction because the difference in polarity between the two solvents results in higher refined wax yields. Zigoneanu et al. (2007) found that isopropanol-extracted RBO contained more antioxidant components. Evaluating compositional differences in the RBO extracted with different solvents requires

further study.

Several scientists studied the winterization and crystallization of RBO or sunflower seed oil. De and Bhattacharyya (1998) studied wet winterization of RBO, and found that high temperature (65-70°C) degumming and low temperature (10°C) dewaxing by centrifugation at 15,000 g improved oil quality of the degummed RBO. Ghosh and Bandyopadhyay (2005) performed solvent winterization of RBO, and their results indicate that the maximum size (2.5-4 um) of wax crystal is achieved at 10-15 °C, and after 1 hr of incubation at 10 °C, no crystal growth was observed. A solvent winterization study of sunflower seed oil in hexane:acetone (15:85) solution done by Morrison and Robertson (1975) suggested that more wax can be removed at lower crystallization temperature and at a lower concentration of solvents. They also found that the more solvent that was used, the longer the clouding time. Morrison and Thomas (1976) studied solvent winterization and refining of sunflower seed oil (50% in hexane), and found that refining and then winterization removed a greater amount of wax. Even though those studies were performed to improve oil quality, they provided insight into winterization conditions that could be employed for the purpose of wax recovery.

The higher wax yields from DF RBO were expected because Riceland Foods extracted the oil from the RB in a way that would reduce the crystallizable components in the RBO and in turn increase their levels in the RB. The two extraction methods with hexane showed different wax yields, and MAE with hexane was found to be more efficient than the Soxhlet method. Unlike the Soxhlet method, the mixture in the extraction vessel in the MAE device can be vigorously agitated by a magnetic stirrer, which is programmable. The extraction temperature can also be elevated with MAE because the extraction vessel can endure more radical extraction conditions that generate higher pressures. The agitation and higher extraction temperature explain why

MAE achieved higher wax yields from DF RBO.

The yields of crude RBW and refined RBW from RB in table 2 and 3 were calculated using the assumption that the crude RBO was completely degummed and the refined RBO contains most of the RBW lipid minus phospholipids, polar lipid species, and moisture. However, the emulsion problem that occurred during degumming makes the assumption presumptive, which consequently affected RBW yield data.

Vali et al. (2005) used $NaBH_4$ to remove resinous matter in crude RBW during solvent fractionation. They observed a reddish brown solid in the RBW-isopropanol solution. In our study, there was a similar occurrence just after the addition of sodium borohydride; the reddish precipitate was removed by filtration and water washing. They also achieved more than 98% of WE contents in refined RBW using RBO tank settling as a starting material. In our study, processed RBO was used as the starting material rather than tank settlings.

The term “wax” usually refers to the crystallizable matter in oil at low temperature, and wax esters are the main component of wax. Wax esters include alkyl esters and steryl esters. The former gives fatty acids and alcohols, and the latter sterols and fatty acids, when they are hydrolyzed. Differences in terminology and composition can give rise to misconceptions relative to commercial applications of wax components. This is especially troublesome in RBW studies. RBW or certain of its components are being touted as having potential health benefits (Hargrove et al., 2004). Therefore, an analysis of the relative abundance of wax components during its extraction and refining will help guide commercial applications.

To determine the total amount of wax esters in the processed RBO, the RBO raffinate, IS RBW, and the refined RBW, were passed through two consecutively connected Si columns. The original purpose of the 2SI method was to separate alkyl esters from steryl esters, but this

approach failed to separate them. Moreau et al. (2002) used a temperature-enhanced alumina HPLC method, and they could separate them. Unfortunately, the manufacturer of the analytical normal-phase alumina column, Merck, no longer produces the column.

Before the analytical steps, WE were separated from the processed RBO, the RBO raffinate, IS RBW, and the refined RBW in order to obtain analytical standards for quantification. ELSD is a robust choice of detectors, especially for substances that lack UV-detectable chemical structures (Megoulas and Koupparis, 2005). ELSD detects everything that is not volatile and blocks the light path, which means that the signal intensity depends not only on the number of detectable molecules but also the size of the molecules. Thus, it was necessary to develop individual standard curves for all of the WE fractions. Based on the crystallizing nature of the wax esters, even though the same amount of WE samples are injected, the signal intensity may vary when the WE compositions are different. Megoulas and Koupparis (2005) pointed out that one of the deficiencies of ELSD is non-linear response, which was shown in the standard curve of WE from the refined FF RBO in fig.1.4. All the standard curves drawn in this study show different signal intensities and different standard regression curves.

In the HPLC analysis, the RBO raffinates contain most of the WE, with the exception of the DF RBO raffinate from hexane extractions. Comparing the weight data and the WE content data, it is evident that not all the crystallizable material in RBO is WE and not all the WEs are crystallizable. WE components with longer chain lengths are more likely to crystallize in RBO. Therefore, there can be clear differences in the compositions of WE separated from the different final products. RBW and RBWE are definitely different entities as much as they are different materials even though they make up a significant portion of the refined RBW.

The WE contents in the refined RBW samples appeared to be similar, which indicates that the

WE and the acylglycerides in the refined RBW may serve as a seed for RBO crystallization. They can be called “crystallizable wax” at room or even higher temperature. The WE contents in the crude RBW showed different values, but they are still crystallizable at room or lower temperature (<10°C). The WE in the RBO raffinate can be considered noncrystallizable. It is predictable that there must be compositional difference between crystallizable and noncrystallizable WE, which can be identified by GC analysis.

1.7. CONCLUSION

The resulting data indicates that hexane is more efficient for wax extraction than isopropanol, and that MAE with hexane rather than conventional Soxhlet extraction method achieved higher WE contents in the processed RBO and in the refined RBW. HS and HWS methods for crude RBO recovery did not affect the RBW yields but did influence the crude RBO yields. The higher extraction temperature of MAE with isopropanol increased the yields of DF RBO, but it did not improve the FF RBO yield and the RBW yields. DF RB contains significant amounts of RBW, and the refined RBW contents were not significantly different from FF RB. The HPLC analysis proved that not all RBW is WE and not all RBWE is crystallizable.

The results also established an efficient procedure of RBW preparation for GC analysis, which includes extraction with MAE, winterization, solvent fractionation, and HPLC separation. This method can be used not only for RBW analysis but also for analysis of WE from other edible oils.

MAE with hexane is the most efficient method for generation of higher amounts of RBW as well as higher WE contents. MAE requires less time and less solvent than Soxhlet extraction. The next studies will focus on the WE composition analysis using GC or GC-MS and the potential applications of the components of RBWE in biomedical fields.

1.8. REFERENCES

Belavadi VK and Bhowmick DN. An investigation of rice bran oil tank settlings. JAOCS. 65(2):241-245.

Bennett H. Industrial waxes, volume 1. Natural and Synthetic waxes. 1975, Chemical Publishing Company, INC., New York, NY.

Cousins ER, Fore SP, Janssen HJ, and Feuge RO. Rice bran oil. VIII. Tank settlings from crude rice bran oil as a source of wax. JAOCS, 1953, 30:9-14.

De BK and Bhattacharyya DK. Physical Refining of rice bran oil in relation to degumming and dewaxing. JAOCS. 1998, 75(11):1683-1686.

Dulin MF, Hatcher LF, Sasser HC, Barringer TA. Policosanol is ineffective in the treatment of hypercholesterolemia: a randomized controlled trial. Am J Clin Nutr. 2006. 84(6):1543-8.

Francini-Pesenti F, Beltramolli D, Dall'acqua S, Brocadello F. Effect of sugar cane policosanol on lipid profile in primary hypercholesterolemia. Phytother Res. 2008. 22(3):318-22.

Garcia A, De Lucas A, Rincon J, Alvarez A, Gracia I, and Garcia MA. Supercritical carbon dioxide extraction of fatty and waxy material from rice bran. JAOCS,1996, 73(9):1127-1131.

Ghosh M and Bandyopadhyay S. Studies on the crystal growth of rice bran wax in a hexane medium. JAOCS, 2005, 82(4):229-231.

Gouni-Berthold I, Berthold HK. Policosanol: clinical pharmacology and therapeutic significance of a new lipid-lowering agent. Am Heart J. 2002. 143(2):356-65. Review.

Granja AL, Hernandez JM, Carbajal D, Arruzazabala L, Mas R, Garcia M. Mixture of higher primary aliphatic alcohols, its obtention from sugar cane wax and its pharmaceutical uses. United States Patent 5,663,156. 1997.

Gunawan S, Vali SR, and Ju Y-H. Purification and Identification of Rice Bran Oil Fatty Acid Steryl and Wax Esters. JAOCS, 2006, 83(5):449-456.

Hansen IA, Mead JF. Fate of dietary wax esters in the rat. Proc Soc Exp Biol Med, 1965,120:527–532.

Hargrove JL, Greenspan P, Hartle DK. Nutritional significance and metabolism of very long chain fatty alcohols and acids from dietary waxes. Exp Biol Med (Maywood). 2004. 229(3):215-26. Review.

Hermann L, Mailer R, and Robards K. Sedimentation in canola oil:a review. Australian Journal of Experimental Agriculture, 1999, 39:103-113.

Ito S, Suzuki T, and Fujino Y. Wax lipid in rice bran. Cereal Chemistry, 1983, 60(3):252-253.
Jacoby D and Mohler ER. Drug treatment of intermittent claudication. Drugs. 2004. 64(15):1657-1670. Review.

Janikula M. Policosanol: a new treatment for cardiovascular disease? Altern Med Rev. 2002. 203-17. Review.

Juliano BO and Bechtel DB. The rice grain and its gross consumption. In: Juliano BO, ED., "Rice: Chemistry and Technology". 1985, pp17-57. American Association of Cereal Chemists, Inc., St.Paul, MN.

Juliano BO. Rice Chemistry and Technology. American Association of Cereal Chemists, 1985 St. Paul, Minnesota

Kassis AN and Jones PJ. Lack of cholesterol-lowering efficacy of Cuban sugar cane policosanols in hypercholesterolemic persons. Am J Clin Nutr. 2006. 84(5):1003-8.

Kassis AN, Marinangeli CP, Jain D, Ebine N, Jones PJ. Lack of effect of sugar cane policosanol on plasma cholesterol in Golden Syrian hamsters. Atherosclerosis. 2007. 194(1):153-8.

Kerckhoffs DA, Brouns F, Hornstra G, Mensink RP. Effects on the human serum lipoprotein profile of beta-glucan, soy protein and isoflavones, plant sterols and stanols, garlic and tocotrienols. J Nutr. 2002 Sep;132(9):2494-505.

Kolattukudy PE. Chap.1 Introduction to natural waxes, In: Kolattukudy PE, ed., Chemistry and biochemistry of natural waxes. 1976. Elsevier, Amsterdam, The Netherlands, pp1-15.

Kreulen HP. Fractionation and winterization of edible fat. JAOCS. 1976. 53:393-396.

Krishna AGG. Influence of viscosity on wax settling and refining loss in rice bran oil. JAOCS. 1993, 70(2):895-898.

Leibovitz Z and Ruckenstein C. Winterization of sunflower oil. JAOCS, 1984. 61(5):870-872.

Lu S and Luh BS. Properties of the rice caryopsis. In: Luh BS, ed., "Rice:Production", 1991. Van Nostrand Reinhold, NY. pp389-419.

McCarty MF. An ezetimibe-policosanol combination has the potential to be an OTC agent that could dramatically lower LDL cholesterol without side effects. Med Hypotheses. 2005;64(3):636-45. Review.

McCarty MF. Policosanol safely down-regulates HMG-CoA reductase - potential as a component of the Esselstyn regimen. Med Hypotheses. 2002. 59(3):268-79. Review.

McCaskill DR and Zhang F. Use of rice bran oil in food. Food Technology, 1999, 53(2):50-53.

Megoulas NC and Koupparis MA. Twenty Years of Evaporative Light Scattering Detection. Critical Reviews in Analytical Chemistry. 2005, 35:301–316.

Meijer GW, Bressers MA, de Groot WA, Rudrum M. Effect of structure and form on the ability of plant sterols to inhibit cholesterol absorption in hamsters. Lipids. 2003. 38(7):713-21.

Moreau RA, Kohout K, Singh V. Temperature-enhanced alumina HPLC method for the analysis of wax esters, sterol esters, and methyl esters. Lipids, 2002, 37(12):1201-1204.

Morrison WH and Thomas J. Removal of waxes from sunflower seed oil by miscella refining and winterization. JAOCS, 1976. 53:485-487.

Morrison WH III and Robertson JA. Solvent winterization of sunflower seed oil. JAOCS. 1975, 52(5):148-150.

Moruisi KG, Oosthuizen W, Opperman AM. Phytosterols/stanols lower cholesterol concentrations in familial hypercholesterolemic subjects: a systematic review with meta-analysis. J Am Coll Nutr. 2006 Feb;25(1):41-8.

No author listed. Amended Final Report on the Safety Assessment of Oryza Sativa (Rice) Bran Oil, Oryza Sativa (Rice) Germ Oil, Rice Bran Acid, Oryza Sativa (Rice) Bran Wax, Hydrogenated Rice Bran Wax, Oryza Sativa (Rice) Bran Extract, Oryza Sativa (Rice) Extract, Oryza Sativa (Rice) Germ Powder, Oryza Sativa (Rice) Starch, Oryza Sativa (Rice) Bran, Hydrolyzed Rice Bran Extract Hydrolyzed Rice Bran Protein, Hydrolyzed Rice Extract, and Hydrolyzed Rice Protein. International Journal of Toxicology. 2006. 25(Sppl.2): 91-120.

No authors listed. Monograph. Policosanol. Altern Med Rev. 2004. (3):312-7. Review.

Norton RA. Quantitation of steryl ferulate and p-coumarate esters from corn and rice. Lipids. 1995. 30(3):269-74.

Pepping J. Policosanol. Am J Health Syst Pharm. 2003. 60(11):1112-5. Review.

Personal correspondence with Riceland Foods Company, Arkansas.

Rajam L, Soban Kumar DR, Sundaresan A, and Arumughan C. A Novel Process for Physically Refining Rice Bran Oil Through Simultaneous Degumming and Dewaxing. JAOCS. 2005. 82(3):213-220.

Ramakrishina P, Venkatesh KVL, Poornima TC, and Manohar B. Effect of wax content on flow properties of rice bran oil. JAOCS, 1987. 64(6):859-861.

Reiner Z, Tedeschi-Reiner E, Romić Z. Effects of rice policosanol on serum lipoproteins, homocysteine, fibrinogen and C-reactive protein in hypercholesterolemic patients. Clin Drug Investig. 2005. 25(11):701-7.

Reiner Z, Tedeschi-Reiner E. Rice policosanol does not have any effects on blood coagulation factors in hypercholesterolemic patients. Coll Antropol. 2007. 31(4):1061-4.

Sah A, Agrawal BKD and Shuka LS. A new approach in dewaxing and refining rice bran oil India. JAOCS.1983, 60(2):467.

Saito N, Ikushima Y, Hatakeda K, Ito S, Asano T, and Goto T. Fractional extraction of rice bran oil and its esters with supercritical carbon dioxide. International Chemical Engineering, 1993. 33(2):307-314.

Shen Z, Palmer MV, Ting SST, and Fairclough RJ. Pilot Scale Extraction and Fractionation of Rice Bran Oil Using Supercritical Carbon Dioxide. J. Agric. Food Chem. 1997. 45:4540-4544.

Taylor JC, Rapport L, Lockwood GB. Octacosanol in human health. Nutrition. 2003. 19(2):192-5. Review.

Trautwein EA, Schulz C, Rieckhoff D, Kunath-Rau A, Erbersdobler HF, de Groot WA, Meijer GW. Effect of esterified 4-desmethylsterols and -stanols or 4,4'-dimethylsterols on cholesterol and bile acid metabolism in hamsters. Br J Nutr. 2002. 87(3):227-37.

Vali SR, Ju Y, Kaimal TNB, and Chern Y. A Process for the Preparation of Food-Grade Rice Bran Wax and the Determination of Its Composition. JAOCS, 2005, 82(1):57-64

Vissers MN, Zock PL, Meijer GW, Katan MB. Effect of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum lipoprotein concentrations in humans. Am J Clin Nutr. 2000. 72(6):1510-5.

Wang Y, Ebine N, Jia X, Jones PJ, Fairow C, Jaeger R. Very long chain fatty acids (policosanols) and phytosterols affect plasma lipid levels and cholesterol biosynthesis in hamsters. Metabolism. 2005. 54(4):508-14.

Wang YW, Jones PJ, Pischel I, Fairow C. Effects of policosanols and phytosterols on lipid levels and cholesterol biosynthesis in hamsters. Lipids. 2003. 38(2):165-70.

Xu Z and Godber JS. Comparison of supercritical fluid and solvent extraction methods in extracting γ-oryzanol from rice bran. JAOCS, 2000, 77(5):547-551.

Yoon SH and Rhee JS. Composition of waxes from crude rice bran oil. JAOCS. 1982. 59:561-563.

Zhao W, Shishikura A, Fujimoto K, Arai K and Saito Shozaburo. Fractional extraction of rice bran oil with supercritical carbon dioxide. Agric. Biol. Chem. 1987. 51(7):1773-1777

Zigoneanu IG, Williams L, Xu Z, and Sabliov CM. Determination of antioxidant components in rice bran oil extracted by Microwave-assisted method. Bioresource Technology. 2007. (in press).

CHAPTER 2.
METHOD DEVELOPMENT FOR ANALYSIS OF WAX ESTERS IN RICE BRAN OIL USING HPLC

2.1. INTRODUCTION

Wax analysis has been performed by chromatographic methods such as liquid column chromatography, HPLC, TLC and GC. Column chromatography with silica gel, Florisil, and alumina is commonly used for initial fractionation of crude oils, leaf waxes, and total lipids (Tulloch, 1976). The substances loaded onto the column are eluted with a gradient of two or three organic solvents, each of which has a different polarity to dissolve specific substances. The resulting fractions are usually subjected to TLC for identification of the fractionated substances by comparison of TLC of each fraction and TLC of standard materials. Depending on the materials expected to be separated by TLC, the mobile phase should be devised to show the most discrete spots, and many solvent systems have been used for one- or two-dimensional TLC (Shantha and Napolitano, 1998). Sometimes, wax analysis can be done only with TLC and GC if significant amounts of wax components are not required for a specific purpose. HPLC methods for wax analysis have been recently developed and actively applied to the separation of specific substances, which are not separated by common column chromatography (Amelio et al., 1998; Moreau et al., 2002). If wax analysis is the only consideration, TLC and GC are enough for the purpose. Some studies on wax analysis used HPLC for separation (Amelio et al., 1993, 1998; Rezanka, 1998; Moreau et al., 2002; Busson-Breyss et al., 1994), and HPCL can employ gradients that give more consistent results. When further analysis is required, sufficient material could be obtained by preparative HPLC methods. This study will comprise not only wax ester analysis but also analyses of its saponified products, so silica gel column chromatography coupled with HPLC will be suitable for that purpose.

2.2. LITERATURE REVIEW

RBW studies introduced in Sec.1.2.1 (Cousins et al., 1953; Yoon and Rhee, 1982; Belavadi and Bhowmick, 1988; Ito et al., 1983; Garcia et al., 1996; Vali et al., 2005) all utilized column chromatography, TLC or GC. They studied RBW or RBWE composition without HPLC. Non-polar lipid species like wax esters (WE) do not have a specific chemical structure that can be detected by UV. Refractive Index (RI) detectors can detect WE, but when a gradient elution or an elevated temperature is required, IR detectors are not a good choice. The choice of a stationary phase has also been a quandary in wax analysis by HPLC. The polarities of hydrocarbons, alkyl esters, and steryl esters are too similar to be separated. Hamilton (1995) indicated that HPLC methodology for wax analysis has been developing slowly because of the need to find a suitable detector, since waxes have no useful UV chromophore. Another reason for the slow progress likely has been solidification of wax in HPLC lines and columns, resulting in unreliable data. If HPLC methodology develops, quantification will be much easier and more timely than using column chromatography (Hwang et al., 2002).

Evaporative light scattering detectors (ELSD) are a robust choice of detectors, especially for substances that lack UV-detectable chemical structures (Megoulas and Koupparis, 2005). ELSD detects everything that is not volatile and blocks the light path, which means that the signal intensity depends not only on the number of detectable molecules but also the size of the molecules. Moreover, solvent gradients or temperature do not affect ELSD detection, so it is an ideal detector for wax analysis.

Nordback and Lundberg (1999) and Moreau et al. (2002) used ELSD as well as normal phase alumina columns for their wax analysis using HPLC. Especially Moreau et al. (2002) reported that high column temperature greatly improved the column resolution as much as it could

separate hydrocarbons, alkyl esters, and steryl esters. Alumina columns and ELSD may be the perfect tool for wax analysis. Unfortunately, it is difficult to find commercially available alumina column these days. In both studies, Aluspher alumina columns were used, but its manufacturer, Merck, no longer produces the column (Personal correspondence, 2007)

Amelio et al. (1993) separated WEs from Olive oil using Supelcosil LC-Si (Supelco, 15cm x 4.6 mm, 5 um) and a UV detector (203.5 nm). Injected lipids were eluted with Hexane/diethyl ether gradient (100:0, 2.5 min; 92:8 for 13.5 min; 0.100, for 11.5 min; 100:0 for 25 min) at 1ml/min. WE was eluted at 4.5-8.0 min, but WE and triacylglycerides (TAG) were not completely separated. Busson-Breysse et al. (1994) studied WEs in Jojoba wax using Lichrosorb reverse-phase RP 18 (Merck, 25cm x 4 mm, 5 um) and an RI detector. The WE were isocratically eluted with propionitrile at 0.8 ml/min, and the retention time was 9-16 min. Their chromatogram showed four discrete peaks, but they did not specify the peak identification. Amelio er al. (1998) tried an HPLC method with Supelcosil LC-Si (Supelco, 15cm x 4.6 mm, 5 um) and a UV detector (217.6 nm) to separate WEs from Olive oil. The injected oil was eluted with a gradient of Hexane/diethyl ether (100:0, 17 min, 100:0 to 92:8, 1min; 92:8, 1.5 min; 92:8 to 0:100, 11 min; 0:100 to 100:0, 24 min). WE was eluted at 17-30 min, but the resolution was still ambiguous between hydrocarbons and WE. Nordback and Lundberg (1999) separated non-polar lipids from zooplanktons using Aluspher Al particles (LiChroCART, Merck, 125 mm x 4.0 mm i.d 5 um diameter) and an ELSD detector. The column temperature was kept constant at 30^{o}C. The solvent system was composed of 0.5% THF in hexane (A) and 20% THF and 20% isopropanol in hexane (B). Hydrocarbons were eluted at 2-2.5 min, alkyl esters at 3.5 min, and steryl esters at 4.5 min. Moreau et al. (2002) successfully separated squalene, stearyl stearate, stearate methyl ester and cholesterol stearate using Aluspher Al 100 column (Merck, 12.5 cm x 4

mm, 5 um) and an ELSD. The solvent system consisted of hexane:THF, 1000:1 (A) and Isopropanol (B) (A:B =100:0, 20 min; 95:5, 1 min; 100:0, 39 min). They maintained the column temperature at 25, 50, or 75 ^{o}C. The standards appeared as 4 discrete peaks, and the resolution was highest at 75 ^{o}C. They also tried a photodiode-array detector and a UV detector, which were not as sensitive as ELSD.

2.3. OBJECTIVES OF STUDY

So far, only a few analytical methods have been developed for wax esters (WE) in edible oils. Only the method using a normal-phase alumina column and an ELSD showed satisfactory results. As long as an alumina column is not available, alternative methods should be developed.

The purpose of this research was 1) to develop normal-phase HPLC methods for WE analysis, 2) to establish suitable solvent systems for reverse-phase HPLC analysis for WE, and 3) to determine the optimum condition for preparative WE separation.

2.4. MATERIALS AND METHODS

2.4.1. RBO Extraction

One kilogram of FF RB was mixed with 1 pound (450g) of glass bead (5 mm i.d.), placed in a cotton pouch, and then weighed. The pouch was inserted into the extraction vessel of the extractor (SFE-3000 System, Thar Designs, Pittsburg, PA), and the vessel was heated at 80°C for 20 min before CO_2 gas flow commenced. The CO_2 flow rate was set to 80 g/min for the first 5 min, changed to 120 g/min until the pressure reached the desired pressure (400 bar), and fixed at 80 g/min after the pressure was reached. The total extraction time was 5 hr. The temperature of the oil collection vessel was set to 50°C so that the extracted oil would remain in liquid state.

After extraction, the weight of the pouch was measured and RBO was removed to a clean flask for the weight measurement. The crude RBO yield was about 14%. After it was weighed, the crude RBO was stored at –20°C.

2.4.2. Crude RBO Processing

The crude RBO was degummed with water. The same volume of hot distilled water was added to 100 g of the crude RBO in a round-bottom flask, which was then heated at 80 °C for 20 min with vigorous agitation, and the water layer was removed by suction. The same procedure was done with 50 ml of 1% aqueous phosphoric acid and then with 50 ml of 0.1% aqueous CaO. The RBO was washed with water one more time. After degumming, 10 g of sodium sulfate (10-60 mesh, Fisher Scientific, Fair lawn, NJ, USA) was added to remove the residual water. The oil was filtered through a Pyrex glass-frit filter (10 – 15 μm) containing 5 g of activated carbon (100-200 mesh, Eastman organic chemicals, Rochester, NY, USA) and 5 g of Fuller's earth (100 – 200 MESH, Sigma, St. Louis, MO, USA) for bleaching. During all processes, the oil temperature was maintained at 70 - 80 °C. The processed RBO was then dissolved in 500 ml of hot hexane, and filtered through a 0.2-μm membrane filter (Whatman, Florham, NJ, USA). After hexane was evaporated by a rotary evaporator, the oil sample was stored at -20 °C.

2.4.3. Preparation of RBW

One hundred grams of the processed RBO was mixed with the same volume of hexane, and the solution was heated at 60 °C until the solution became transparent. It was cooled to room temperature, and then placed in a low temperature incubator at 20 °C for 1 hr. The temperature of the incubator was further reduced to 18, 16, 14, and 12 °C, and each temperature was

maintained for 1 hr. Finally, the solution was cooled at 10 °C, and left overnight for the completion of wax crystallization. After the winterization was finished, the yellowish crude RBW was recovered by filtration (0.2-μm membrane filter), and then dried at 50 °C. This hexane winterization was done twice. The hexane-washed RBW was dissolved in the same volume of isopropanol and refluxed at 80 °C for 1 hr. During reflux, 2 ml of 10% aqueous sodium borohydride (Sigma, St. Louis, MO) was added to the solution. After reflux, the isopropanol solution was filtered through a 0.2-μm membrane filter when it was still hot, and then winterized. After winterization, the refined RBW was recovered by filtration and washed with water on the same filter. The RBW was dried at 80°C, weighed, and stored at -20°C. The yield of the refined RBW from the processed RBO was 1.25%.

2.4.4. HPLC System

HPLC analysis of RBO samples was performed with a Waters Delta Prep 4000 preparative chromatography system (Waters Corporation, Milford, Massachusetts, USA). Two detectors were used: a UV detector (Waters Lamda-Max Model 481 LC spectrophotometer, Waters Corporation, Milford, Massachusetts, USA) and an ELSD detector (Shimadzu low temperature evaporative light scattering detector ELSD-LT2, Shimadzu, Kyoto, Japan). The data was processed with the Shimadzu GC solution program (Shimadzu Corporation, Kyoto, Japan). Fractions were collected with a Waters fraction collector (Waters Corporation, Milford, Massachusetts, USA). All columns were purchased from Supelco (Bellefonte, PA, USA); Supelcosil LC-SI column (250 mm x 4.6 mm, 5 μm) coupled with a guard column (50 mm x 2 mm) which was manually packed with TLC-grade silica gel (SilicAR TLC-7, 35 – 60 μm, Mallinckrodt, Phillipsburg, NJ, USA), and Discovery C18 column (250 mm x 4.6 mm, 5 μm)

coupled with a guard column (Supelguard, 20 mm x 4 mm, 5 μm). Only HPLC-grade solvents were used for all analyses. The standard materials for HPLC analysis, octacosane (C28), stearyl stearate (SS), stearate methyl ester (SME), behenate methyl ester (BME), cholesteryl stearate (CS), and tristearine (TS) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4.5. HPLC Methods

For the normal phase analysis, the solvent temperature and the column temperature were maintained at 50°C. The evaporation temperature for ELSD was set to 40°C, and nitrogen was used for solvent evaporation at 30 psi. The sensitivity of ELSD was set to 5 (highest) for μg-level samples, and 3 (middle) for 1-mg levels. ELSD data acquisition started 2 min after injection. The UV detector was used at various wavelengths, mainly for fraction collection. Two analytical silica (2SI) columns connected with a 5-cm stainless steel tubing were used for the analysis. RBO samples were prepared at a concentration of 10 mg/ml solution in hexane, and 100 μg (10 μl) or 1 mg (100 μl) of the solution was manually injected into a 2-ml sample loop after being slightly heated. The standards were prepared in hexane at 1 mg/ml, and 1-25 μg (1-25 μl) was injected.

Method 1: The injected lipids were eluted with Hexane (Solvent A) for the first 4 min, and for the next 11 min, hexane:diethyl ether (20:1, v/v, Solvent B) was used. The columns were washed with methanol for 5 min and acetone for 5 min after elution. During operation, the flow rate was fixed at 2.5 ml/min.

Method 2: To visualize the amount of wax esters (WE) and triacylglycerides (TAG) in each fraction, the mixture of hexane:diethyl ether (10:1, v/v) was used to wash the column instead of methanol and acetone.

For the reverse-phase analysis, the solvent temperature was maintained at 50°C between the sample loop and the pump. Before the column, the solvents were cooled to 30°C, and the analytical C18 column was not heated. The evaporation temperature for ELSD was set to 60°C, and nitrogen was used for solvent evaporation at 30 psi. The sensitivity of ELSD was set to 5 (highest) for μg-level samples, and 3 (middle) for 1-mg samples. ELSD data acquisition started 3 min after injection. RBO samples were prepared at a concentration of 10 mg/ml solution in isopropanol, and 100 μg (10 μl) or 1 mg (100 μl) of the solution was manually injected after being heated until the solution became transparent. The standards were prepared in isopropanol at 1 mg/ml, and 1-25 μg (1-25 μl) was injected.

Method 3: The injected lipids were eluted with acetonitrile (Solvent A): propanol (Solvent B) (65:35, v/v) for the first 14 min, A40:B60 for the next 5 min, and A30:B70 for the last 10 min. During operation, the flow rate was fixed at 2 ml/min.

Fractions were collected to identify each peak. When method 1 was used, the WE fraction was directly collected from the column right before ELSD. Two milligrams of RBO was injected into the silica column, and the WE fraction was collected. The solvent was removed with a centrifugal evaporator and the WE sample was dissolved in 1 ml of isopropanol, all of which was injected into a C18 column. When method 3 was used, 1 mg of RBO was injected and fractions were collected at 1-min intervals (2 ml/min). Two hundred-fifty microliters of each fraction was reinjected into the C18 column to visualize the corresponding peak for each fraction. After the solvent was evaporated, each of the fractions was dissolved in 1 ml of hexane. One hundred microliters of the solution was injected into 2SI columns, and the lipid was eluted by method 2.

2.5. RESULTS

The chromatograms of method 1 have already been depicted in fig.1.6. Wax esters (WE) were eluted at 5.5 to 6.0 min. When 100 µg of RBO was injected, the hydrocarbon peak was almost not visible, indicating the hydrocarbon content in RBO is very small. Method 1 eluted WE as a single peak, facilitating the WE measurements in RBO samples.

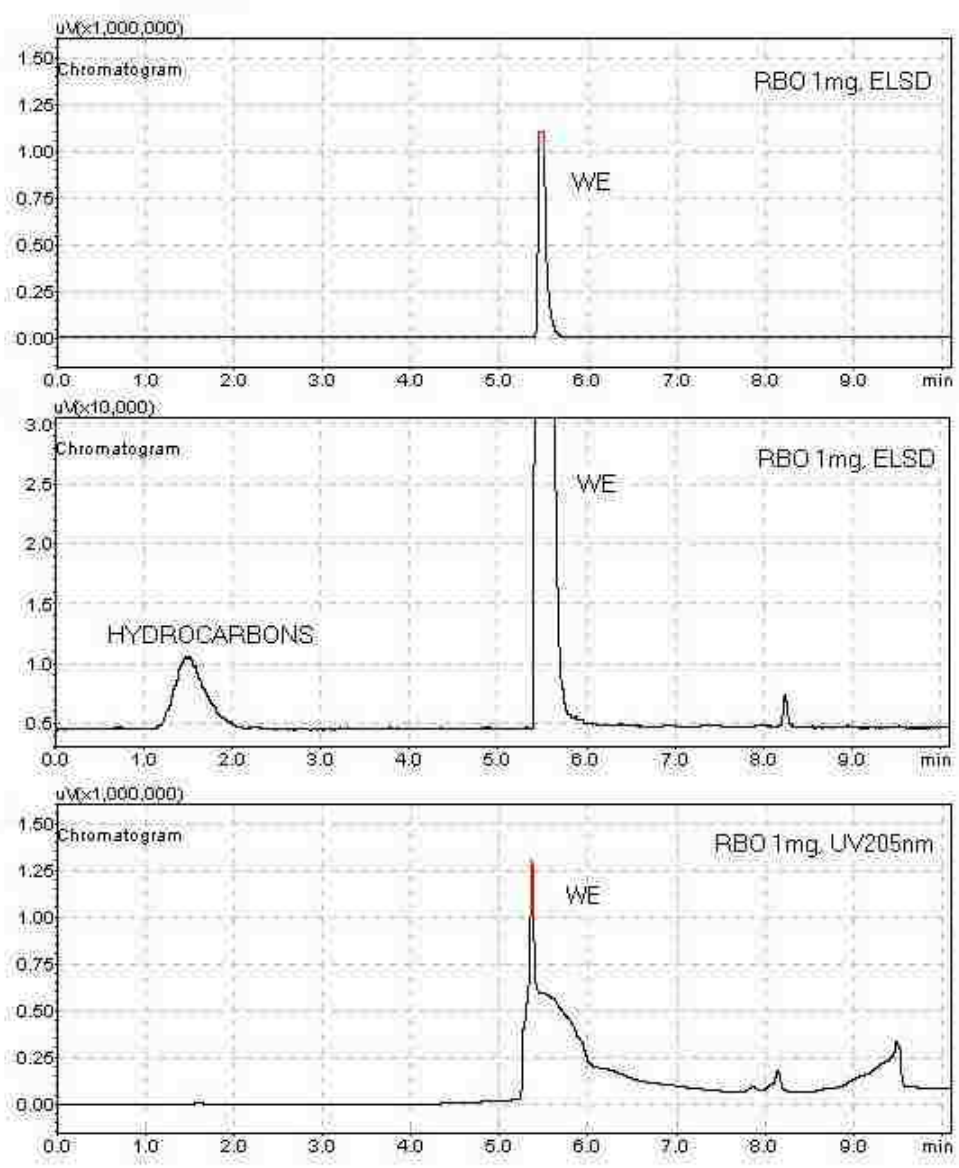


Figure 2.1. Chromatograms of Method1. RBO (1 mg) injection (upper), enlarged view of the upper chromatogram (middle), and chromatogram from UV detector (bottom). WE: wax esters.

When 1 mg of RBO was injected, the hydrocarbon peak appeared in fig.2.1. The solvent system of method 1 also allowed UV detection, which can be used to collect the fractions. For method 1, the column condition was really important, and when the column was not fully activated or washed, the WE appeared in two peaks (fig.2.2). The WE peak was divided into 2 peaks at 3.0 min and at 5.5 min. Compared with the WE peak height in fig.1.6, the peak at 5.5 min is much smaller.

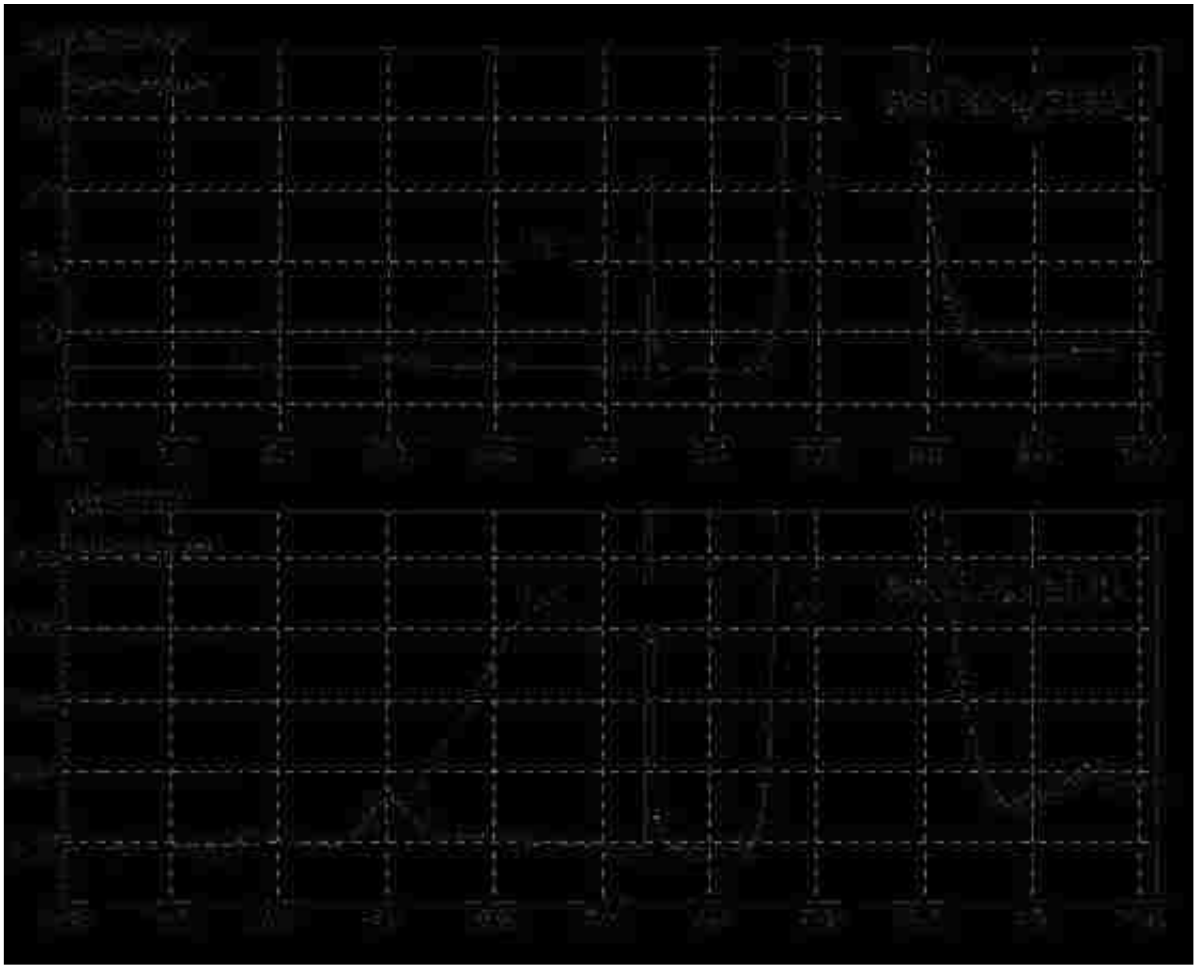


Figure 2.2. Chromatograms of Method1 when the columns are not fully activated. Injection of 100 μg of RBO (upper) and its enlarged view (bottom). WE: wax esters, AG: acylglycerides.

The chromatograms of RBO by C18 column (method 3) are shown in fig. 2.3. Numerous, well resolved peaks appeared when 100 μg of RBO was injected (fig.2.3-A). The methyl ester standard, behenate methyl ester, appeared at the front, which indicates the elution from C18 column is not solely dependent on the polarity of the substance. Based only on the standard injection (fig.2.3-B), WE was eluted after 17.5 min.

Fig.2.4 shows the chromatograms from ELSD and UV detector at several wavelengths when 1 mg of RBO was injected. Only at 205 nm the UV detection was similar to ELSD before the solvent composition was changed at 19 min. UV at 205 nm was not as sensitive as ELSD from 13 to 18 min (fig.2.4-A and C), but the use of UVD allowed fraction collection. Relatively polar lipids were detected by UVD at other wavelengths.

In order to determine which peak in the chromatogram from method 3 was WE, the WE fraction was collected from 2SI columns after 2 mg of RBO was injected, and the WE fraction was injected into the C18 column (Fig.2.5). The result shows the peak positions of WE in the chromatogram, and WE appeared to elute from 13 min to the end.

After 1 mg of RBO was injected into the C18 column, the fractions were collected based on the chromatogram (fig. 2.6-upper) at 1-min intervals, and they were reinjected into the same column again to see which fraction contained which peak (fig. 2.6). The fractions were also injected into 2SI columns and eluted by method 2 (fig. 2.7). Surprisingly, all the fractions from C18 column showed the WE peak at 6 min and triacylglyceride (TAG) peak at 13 min (fig. 2.7), and the heights of WE peak in each fraction did not change much while those of TAG started to decrease from fr.18. This indicates that peak 5 and 6 in fig. 2.6 (upper) may be at the border between TAG and WE, which is well supported by the chromatogram of the standard injection in fig. 2.6 (middle).

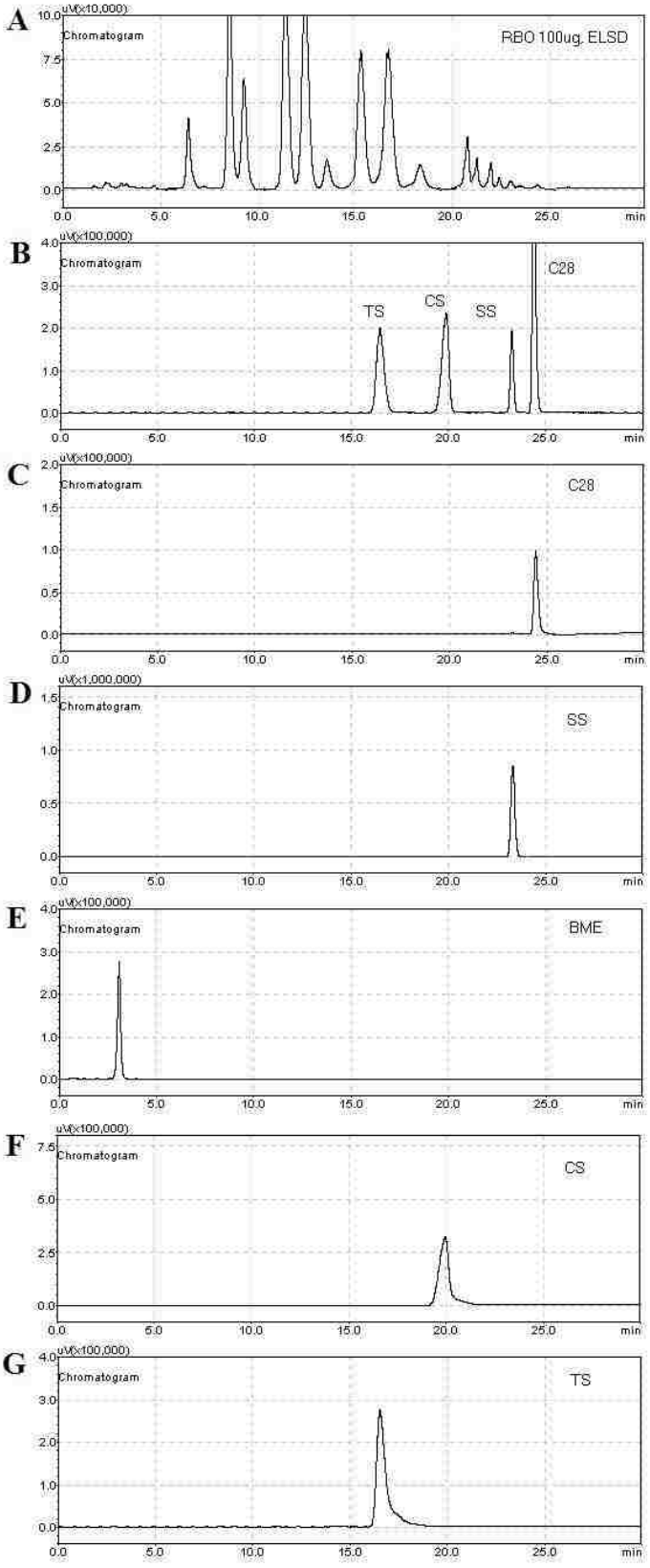


Figure 2.3. Chromatograms of Method3. RBO injection (A), standard mixture (B), and individual injections of each standard (C-G). C28: octacosane, SS: stearyl stearate, BME: behenate methyl ester, CS: cholesteryl stearate, TS: tristearine.

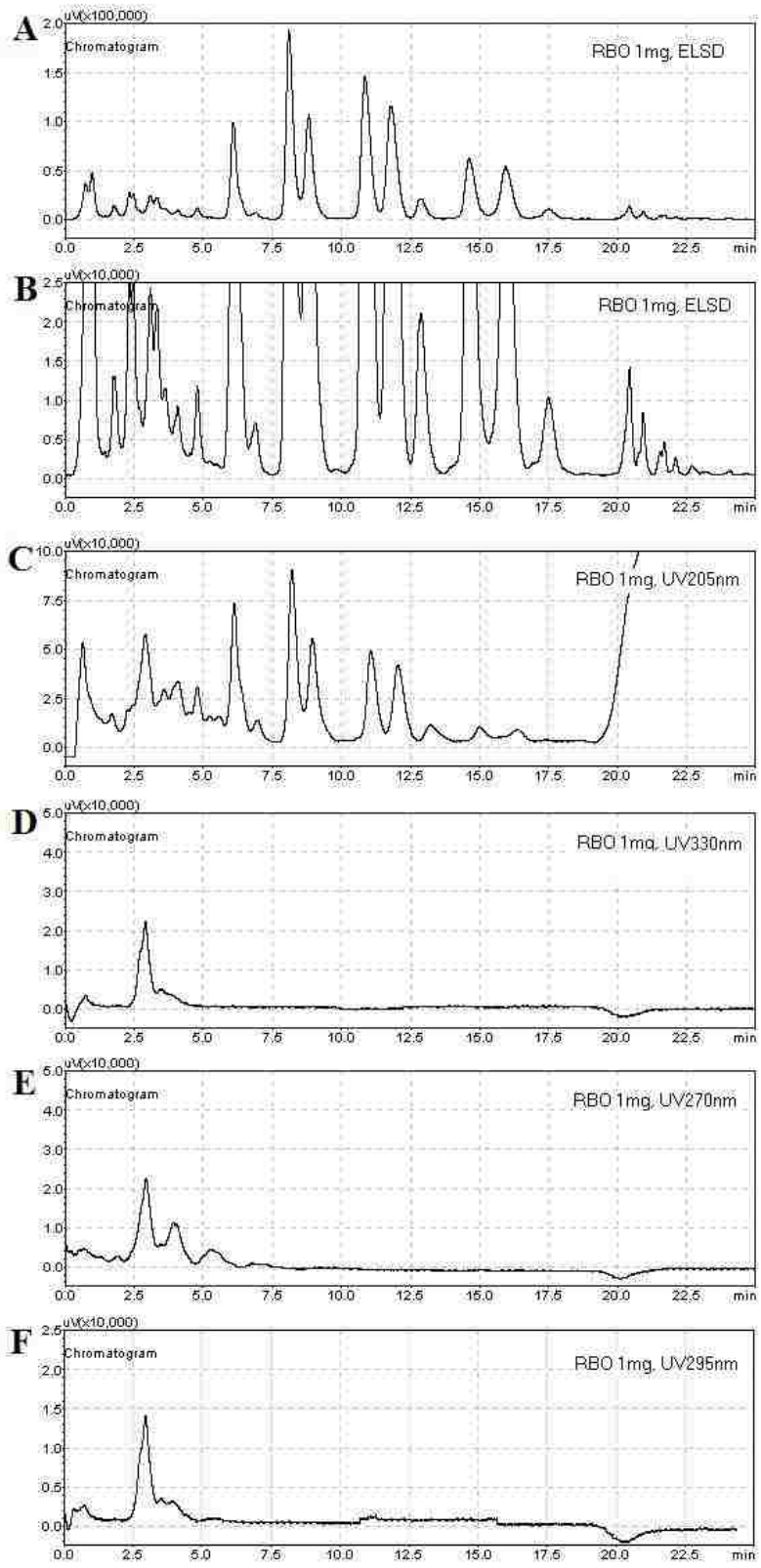


Figure 2.4. Chromatograms of Method3 with ELSD and UV detector (UVD). RBO injection (A), enlarged view of A, and chromatograms from UVD at various wavelengths.

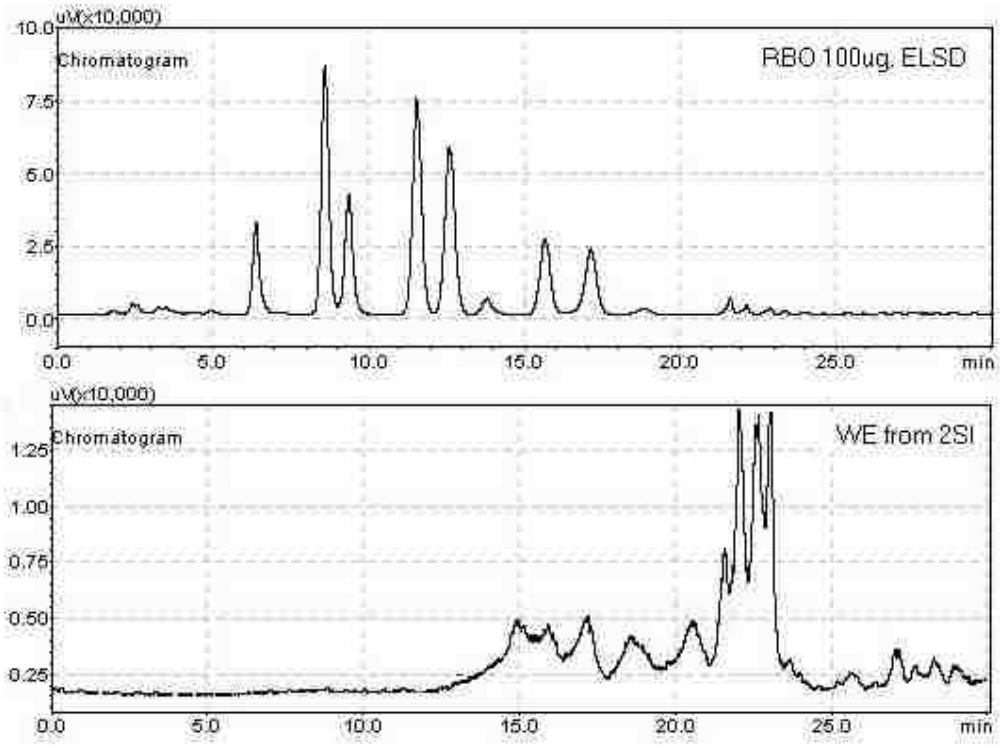


Figure 2.5. Chromatograms of WE fraction by Method3. RBO injection (upper) and WE fraction collected from Method1 (bottom)

RBW was injected into the C18 column and eluted by method 3 (fig. 2.8). The peak pattern was similar to that of RBO at 5 to 20 min. The elution of RBO was almost finished after 25 min, but RBW showed numerous substances which were eluted after 25 min. The result suggests that some species among WEs, especially fatty acid methyl esters, are eluted early and other WEs are eluted late.

2.6. DISCUSSION

It is difficult to determine the exact position of WE in the C18 chromatogram, indicating that the separation or the determination of total WE by C18 column is also difficult or impossible.

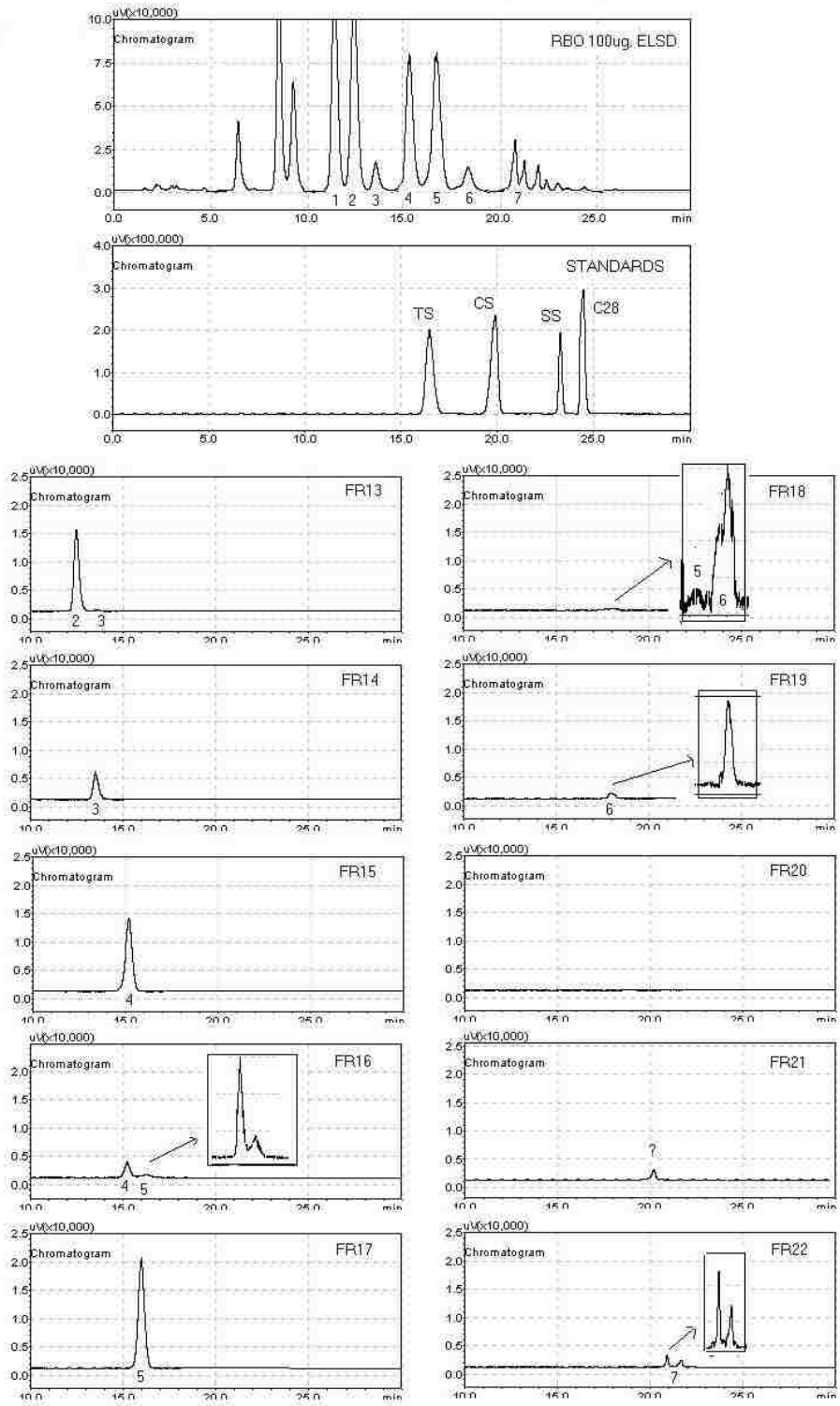


Figure 2.6. Chromatograms of Method 3 for fractionation. Fractions were collected and reinjected into the same column

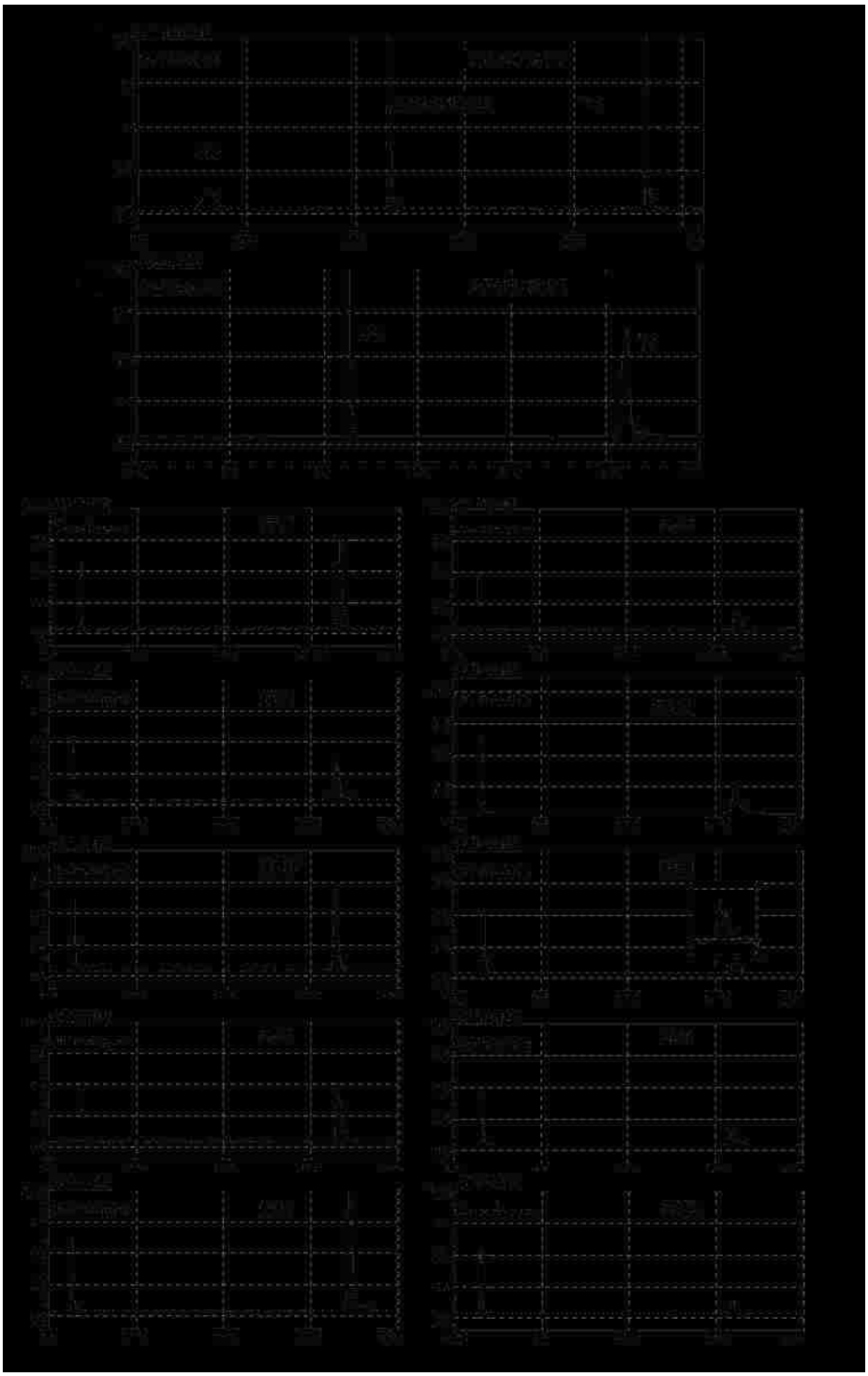


Figure 2.7. Chromatograms of fractions from Method 3 injected into 2SI column. Standard mixture with Method1 (upper), CS and TS with Method 2 (middle), and individual injections of each fraction. C28: octacosane, SS: stearyl stearate, BME: behenate methyl ester, CS: cholesteryl stearate, TS: tristearine.

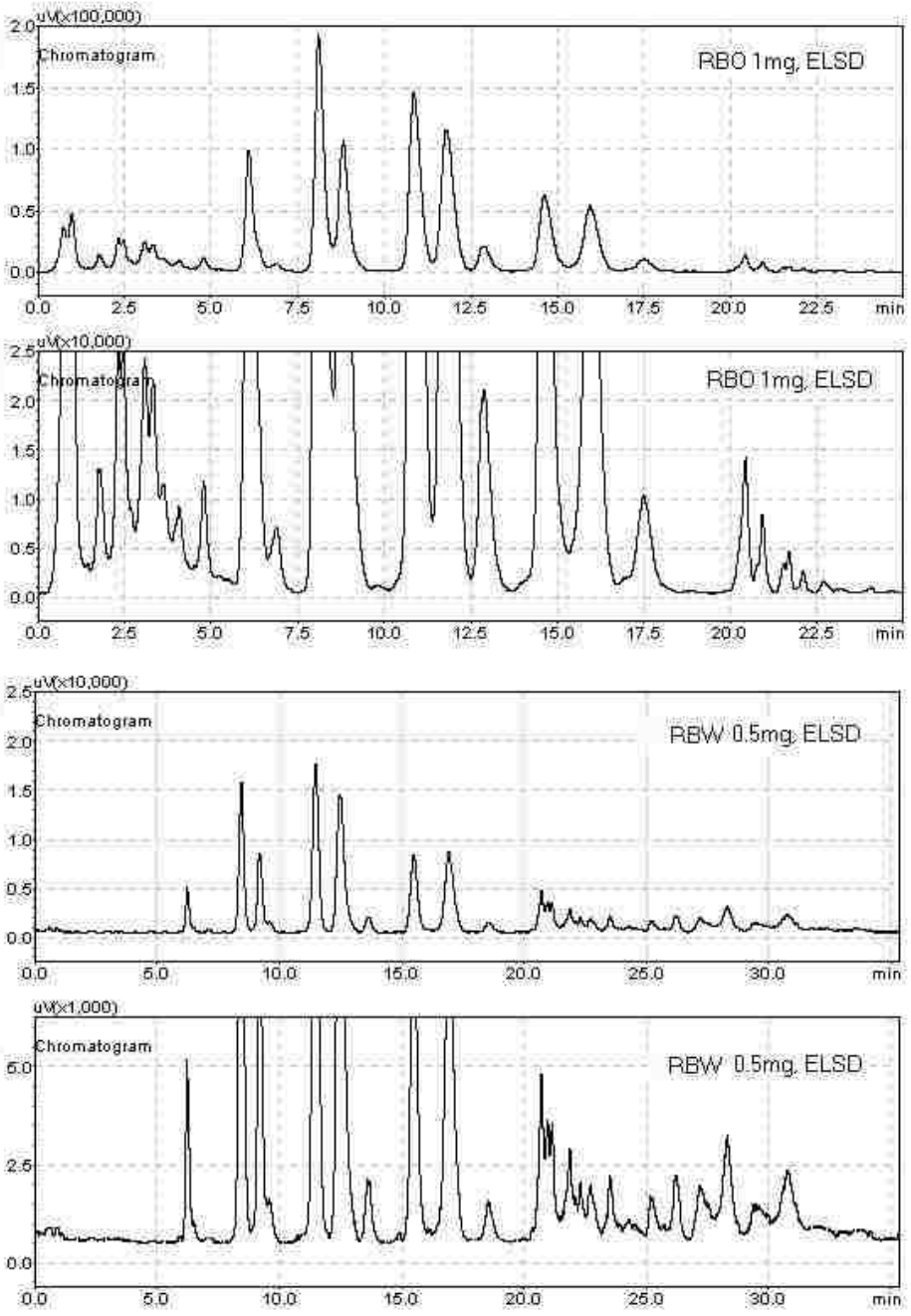


Figure 2.8. Chromatograms of RBO and RBW by Method 3. The second and fourth chromatograms are the enlarged views of the first and third one, respectively.

When WE from 2SI columns was injected into a C18 column, the WE was eluted from 13 min (fig. 2.5). When the standards were injected into a C18 column, WE started to appear at 20 min

(fig. 2.3). When the fractions from C18 column were injected into the 2SI column, nearly all the fractions showed a certain amount of WE (fig. 2.7). All this evidence indicates that the C18 column does not release all the WE at a certain point of time like Si column, and that some kinds of WE substances such as methyl esters are eluted early. BME was eluted at around 3 min (fig. 2.3). Stearate methyl ester (SME) was injected into C18 column, and it was also eluted early (fig. 2.9). In addition to the early elution, methyl esters caused another problem in which the signal intensity was very weak. When 1 μg was injected with Method 3, nothing appeared in the chromatogram (fig. 2.9). The second chromatogram in fig. 2.9 shows 3 consecutive injections of SME eluted isocratically with acetonitrile:propanol (30:70, v/v). More than 5 μg injection barely showed a small peak (ELSD sensitivity = 3). From the SI columns, the substances are eluted in the order of their polarities, but it is not likely that the same principles are applied to C18 column chromatography.

The method development was started with an analytical C18 column (fig. 2.10). The solvent system consisted of Solvent A (acetonitrile:isopropanol:acetone:methanol = 5:1:1:3) and Solvent B (propanol), and the lipids were eluted with A100% for the first 32 min, A90:B10 for the next 10 min, and A70:B30 for the remainder of the chromatogram. Fig. 2.10 shows the chromatogram when 1 mg of FF RBO was injected. This method exhibits a good resolution but the running time was too long, which is why Method 3 was developed. The peak pattern was similar to that of Method 3. The second chromatogram in fig. 2.10 shows a 1-mg injection of RBO into 1 Si column. The lipids were eluted with 1% diethyl ether in hexane at 2 ml/min while the column was heated at 70°C, and methanol was used to wash the column starting at 12 min. A group of peaks appeared between 1 – 4 min, and they were collected and re-injected into the C18 column (fig. 2.10-last panel) with the solvent system explained above. The fraction shows many different

lipids but no more polar species, which means that this normal phase method failed to separate each component of WE. This method also failed to separate hydrocarbons and WE. Figure 2.10-third panel shows the chromatogram of the standards.

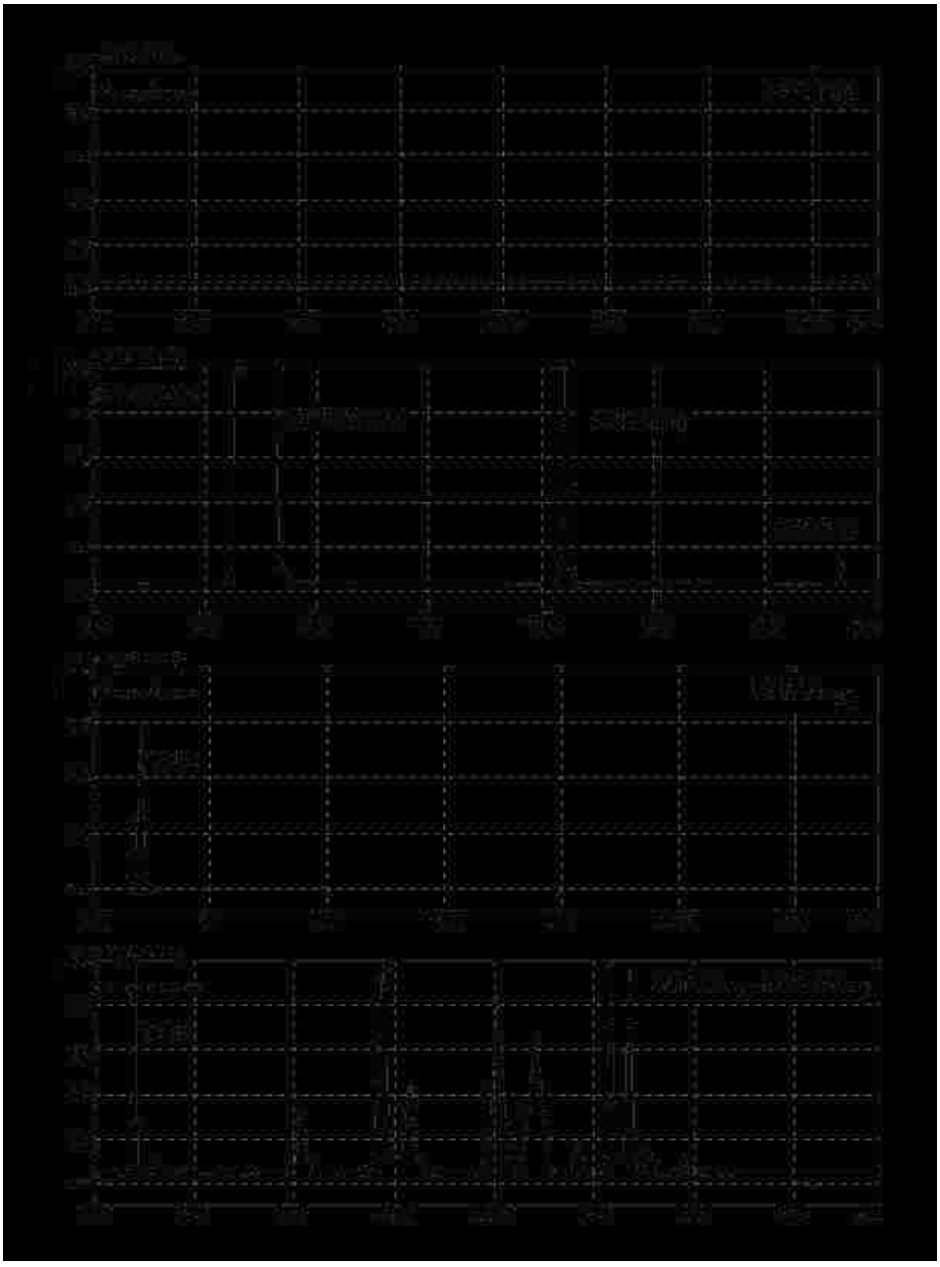


Figure 2.9. Chromatograms of SME (stearate methyl esters) with Method3. The second chromatogram shows 3 consecutive injections of SME eluted isocratically with acetonitrile:propanol (30:70, v/v).

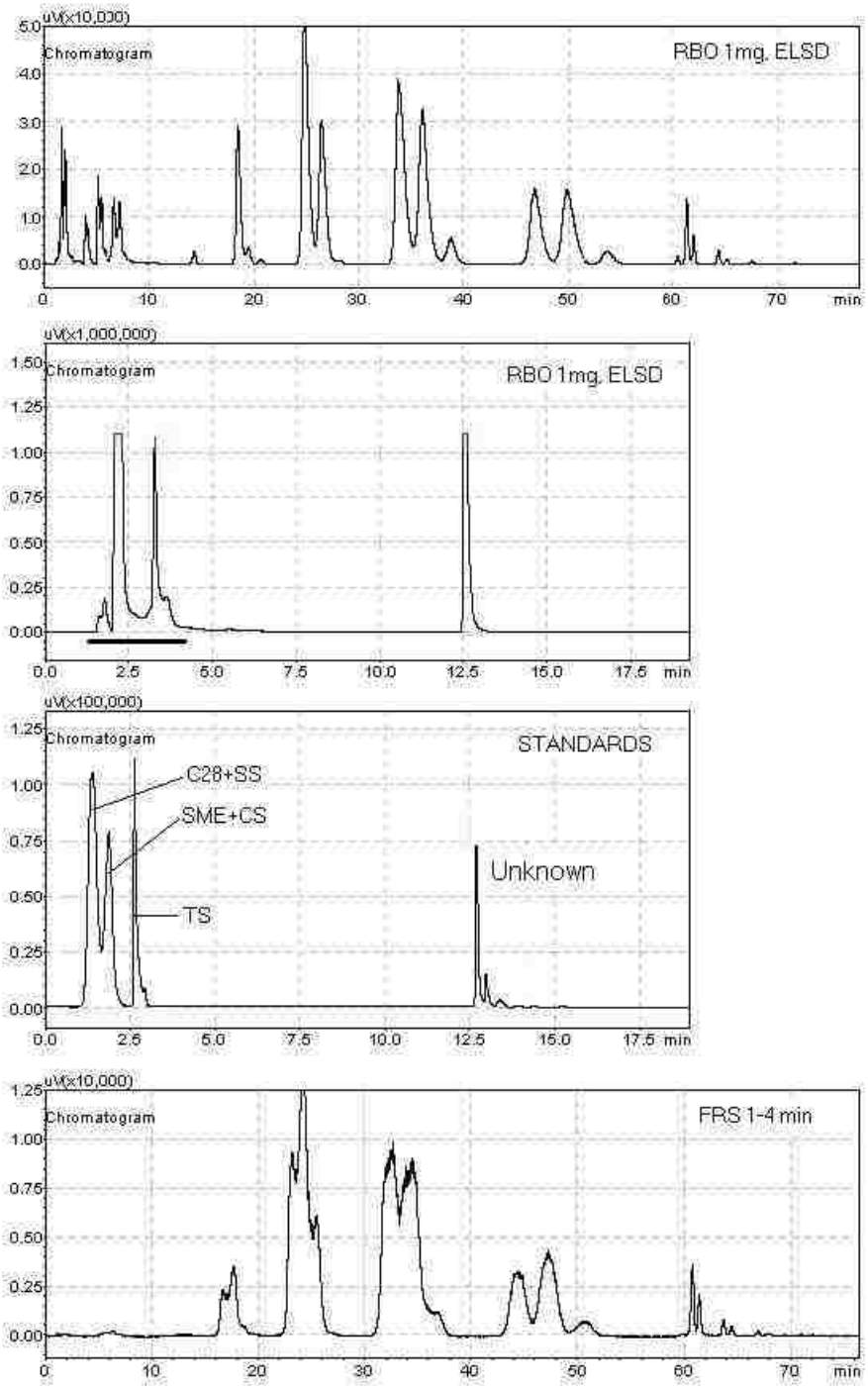


Figure 2.10. Chromatograms of other HPLC methods. The first and last pictures are from a C18 column method, and the second and third ones are from a Si column method. C28: octacosane, SS: stearyl stearate, SME: stearate methyl ester, CS: cholesteryl stearate, TS: tristearine.

To obtain discernable resolutions, two analytical silica (2SI) columns, connected consecutively, were used (fig. 2.11). The solvent system consisted of hexane (Solvent A) and hexane:diethyl ether (100:2, v/v, Solvent B). The injected lipids were eluted with A for the first 6 min and with B for the next 15 min. The column was washed with methanol after elution. The flow rate was set to 2 ml/min. ELSD data acquisition started 2 min after injection. The solvent was heated at 50 °C while it was passing through the tubing between the sample loop and the pump, and the column was also heated at 50 °C. The peaks of hydrocarbons and long chain alkyl esters appeared separate, but it failed to separate SME and CS. To separate SME from CS, the solvent with higher polarity (hexane:diethyl ether = 100:3, v/v) was used as Solvent B (fig. 2.12), but the peaks appeared almost the same as in fig. 2.11. The elution condition was the same as above.

Another solvent system was developed in which the injected lipid was eluted isocratically with hexane:diethyl ether (100:2) from the start of the run. The flow rate was set to 2 ml/min, and the solvent and the column were heated to 50 °C. This method caused the WE to appear as one peak, but the distance between hydrocarbons and alkyl esters (indicated by an arrow) was too narrow (fig. 2.13). SME and CS still appeared as one peak.

Method 1 was developed to separate SME and CS, but it failed. However, the increased flow rate (2.5 ml/min) and the higher polarity of the solvent made the WE peaks appear as one peak, which made it easier to separate the total WE from RBO. Method 2 was the same as method 1 except the washing solvent. When the column was washed with methanol, it eluted all remaining material including polar lipid species. The washing solvent of method 2 (hexane:diethyl ether = 10:1) enabled the visualization of the TAG peak.

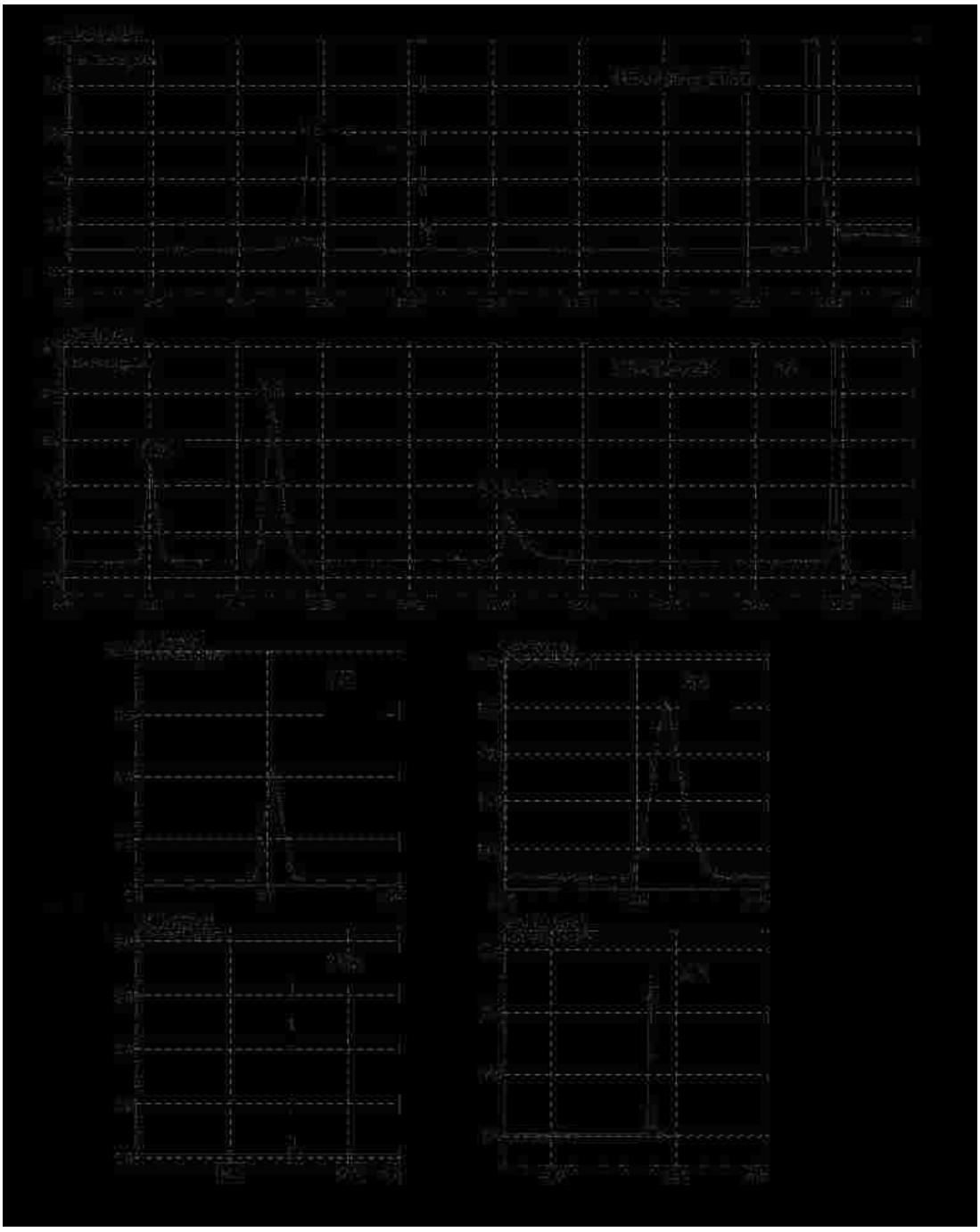


Figure 2.11. Chromatograms of a 2SI method with hexane:diethyl ether (100:2). The first picture shows RBO injection, and the rest show the standard injection. C28: octacosane, SS: stearyl stearate, SME: stearate methyl ester, CS: cholesteryl stearate, TS: tristearine.

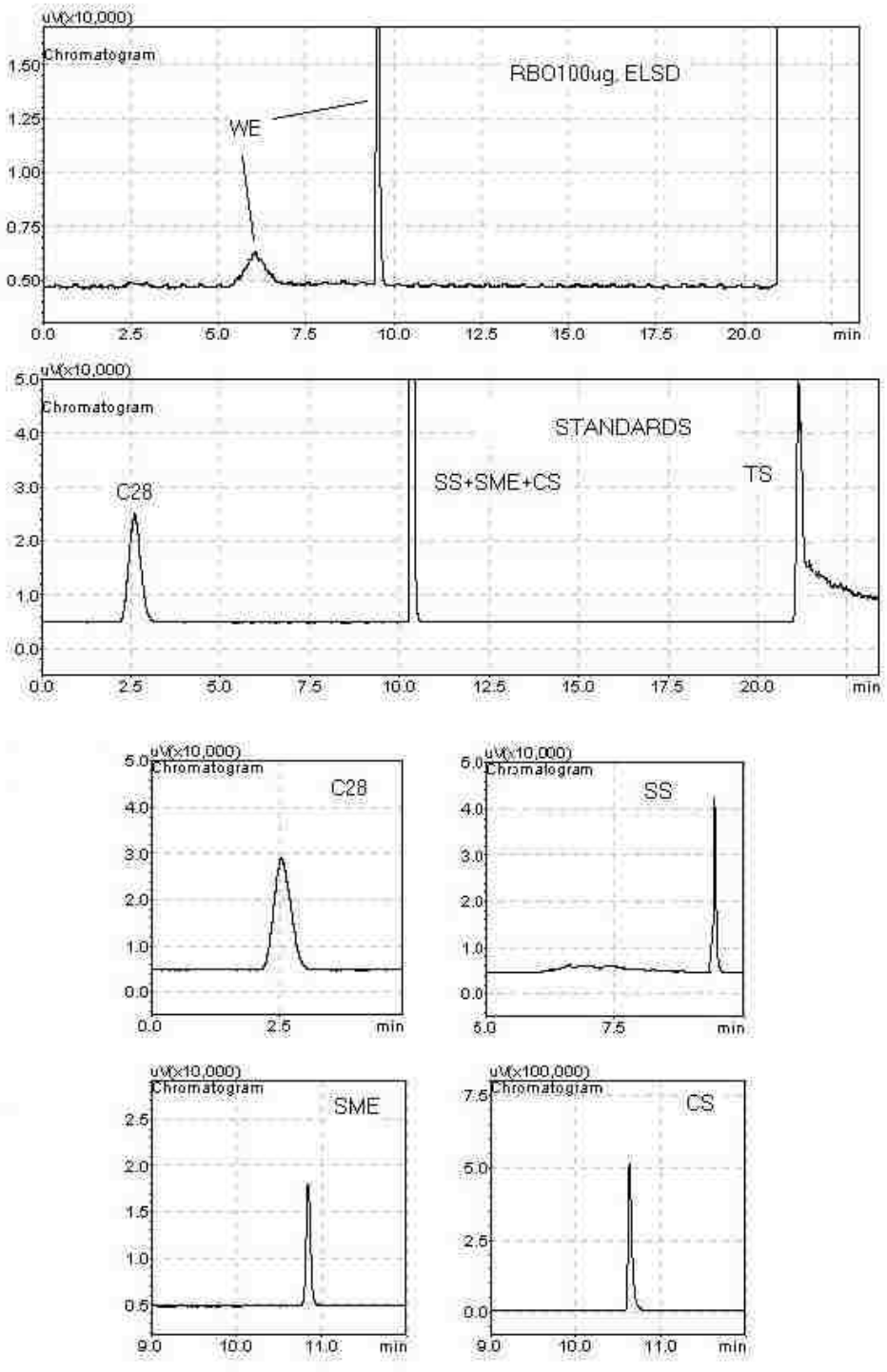


Figure 2.12. Chromatograms of a 2SI method with hexane:diethyl ether (100:3). The first picture shows RBO injection, and the rest show the standard injection. C28: octacosane, SS: stearyl stearate, SME: stearate methyl ester, CS: cholesteryl stearate, TS: tristearine.

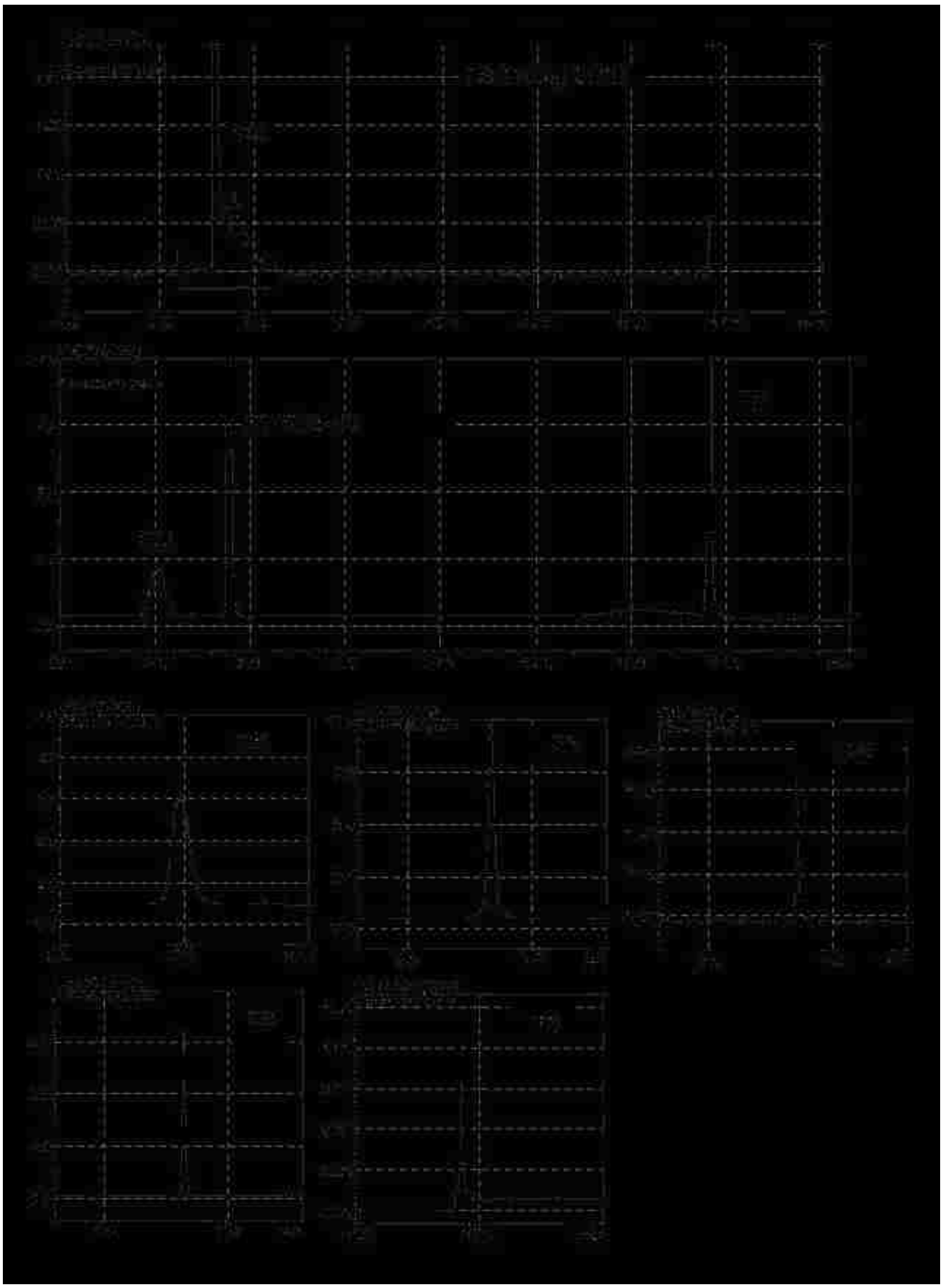


Figure 2.13. Chromatograms of a 2SI method with hexane:diethyl ether (100:2, isocratic). The first picture shows RBO injection, and the rest show the standard injection. C28: octacosane, SS: stearyl stearate, SME: stearate methyl ester, CS: cholesteryl stearate, TS: tristearine.

A preparative process with a C18 column (Discovery C18 preparative column, 250 mm x 212 mm, 5 μm, coupled with Supelguard, 20 mm x 4 mm, 5 μm) method was also developed. The solvent temperature and the ELSD condition were the same as Method 3, except the sensitivity was set to 1 (the lowest). Soxhlet-extracted FF RBO and DF RBO samples were prepared in isopropanol at 500 mg/ml, and 100 mg (about 200 μl) of the solution was injected after the sample was heated until it became clear. ELSD data acquisition started 4 min after injection. In the chromatogram (fig. 2.14), the injected lipids were eluted with acetonitrile (Solvent A): chloroform (Solvent B) (75:25, v/v) for the first 18 min, A60:B40, for the next 3 min, A30:B70, for the next 4 min, and A10:B90 for the last 4 min. The flow rate was fixed at 15 ml/min, and the eluent was distributed to the ELSD and the waste. Both of the chromatograms (fig. 2.14) produced similar peak pattern except the peak at 30 min was much stronger with DF RBO. The occurrence of three conspicuous peaks at 24, 26, and 30 min requires further analysis for identification and composition.

This preparative method was developed for preparation of WE that could be used as a weight standard in RBO analysis. For a preparative silica column, the oil should be carefully dehydrated in order to maintain the resolution, and polar substances should be removed before injection, which means the unprocessed crude RBO is not suitable for the silica column. However, the unprocessed crude RBO can be injected into a C18 column after a short filtration. This is a great advantage over a preparative silica column.

In the prep C18 method, chloroform was used instead of propanol. The polarity indices of chloroform and propanol were almost the same (4.1 and 4.0, respectively), but their boiling points are different (67°C and 97°C, respectively) (See appendix 7). The mixture of acetonitrile and propanol did not block UV 205 nm as much as that of acetonitrile and chloroform, which

makes acetonitrile and propanol more suitable for analytical purpose. In large-scale separation, the boiling point of the solvent is important during the recovery of the eluted material, and solvent evaporation sometimes requires a long time. The sample can deteriorate during high temperature evaporation. At 90% of chloroform in acetonitrile, the preparative column pressure reached 1800 psi. Even at 70% of propanol in acetonitrile, the column pressure appeared to be 2800 psi with the analytical C18 column. The mixture of acetonitrile and chloroform would be a more appropriate solvent for preparative purpose.

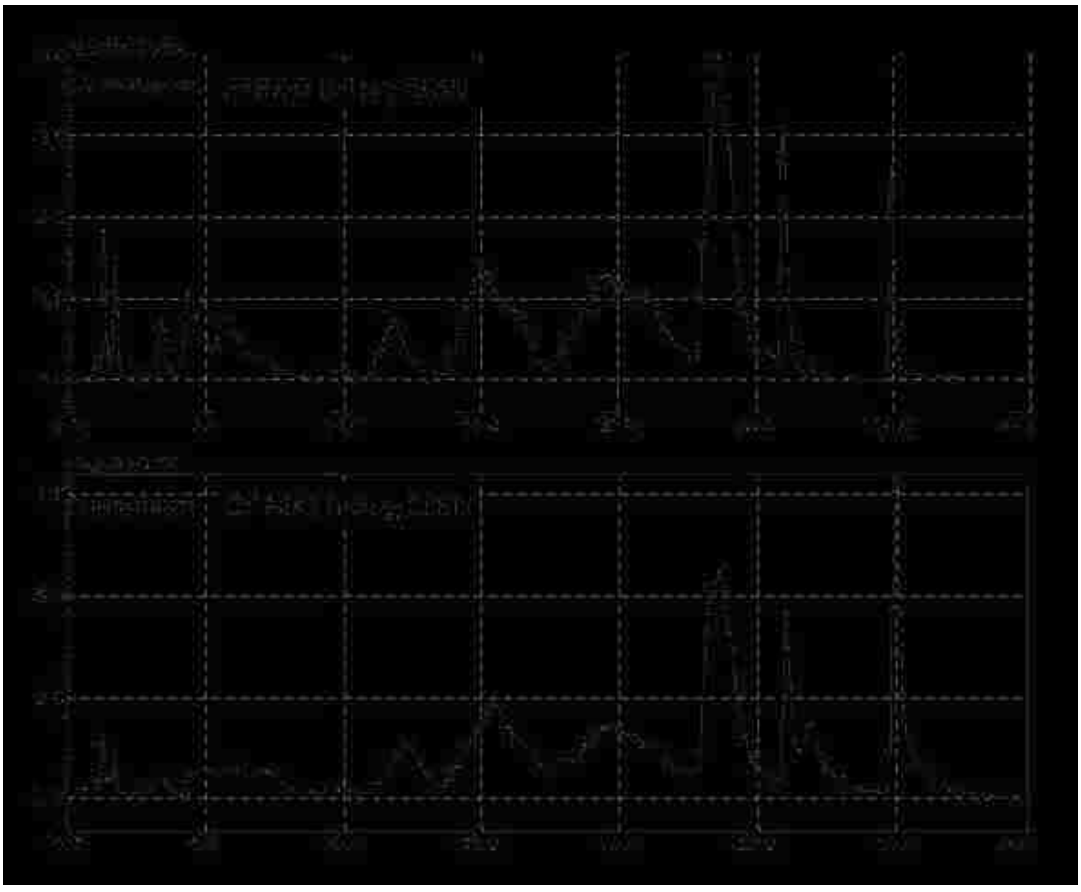


Figure 2.14. Chromatograms of a preparative C18 column method.

2.7. CONCLUSION

Several HPLC methods for analysis and separation of WE in RBO were developed and tested with standards and RBO samples. WE was not separated with one simple solvent system, and 2 or more solvents were required to achieve proper resolution. The 2SI methods, originally designed to separate fatty acid methyl esters from steryl esters, failed to separate them, but was useful for the measurement of total WE in a sample as well as WE separation. Preparative or analytical C18 columns showed several advantages over silica columns, but C18 methods could not elute the WE as one peak or in a short time period, which were not appropriate for the measurement or separation of total WEs in an oil sample. The prep C18 method and all other analytical C18 methods may have a potential for preparation of WEs with specific chain lengths or structures.

2.8. REFERENCES

Amelio M, Rizzo R, and Varazini F. Separation of Stigma-3,5-diene, Squalene Isomers, and Wax esters from olive oil by single High-Performance Liquid Chromatography run. JAOCS. 1998, 75(4):527-530

Amelio M, Rizzo R, and Varazini F. Separation of wax esters from olive oils by high-performance liquid chromatography. JAOCS. 1993, 70 (8):793-796

Belavadi VK and Bhowmick DN. An investigation of rice bran oil tank settlings. JAOCS. 1988, 65(2):241-245.

Busson-Breysse J, Farines M and Soulier J. Jojoba wax: its esters and some of its minor components. JAOCS. 1994. 71(9):999-1002.

Cousins ER, Fore SP, Janssen HJ, and Feuge RO. Rice bran oil. VIII. Tank settlings from crude rice bran oil as a source of wax. JAOCS. 1953, 30:9-14.

Garcia A, De Lucas A, Rincon J, Alvarez A, Gracia I, and Garcia MA. Supercritical carbon dioxide extraction of fatty and waxy material from rice bran. JAOCS. 1996, 73(9):1127-1131.

Hamilton, R.J., Analysis of Waxes, in Waxes: Chemistry, Molecular Biology and Functions,

edited by R.J. Hamilton, Oily Press, Dundee, Scotland, 1995, 6:311–342.

Hwang KT, Cuppett SL, Weller CL, Hanna MA. Properties, Composition, and Analysis of Grain Sorghum Wax. JAOCS, 2002, 79(6):521-527.

Ito S, Suzuki T, and Fujino Y. Wax lipid in rice bran. Cereal Chemistry, 1983, 60(3):252-253.

Megoulas NC and Koupparis MA. Twenty Years of Evaporative Light Scattering Detection. Critical Reviews in Analytical Chemistry. 2005, 35:301–316.

Moreau RA, Kohout K, and Singh V. Temperature-enhanced alumina HPLC method for the analysis of wax esters, sterol esters, and methyl esters. Lipids. 2002. 37 (12):1201-1204.

Nordback J and Lundberg E. High Resolution Separation of Non-Polar Lipid Classes by HPLC-ELSD Using Alumina as Stationary Phase. J. High Resol. Chromatogr. 1999, 22:(9) 483–486

Personal correspondence to representative of Merck in the U.S. 2007.

Rezanka, T. Very-long-chain alkyl esters in Cereus peruvianus wax. Phytochemistry. 1998. 47(6):1145-1148.

Shantha NC and Napolitano GE. Lipid analysis using thin-layer chromatography and the Iatroscan. In: Hamilton RJ, editor. Lipid analysis in oils and fats. 1998. Blackie Academic & Professional (Chapman & Hall), London, UK. pp1-33.

Tulloch AP. Chemistry of Waxes of Higher Plants. In; Kolattukudy PE, editor. Chemistry and Biochemistry of Natural Waxes. 1976, Elsevier Scientific Publishing Company, Amsterdam, The Netherland. p235-287.

Vali SR, Ju Y, Kaimal TNB, and Chern Y. A Process for the Preparation of Food-Grade Rice Bran Wax and the Determination of Its Composition. JAOCS. 2005, 82(1):57-64.

Yoon SH and Rhee JS. Composition of waxes from crude rice bran oil. JAOCS. 1982, 59:561-563.

CHAPTER 3.
COMPOSITIONAL ANALYSIS OF WAX ESTERS FROM RBO AND RBW USING GC-MS

3.1. INTRODUCTION

Wax analysis can be accomplished using TLC and GC when significant amounts of wax components are not required for further specific purposes. Based on the staining methods for TLC, when a non-destructive staining method is used, the stained layer is scrapped, the substance is eluted from the matrix, and then it is directly injected into GC. GC analysis is almost always used for wax analysis, for both identification and composition analysis of wax components (Misra and Ghosh, 1991). Nowadays, most wax studies adopt GC methods with a capillary column, which should be stable at temperatures up to 400 ^{o}C for wax analysis. High resolution of GC makes it possible to identify an unknown material at very low concentrations, to analyze the concentration of an injected substance, and even to confirm the existence of structural isomers in the sample. It also provides information on the purity of an isolated substance whose concentration is too low to be detected by TLC. However, before GC analysis, WE should be saponified or hydrolyzed into fatty acids and fatty alcohols or sterols. Fatty acids should be derivatized into methyl esters (Gunawan et. al., 2006), and both alcohols and sterols into TMS ethers (Lagarda et. al., 2006; Adhikari et. al., 2006). Wax esters are usually injected into a GC without derivatization. Recently, a group of researchers at the USDA-SRRC in New Orleans conducted composition analyses of rice bran policosanol and found that long chain fatty alcohols are resistant to TMS derivatization (BSTPA + TMCS) (Personal correspondence, 2008). Another difficulty in analysis of steryl esters is that they are usually not detected by GC due to their high molecular weights. Ito et. al. (1983) and Gunawan et. al. (2006) provided some information on saponified steryl esters or sterol methyl esters, but not on the intact steryl esters.

3.2. LITERATURE REVIEW

3.2.1. RBWE Composition

In Sec. 1.2.1, several studies on composition analysis of RBW were reviewed. Yoon and Rhee (1982) reported that the hard RBW contained fatty alcohols of C24, C26, and C30, and fatty acids of C22, C24, and C26, while the soft RBW was composed of saturated fatty alcohols of C24 and C30, and saturated fatty acids of C16 and C26. Their study indicates that the hard RBW, which has a higher melting point than that of the soft RBW, contains slightly more long chain species. Ito et al. (1983) identified short chain alkyl esters of C15-C20, and long chain alkyl esters of C38-C58. The main SE was linoleoyl sitosterol. They separated WE from RBO, not from RBW, which explains the existence of short chain alkyl esters. Garcia et al. (1996) identified the composition of fatty acids (C14-C34) and fatty alcohols (C22-C34) in WE from RBW. They also reported that supercritical carbon dioxide (SC-CO2) extracted RBO had more long chain species. Vali et al. (2005) indicated that RBW is mainly a mixture of saturated alkyl esters of C22 and C24 fatty acids and C24 to C40 aliphatic alcohols, with C24 and C30 being the predominant fatty acid and fatty alcohol, respectively. The chain lengths of intact alkyl esters reached C44-C64. They used WE from crude RBO tank settling, not from RBO. Gunawan et al. (2006) found that the major fatty acids in steryl ester fraction are linoleic acid and oleic acid, and that the sterols were campesterol, stigmasterol, sitosterol, stigmastenol, citrostadienol, cycloartenol, and cycloartanol. The major constituents were saturated alkyl esters of C22 and C24 fatty acids and C24 to C40 aliphatic alcohols, with C24 and C30 being the predominant fatty acids and fatty alcohols, respectively. They also identified long chain alkyl esters (C44-C62).

From the studies above, several general aspects of WE from RBO or RBW can be found;

- WE from RBW tends to have more long chain species than that from RBO.
- Alcohols (C24-C40) in RBWE tend to be longer than fatty acids (C16-C26).
- The higher the RBW melting point, the longer are the chain lengths of its alkyl esters.
- The chain lengths of fatty acids in alkyl esters tend to be longer than those of fatty acids in steryl esters.

Unfortunately, the authors of the studies above did not compare the composition of WE from RBO and from RBW which are extracted from the same RB at the same time.

3.2.2. GC Methods for Wax Analysis

Recent studies on wax composition analysis used a nonpolar polysiloxane (DB-5HT) GC column, which is stable at high temperature up to 400 ^{o}C. Nitrogen or helium has been used as carrier gases. Vali et al. (2005) analyzed WE in RBW with a DB-5HT (5%-phenyl)-methylpolysiloxane nonpolar column (15m x 0.32 mm). The temperature was set to 80-380 ^{o}C at 15 ^{o}C/min. Gunawan et al. (2006) used the same column for WE analysis in RBW. The temperature was set to 80-365 ^{o}C at 15 ^{o}C/min. Nota et al. (1999) studied WE in olive oil with a RTX-65TG capillary column (30m x 0.25mm, 0.1 um thick). The temperature was set to 270-360 ^{o}C at 5 ^{o}C/min. Reiter and Lorbeer (2001) analyzed WE in olive oil and sunflower oil. They used a DB-1 fused silica capillary column (12 m x 0.32 mm, 0.25 μm) and the temperature was set to 75-350 ^{o}C at 10 ^{o}C/min. In those studies, they used the same condition for fatty acid and fatty alcohol analysis. Grob and Lanfranchi (1989) determined free sterols, steryl esters, and WEs in oils and fats using LC-GC. They analyzed all compounds in a single run of GC after derivatization of sterols with pivalic anhydride in pyridine. The temperature ramped from 200-350 ^{o}C at 10 ^{o}C/min. The sterols were eluted at 280-310 ^{o}C (8-11 min), alkyl esters (C38-C46) at

320-350 ^{o}C (12-16 min), and steryl esters at 350 ^{o}C (17-20 min). Alkyl esters in RBW tend to be longer than C38-C46, so the same method cannot be applied to RBW analysis. However, their study provides information on a suitable temperature range for WE analysis. Bianchi et al. (1994) studied the chemical structure of alkyl esters from Sansa olive oil using GC-MS with a temperature range of 60 – 350 ^{o}C. They could identify alkyl esters of C19-C46. Hu et al. (1993) characterized wax sediments in refined canola oil, and the temperature range was 240-360 ^{o}C. They identified C36-C56 alkyl esters, and some triacylglyceride species (C48-C60) were also eluted at the same temperature range with the alkyl esters. These studies indicate that for RBW analysis the temperature of GC should be raised up to 400 ^{o}C because it contains C62 alkyl esters and steryl esters. Even though that high temperature can be used, it is likely that some WE species might not be detected. To analyze longer and heavier WE, several analytical methods other than GC-MS were used. Ito et al. (1983) and Belavadi and Bhowmick (1988) used Infrared (IR) spectroscopy for RBW analysis. Nuclear Magnetic Resonance (NMR) spectroscopy was used for alkyl ester analysis (Bianchi et al., 1994). Mass spectroscopy (MS), which is not coupled with GC, can be utilized for WE analysis (Hwang et al., 2002).

3.3. OBJECTIVES OF STUDY

Up to this point, the amounts of WE in RBO samples prepared by MAE and Soxhlet extraction methods were investigated with winterization and HPLC. RBW separated with winterization contains not only WE but also other lipid species. There have been several studies on the compositions of WE in RBW, which consists of alkyl esters and steryl esters, but the composition was not compared with that of WE separated from RBO by HPLC. WE separated from RBO by HPLC contains not only crystallizable species but also non-crystallizable

components, and the chain lengths of the crystallizables are considered relatively longer than those of the non-crystallizables. Therefore, comprehensive studies are required to clarify compositional differences between crystallizable WE and non-crystallizable WE. This can be investigated by GC analysis after WEs are separated from RBW and RBO and saponified.

Therefore, the objectives of this study were; 1) to develop an HPLC method to separate fatty acids, fatty alcohols, and sterols from saponified RBWE, 2) to obtain qualitative compositions of intact WE by injecting it into a mass analyzer, 3) to compare the compositional difference between WE from RBW and RBO, and 4) to compare the compositional difference between WE from FF RB and DF RBO.

The results from these experiments can provide insight into the separation methods for each component of wax esters as well as their compositions.

3.4. MATERIALS AND METHODS

3.4.1. Mass Spectroscopy

WE from FF RBW, FF RBO, DF RBW, and DF RBO, which were all prepared from Soxhlet extraction, were injected into a mass analyzer in order to obtain the molecular weights of intact WE. All WE samples were prepared in isopropanol at 10 mg/ml, and 100 μl of each sample was mixed with the same volume of acetonitrile:methanol:formic acid (50:50:1). The sample solution was directly injected into a Finnigan LTQ Mass Spectrometer (Thermo Electron Corporation, Waltham, MA). The sample flow rate was set to 10 μl/min. The electronspray ionization (ESI) condition was as follows; Nitrogen and helium were used as sheath gas and as collision gas, respectively, which were set at flows of 6 units; ionspray voltage and the Tube Lens Voltage (TLV) were set to 6300 V and 30 V, respectively; capillary temperature was set to 300 ^{o}C and

the capillary voltage of 15 V was used. A full scan mass spectrum between 0 and 2000 Da was acquired at 10 msec/scan. To obtain the spectrum of each sample, only m/z ($M+1^{+}$) range of 0 to 1000 Da was selected. Only ions with relative abundance higher than 10% were considered for this study.

3.4.2. Saponification

WEs from hexane-extracted RBO and RBW were used for saponification and further GC analyses. WE was separated from the processed RBO by the preparative HPLC method and from the refined RBW by the analytical HPLC method described previously. Five milligrams of the refined RBW was injected each time and ELSD sensitivity was set to 1 (lowest). Ten to twenty milligrams of WE was placed in a screw-capped tube, and 2 ml of 30% NaOH in isopropanol was added to each tube. Saponification lasted 6 hr at 100 °C with vigorous agitation. After saponification, isopropanol was dried in a centrifugal evaporator at 80 °C, and 4 ml of ethyl acetate was added to the tube. The mixture was sonicated for 2 min, heated at 60 °C for 10 min, and sonicated again for 2 min. It was then centrifuged at 1500g for 5 min, and the ethyl acetate layer (fatty alcohol fraction, FAL) was carefully removed into another test tube. After this ethyl acetate extraction was done 3 times, the white solid residue was completely dried in a centrifugal evaporator at 80 °C. The dried matter was acidified with 2 ml of 37% HCl in water and heated at 60 °C for 1 hr. During acidification, the mixture was vortexed every 5 minutes. After acidification, 4 ml of ethyl acetate was added to the tube, vortexed, and heated at 60 °C for 1 hr. The mixture was then centrifuged at 1500g for 5 min, and the ethyl acetate layer (fatty acid fraction, FA) was carefully removed into another test tube. This extraction was done 3 times with ethyl acetate and 1 time with hexane. After the solvent was dried in a centrifugal evaporator, the

FAL and FA fractions were dissolved in 4 ml of hexane, dehydrated with 100 mg of sodium sulfate, and filtered through a 0.2-μm syringe filter. After hexane evaporation, the FA and FAL fractions were weighed and stored at -20 °C. The saponification process and recovery of FAL and FA are shown in fig. 3.1.

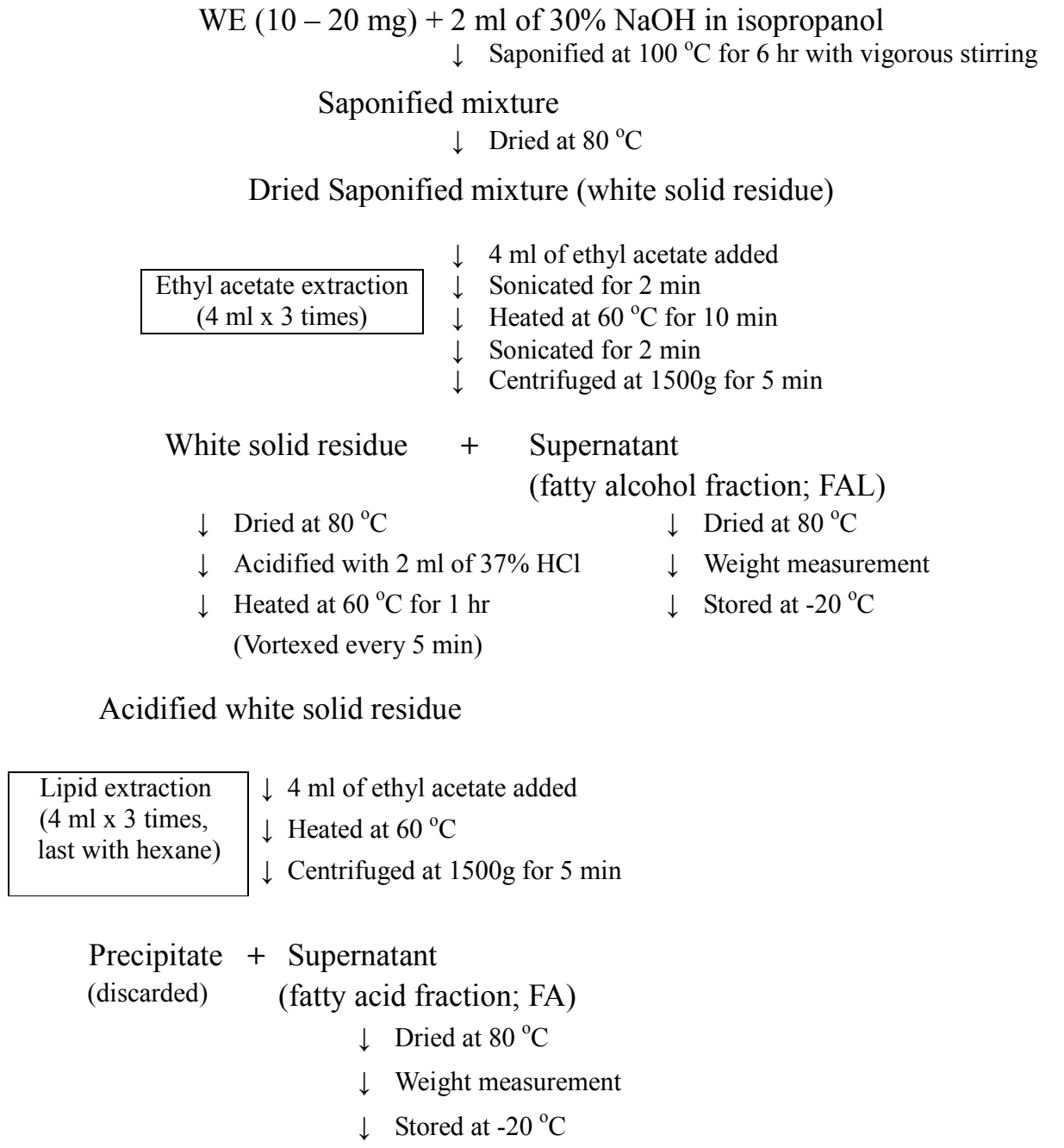


Figure 3.1. Saponification Procedure and Recovery of Fatty Acid and Alcohol Fractions

3.4.3. HPLC Separation of Fatty Acids, Fatty Alcohols and Sterols from FA and FAL

Fatty acids were separated from FA and fatty alcohols and sterols were separated from FAL by an HPLC method. This separation was performed with a Waters Delta Prep 4000 preparative chromatography system (Waters Corporation, Milford, Massachusetts, USA), an ELSD detector (ELSD-LT2, Shimadzu, Kyoto, Japan), and an analytical HPLC column (Supelcosil LC-SI column, 250 mm x 4.6 mm, 5 μm, Supelco, Bellefonte, PA) coupled with a guard column (50 mm x 2 mm), which was manually packed with TLC-grade silica gel (SilicAR TLC-7, 35–60 μm, Mallinckrodt, Phillipsburg, NJ). The data was processed with a Shimadzu GC solution program (Shimadzu, Kyoto, Japan). All the stainless steel tubing was heated at 50 °C, but the column was not heated. The ELSD sensitivity was set to 3 (middle). Fractions were collected with a Waters fraction collector (Waters Corporation, Milford, Massachusetts, USA). The standard materials, behenate methyl ester (BME), octadecanol (OCT), cholesterol (CHOL), and stearic acid (SA) were all purchased from Sigma -Aldrich (St. Louis, MO, USA).

The solvent system consisted of hexane:diethyl ether (20:1, Solvent A) and hexane:acetone (5:1, solvent B), and the lipid was eluted with A100% for 5 min, A85%B15% for 10 min, and B100% for 5 min. The standards were prepared at 5 mg/ml in hexane, and 10 – 200 μg was injected. The FAL samples were prepared at 1 or 2 mg/ml in hexane, and 1 ml of each sample was injected. For the fraction collection, the eluent was bypassed directly to the fraction collector, rather than to the ELSD. Fatty acids (A) were collected from FA, and fatty alcohol (AL) and sterols (S) were collected from FAL. After solvent evaporation, A, AL, and S were stored at -20 ^{o}C.

3.4.4. GC Analysis

The fatty acid (A) and the FA samples were derivatized with 1 ml of BCL3-methanol and 1 ml of 2,2-dimethoxypropane (Sigma-Aldrich, St. Louis, MO, USA), and the fatty alcohols (AL) and sterols (S) were derivatized with BSTFA + TMCS (99:1, SYLON BFT) (Sigma-Aldrich, St. Louis, MO, USA). Fatty acid methyl esters standards, which correspond to lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), nonadecanoic acid (C19:0), arachidic acid (C20:0) and behenic acid (C22) fatty acids, and fatty alcohol standards, which correspond to hexadecanol (C16), octadecanol (C18), eicosanol (C20), docosanol (C22), tetracosanol (C24), hexacosanol (C26), octacosanol (C28), and triacontanol (C30) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The alcohol standards were derivatized as described above before injection. The regression lines of fatty acid and alcohol standards appear in fig. 3.2.

Two microliters of each sample was injected into a Hewlett Packard 5890 series II GC (Hewlett-Packard Company, Palo Alto, CA, USA) coupled with a flame ionization detector (FID) and SGE HT-5 high temperature GC column (25 m x 0.32 mm, 0.1 μm, SGE Incorporated, Austin, TX,` USA). Nitrogen was used as a carrier gas and the column pressure was maintained at 10 psi. The split ratio was set to 1:10. The data was processed with the Agilent Chemstation program (Agilent techonolgies, Santa Clara, CA, USA). The injector temperature and the detector temperature were set to 350 ^{o}C and 400 ^{o}C, respectively. The oven temperature was started at 150 ^{o}C for the first 3 min, raised to 400 ^{o}C at 15 ^{o}C/min, and maintained at 400 ^{o}C for 15 min. The total running time was 34.7 min. The peaks of FAME and alcohol samples were identified by comparison with authentic standards. To identify each sterol peak, the sterol samples were also injected into GC-MS. The GC-MS system consisted of Agilent GC 6890 system (Agilent techonolgies, Santa Clara, CA, USA) coupled with Hewlett Packard 5973 Mass

Selective Detector (Hewlett-Packard Company, Palo Alto, CA, USA), a Gerstel Multipurpose autosampler (Gerstel Inc., Baltimore, MD, USA), and an Agilent J&W DB-5MS capillary column (30m x 250 μm i.d., 0.5 μm; Agilent techonolgies, Santa Clara, CA, USA). The data was processed with Agilent Chemstation program (Agilent techonolgies, Santa Clara, CA, USA). The flow rate of helium gas was maintained at 1.0 ml/min, and one microliter of each sample was injected splitlessly. The injector and detector temperatures were kept at 350 ^{o}C. The oven temperature started at 150 ^{o}C for 5 min, increased to 350 ^{o}C at 10 ^{o}C/min, and stayed at 350 ^{o}C for 10 min.

3.4.5. Data Analysis

The data were analyzed with SAS One-way ANOVA program ($p<0.05$), and Tukey's studentized range test was used for the comparison of any significant differences ($p<0.05$).

3.5. RESULTS

The mass spectra of intact WE from FF RBW, FF RBO, DF RBW and DF RBO, which were prepared by Soxhlet extraction, appear in fig. 3.3, 3.4, 3.5, and 3.6, respectively. WE from FF RBW contained more long chain WE than that from FF RBO. In fig 3.6, the spectrum of WE from FF RBW presented the ions with more than 50% of relative abundance with the M+1 ranging from 553.33 to 845.42 and the most abundant ions were 713.42 and 757.42, while the prominent ions with the same relative abundance ranged from 518.79 to 713.41 in the spectrum of WE from FF RBO (fig. 3.3), in which the most abundant ion was 553.33. The left and right parts of the ion 553.33 in both spectra showed a clear difference between the compositions of the 2 WE samples. The ion peak patterns in the mass spectra of WEs from DF RBW and DF RBO

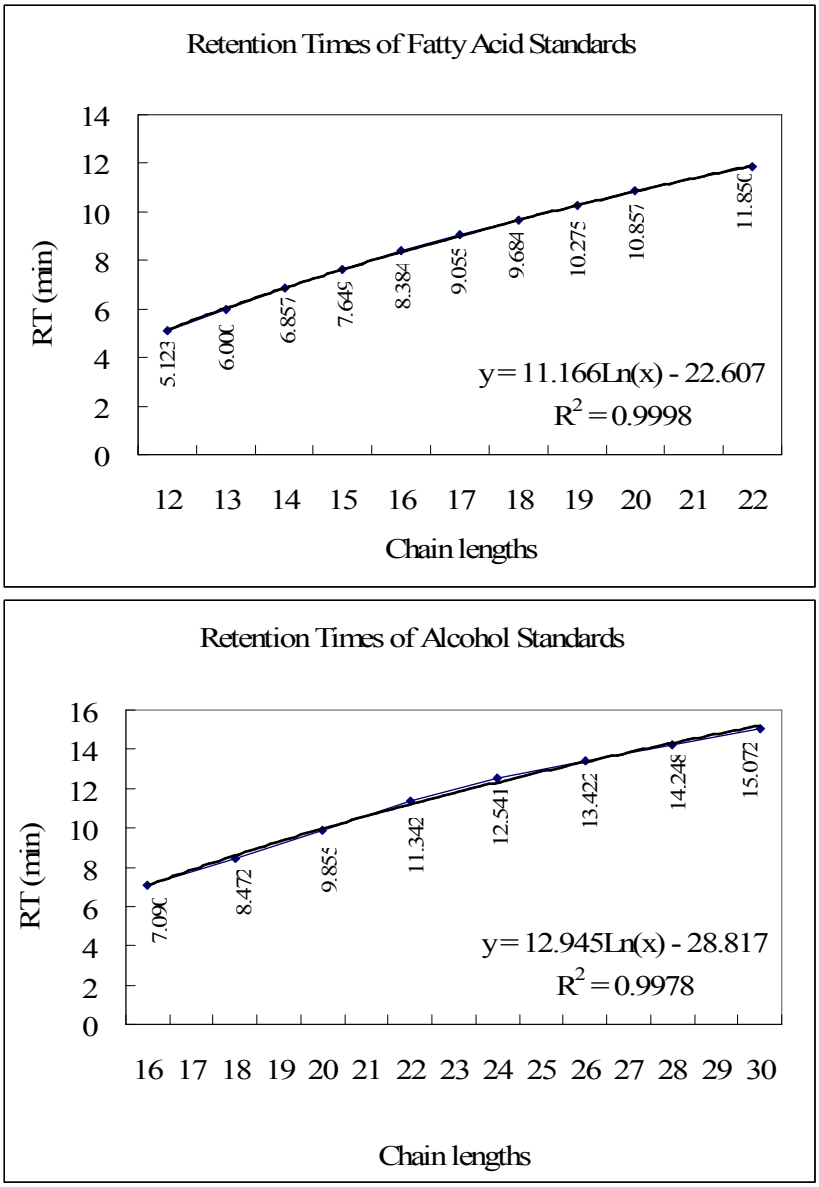


Figure 3.2. Regression lines of retention times of fatty acid and alcohol standards. The peaks in the chromatogram were identified with the regression lines.

were relatively similar (fig. 3.5 and 3.6), but the ions with M+1 ranging from 700 to 900 were more abundant in WE from DF RBW. The molecular weights of alkyl esters and steryl esters are presented in Appendix 8 and 3. In fig. 3.3, the ions were concentrated in the M+1 range between

600 and 900, which corresponds to alkyl esters with chain lengths from C40 to C62 (Appendix 8) and most of steryl esters (Appendix 9). In the region of M+1 over 600 in all the spectra, the groups of two peaks, whose M+1 difference was 14, appeared regularly, and the M+1 difference between two groups was 44, which correspond to the ions of a methyl group ($–CH_2$-) and a ester bond (-COO-), respectively. When fragmented, WE is expected to generate ions that the M+1 are close to the molecular weights of the fatty acids, alcohols, or sterols in the WE. As shown in Appendix 8 and 9, the molecular weights of fatty acids, alcohols, or sterols mostly range from 200 to 600. In that range, only the ions of 308.33 and 553.33 were abundant in the mass spectrum of WE from FF RBW (fig. 3.4), but more than half of the ions with WE from FF RBO belonged to the range.

Fatty acids, fatty alcohols and sterols were separated by HPLC from the saponification products, FAL and FA. Figure 3.7 shows the chromatograms of standard injections. Compared to the signal intensities of BME and CHOL, the peak heights of C18 standards such as OCT and SA were very low when the same amount was injected. Intact WE and its saponified products, FAL and FA, were injected, and their chromatograms appear in fig. 3.8 and 3.9. Intact WE from the processed FF RBO appeared at 2.5 min (fig. 3.8). The FAL fraction contained unsaponified WE at 2.5 min, fatty alcohols (AL) at 7.5 min, and sterols (S) at 10.5 min. The FA fraction showed unsaponified WE at 2.5 min, which was much smaller than that in FAL, and fatty acids (A) at 15 min. In fig. 3.9, the chromatograms of WE from the refined FF RBW are presented, and the results were similar to fig. 3.8. However, the FAL fraction of WE from FF RBW did not show any sterol peak at 10.5 min, which is different from fig. 3.4. The saponification products of WE from the refined DF RBW and the processed DF RBO also exhibited similar peak patterns, and FAL of WE from DF RBW did not show the sterol peak (chromatograms not shown).

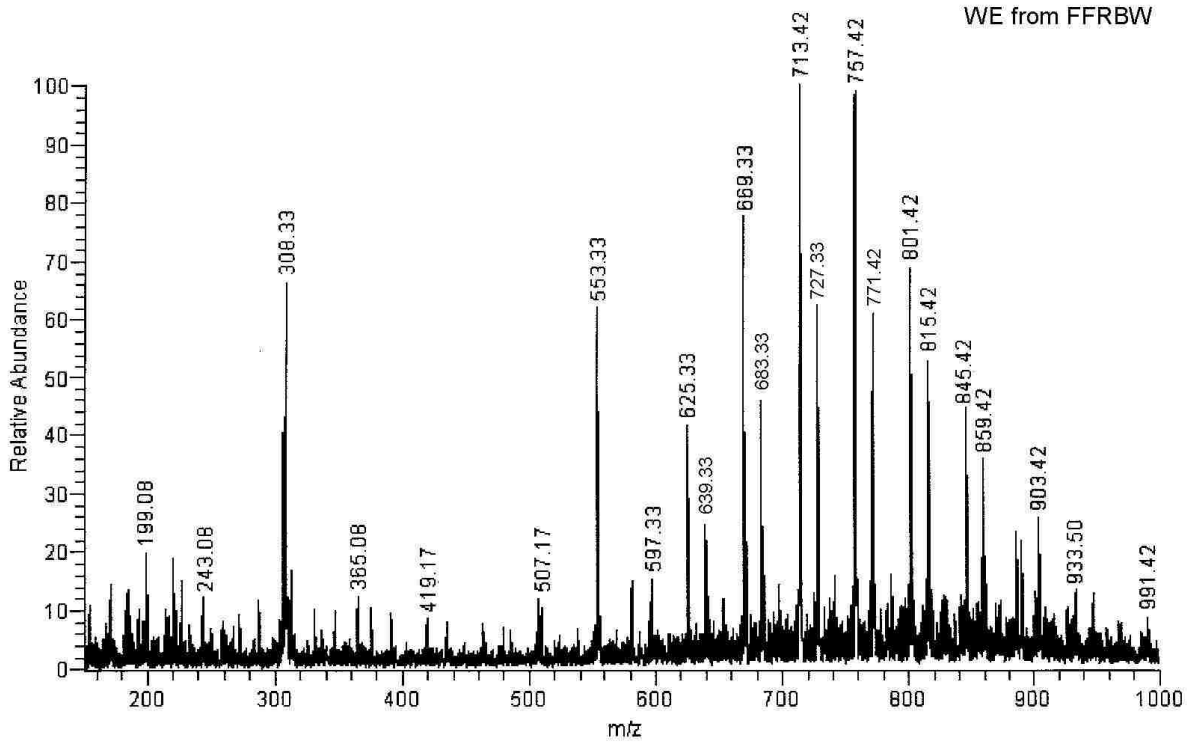


Figure 3.3. Mass spectrum of intact WE from FF RBW

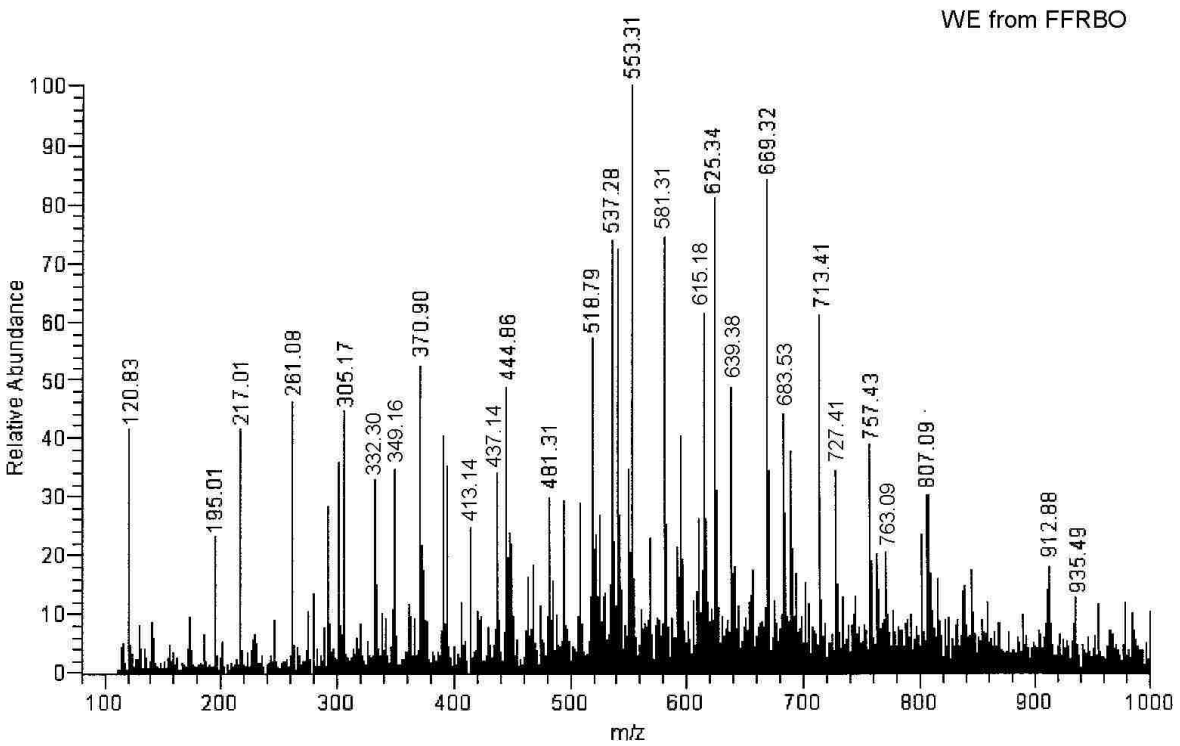


Figure 3.4. Mass spectrum of intact WE from FF RBO.

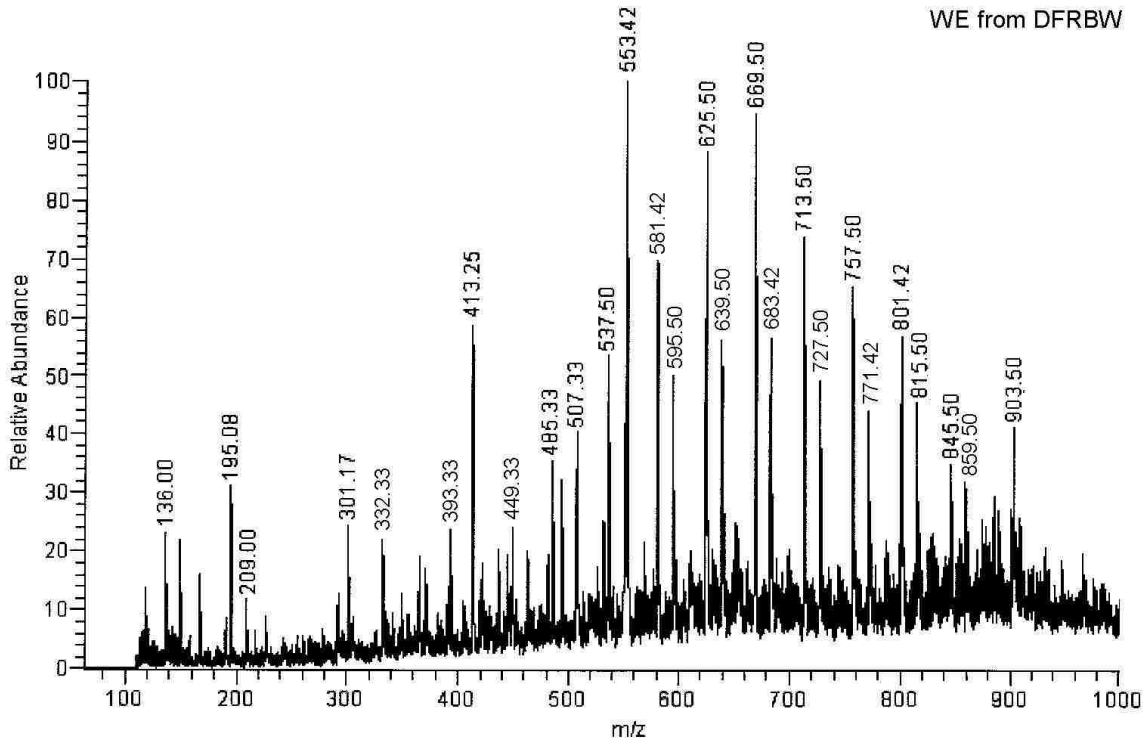


Figure 3.5. Mass spectrum of intact WE from DF RBW.

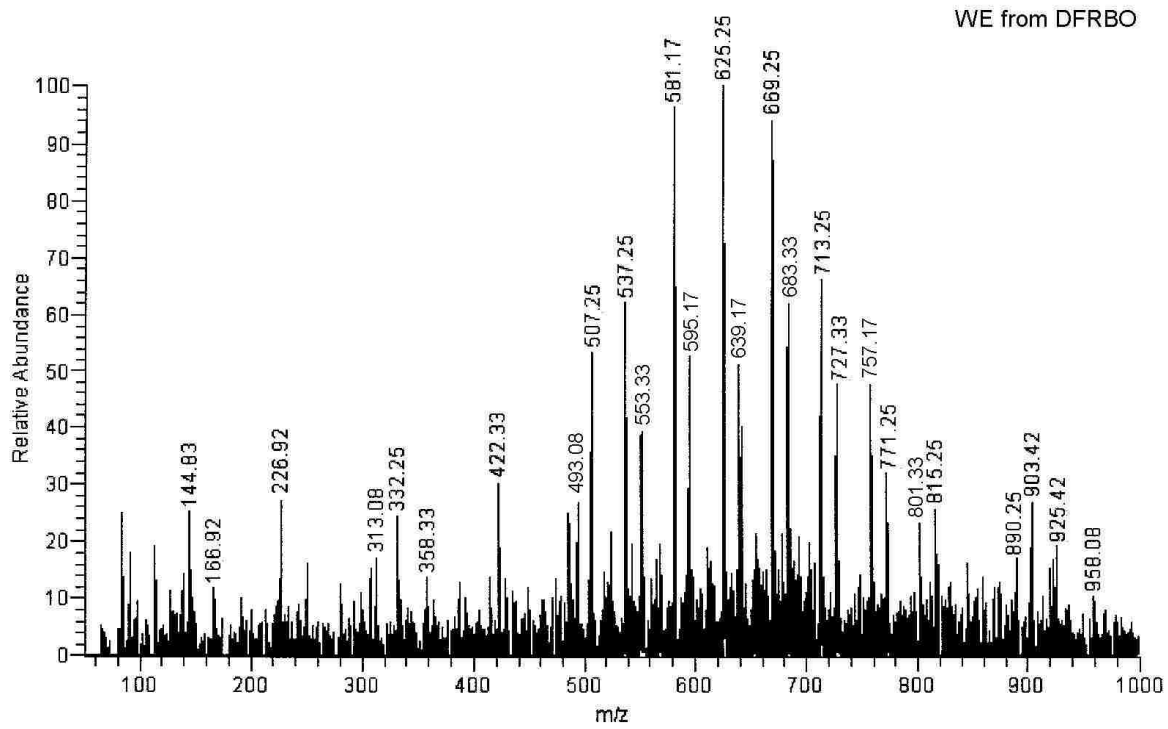


Figure 3.6. Mass spectrum of intact WE from DF RBO.

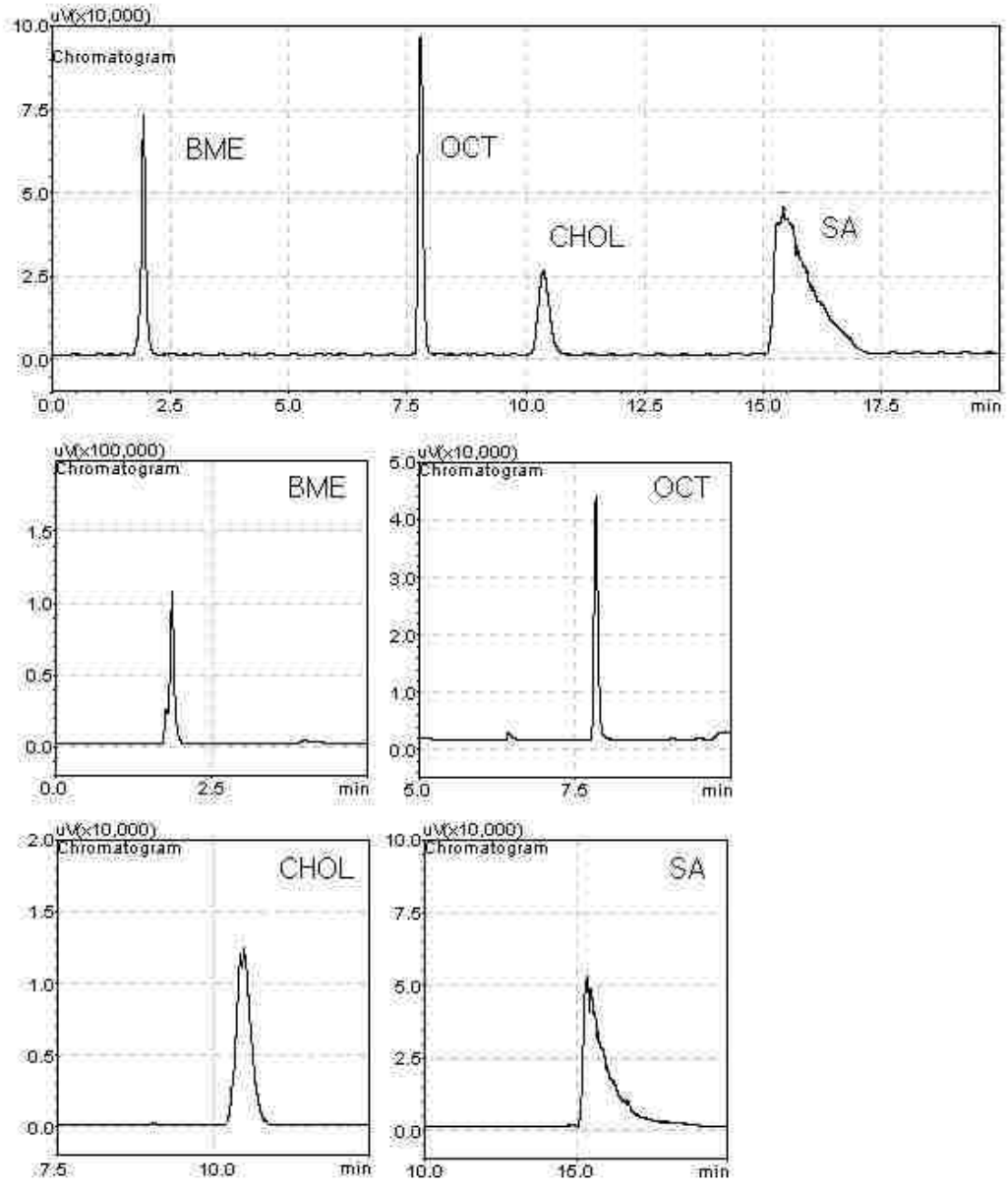


Figure 3.7. Chromatograms of standard injections. Standard mixture (upper; BME 15µg + OCT 150µg + CHOL 10µg + SA 200µg) and individual injections of each standard (lower;. BME 20µg, OCT 100µg, CHOL 5µg, SA 200µg). BME: behenate methyl ester, OCT: octadecanol, CHOL: cholesterol, SA: stearic acid.

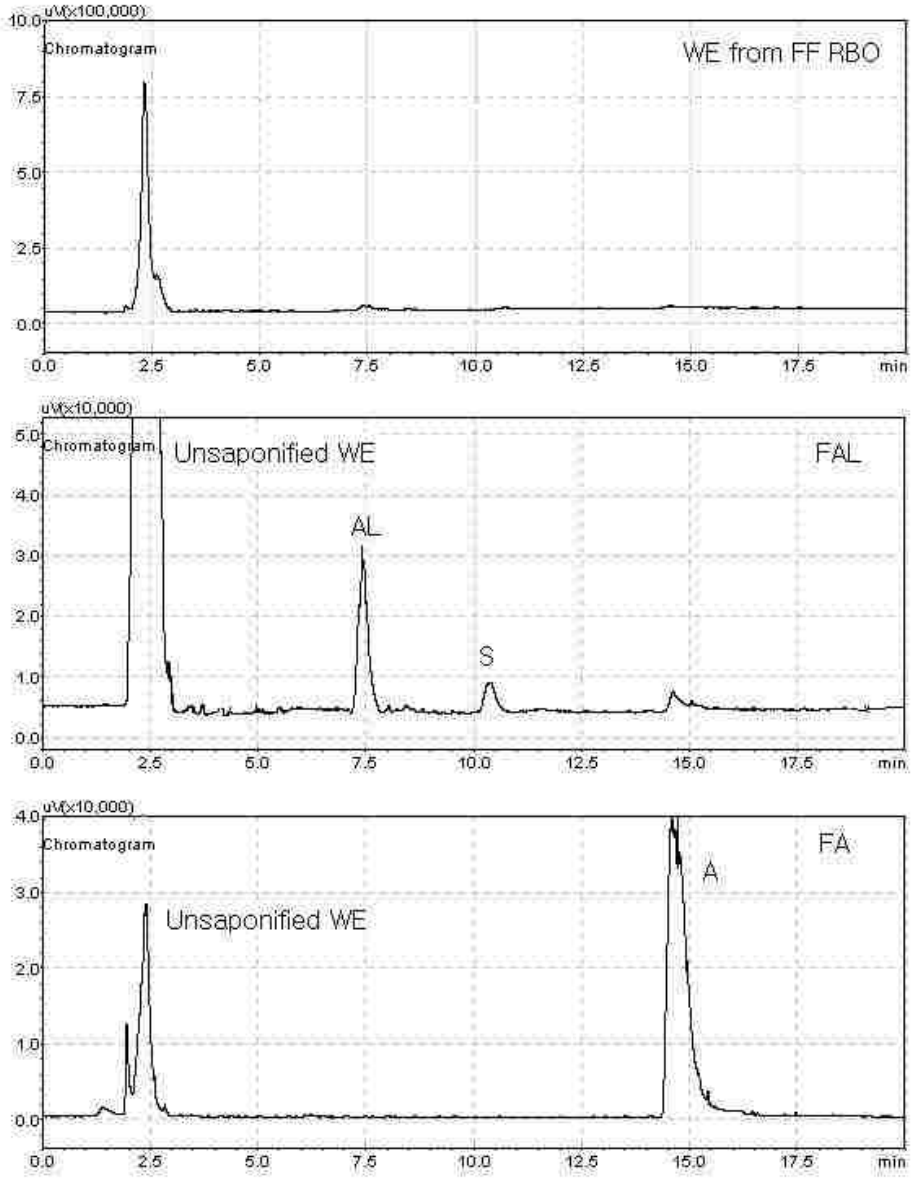


Figure 3.8. Chromatograms of WE from FF RBO and its saponified materials, FAL and FA. Intact WE (50 μg, upper) before saponification, FAL (50 μg, middle) and FA (10 μg, bottom) after saponification. AL: fatty alcohols, A: fatty acids, and S: sterols.

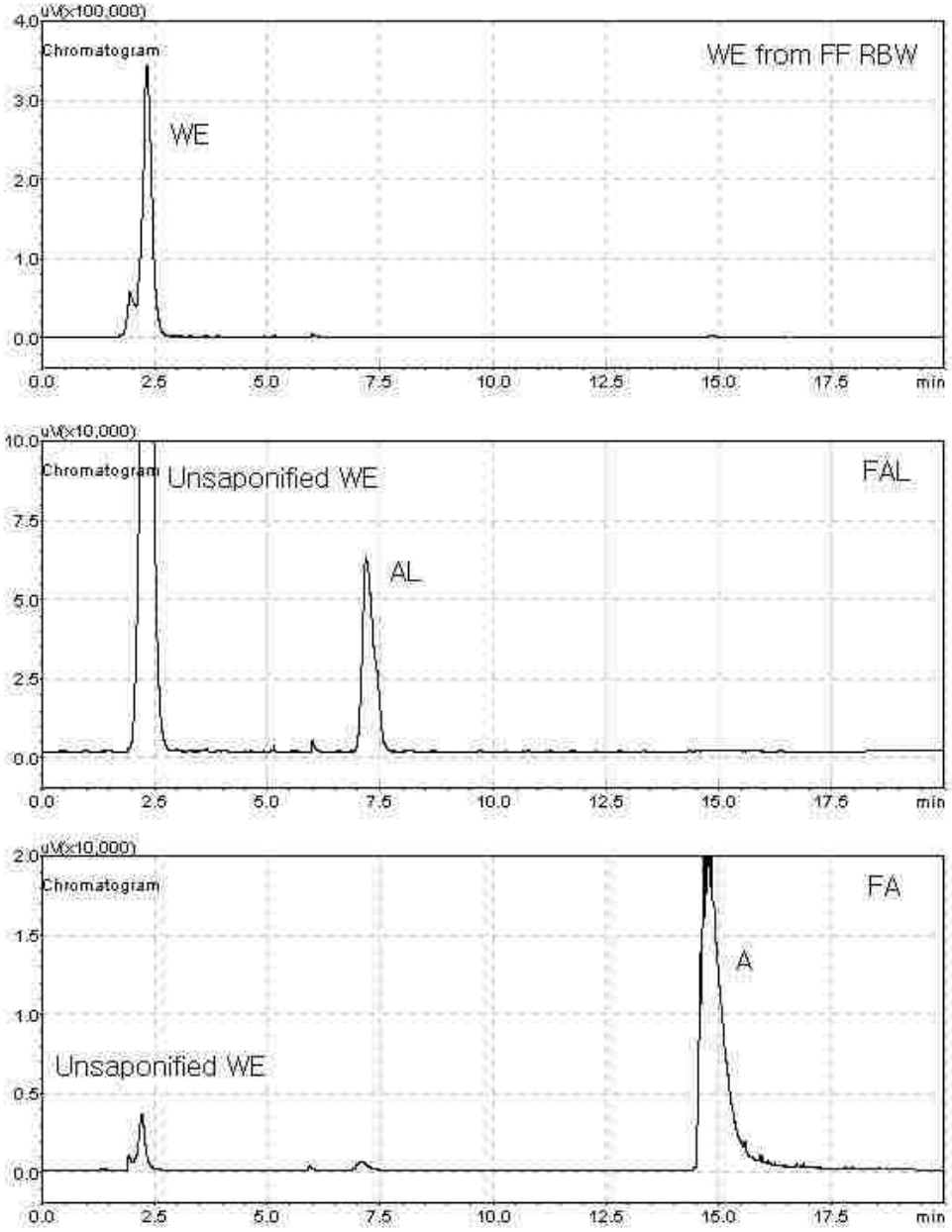


Figure 3.9. Chromatograms of WE from FF RBW and its saponified materials, FAL and FA. Intact WE (50 μg, upper) before saponification, FAL (50 μg, middle) and FA (10 μg, bottom) after saponification. AL: fatty alcohols, A: fatty acids

The GC chromatograms of alcohol samples from WE after saponification are presented in fig. 3. 10. The WE samples from RBO and RBW prepared with hexane extraction were used for GC analysis, and only two samples, of which WE contents were closest to the mean value in Chapter 1, were used after saponification. Alcohol samples of WEs from FF RBW and DF RBW showed similar peak patterns (upper 2 chromatograms in fig. 3.10). Alcohol samples of WEs from FF RBO and DF RBO also appeared similar except for C22 alcohol (lower 2 chromatograms in fig. 3.10). However, the alcohol chromatograms of WEs from RBW and RBO demonstrated that WE from RBW contained more long chain alcohols with chain lengths over C30. Especially C32 and C34 alcohol species appeared as many peaks, which indicates that there may be structural isomers in those alcohols. The chromatograms also exhibited very small peaks between large peaks, which may be odd numbered alcohols. The quantification data of each alcohol species are shown in table 3.1 and 3.2. The major alcohol species were C30, C32, and C34 alcohols (table 3.1), which comprised 9-17%, 25-35%, and 23-33%, respectively. WE from FF RBW contained higher amounts of C32 and C34 alcohols, while the contents of C26, C28, C30, C36, and C38 were higher in WE from DF RBW. In table 3.1, the compositional difference with different extraction methods appeared in C28, C30, C34, and C36, and the differences were prominent in WE from FF RBW. The major alcohol species in WE from RBO were C28 (12-13%), C30 (~20%), C32 (13-15%), and C34 (11-14%) (table 3.2). WE from DF RBO contained slightly higher amounts of C34 and C36, but the content of C22 alcohol was higher in WE from FF RBO. The extraction methods made significant differences in the alcohol contents, but the differences were small (table 3.2). The alcohol contents of WEs from RBW and RBO became obvious when the results of table 3.1 and 3.2 were compared. WE from RBW contained more long chain alcohols (C30-C38), which comprised about 80%, and the contents of short chain

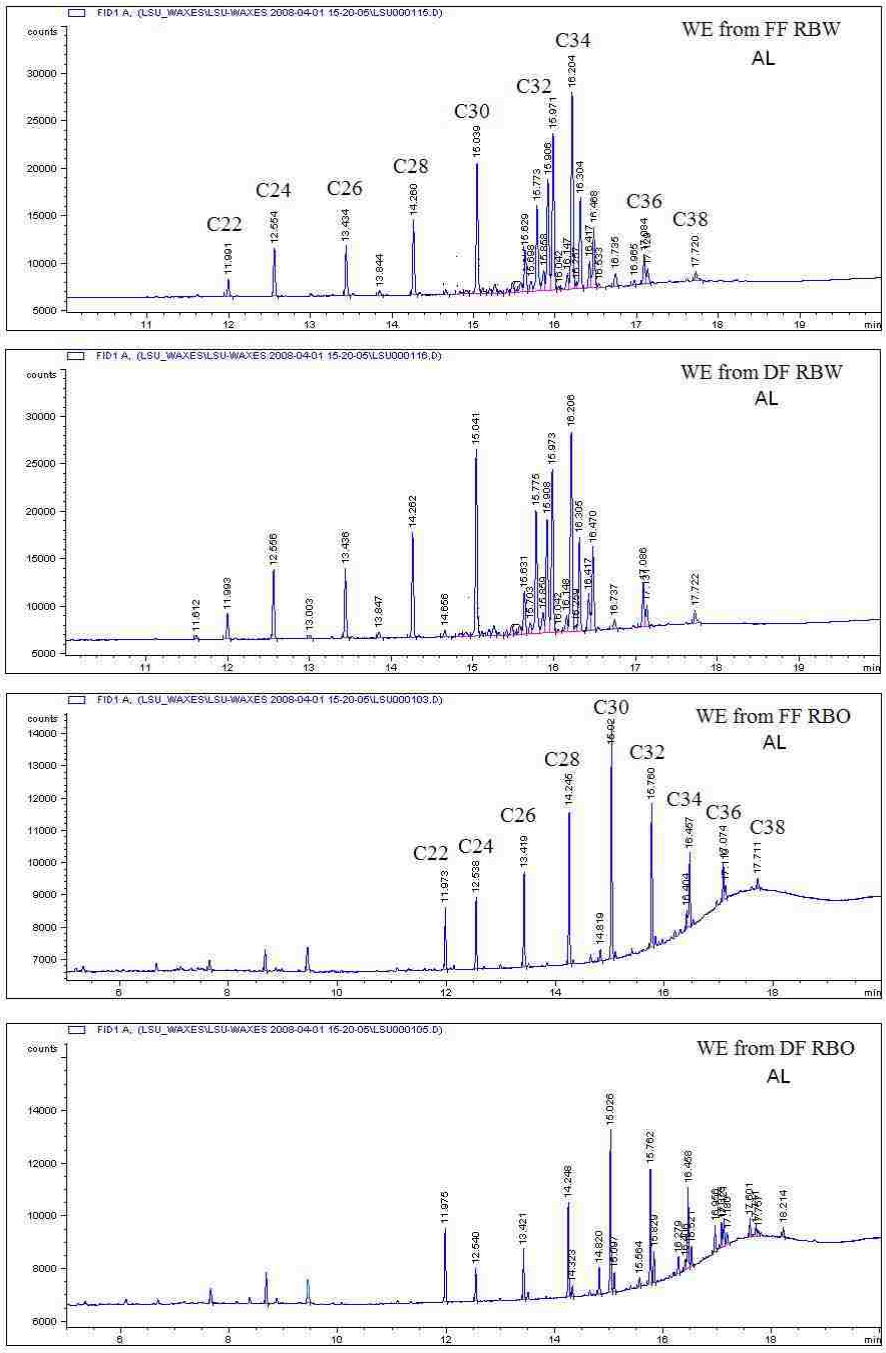


Figure 3.10. GC chromatograms of alcohols (AL) from WE. C22 – C38: Each number indicates the chain length of the saturated 1-alcohol species.

alcohols were less than 20%. Alcohol species shorter than C22 were not detected in the alcohol samples of WE from RBW. WE from RBO contains 6 or 7% of short chain alcohols shorter than C22, and the contents of long chain alcohols over C28 were 62 – 68%.

Table 3.1. Alcohol compositions (%)[a] of WE from RBW.

RB	FF RB		DF RB	
Extractions	SOXHLET	MAE (Hex, 80)	SOXHLET	MAE (Hex, 80)
<C22	n.d.	n.d.	n.d.	n.d.
C22	1.43±0.13^{c,g}	1.22±0.09^{d,g}	0.76±0.06^{e,g}	1.04±0.07^{d,g}
C24	4.19±0.55^{c,h}	3.44±0.16^{d,h}	3.61±0.27^{d,h}	4.13±0.21^{c,h}
C26	3.87±0.17^{c,h}	3.62±0.21^{c,h}	5.37±0.51^{d,i}	5.93±0.36^{d,i}
C28	4.37±0.08^{c,h}	5.42±0.27^{d,i}	8.52±0.40^{e,j}	10.31±0.44^{f,j}
C30	9.08±0.67^{c,i}	14.40±0.67^{d,j}	15.99±0.52^{e,k}	17.39±1.33^{e,k}
C32	35.45±1.27^{c,j}	34.04±1.09^{c,k}	28.83±0.10^{d,l}	25.71±1.45^{d,l}
C34	33.73±1.53^{c,j}	29.62±1.57^{d,l}	24.60±0.54^{e,m}	23.81±0.89^{e,l}
C36	4.68±0.14^{c,h}	4.89±0.24^{c,m}	6.28±0.80^{d,n}	7.05±0.44^{d,m}
C38	0.55±0.01^{c,k}	0.95±0.06^{d,n}	2.15±0.19^{e,o}	1.97±0.02^{e,n}
>C38	tr	tr	tr	tr
Total[b]	97.37±4.55^{c}	97.59±3.46^{c}	96.10±3.37^{c}	97.34±4.35^{c}

a: n=2 and values appear as Mean value ± SD. n.d.: not detected. tr: trace amount. b: The sum of each % is not 100 because there are some odd-numbered alcohol species and those with trace amounts. c-f: Values with different superscripts are significantly different in the same row ($p<0.05$). g-o: Values with different superscripts are significantly different in the same column ($p<0.05$). The graphical illustration of table 3.1 appears in Appendix 10.

GC chromatograms of fatty acid samples showed many peaks (fig. 3.11), which made the quantification of each fatty acid peak almost impossible. In fig. 3.11, several other peaks could be seen around the peak corresponding to a fatty acid standard. Compared with the result of standard injection, fatty acid species ranging from C13 to C22 were identified. However, some of them, especially C18, C19, and C20, exhibited very small peaks among many others, in which case the quantification would be suspected. The GC data could be used qualitatively. The possible causes of such complicated chromatograms and data will be discussed later.

Table 3.2. Alcohol compositions (%)[a] of WE from RBO.

RB	FF RB		DF RB	
Extractions	SOXHLET	MAE (Hex, 80)	SOXHLET	MAE (Hex, 80)
<C22	6.83±0.28^{c,g}	7.44±0.69^{d,g}	6.39±0.59^{c,g}	7.63±0.25^{d,g}
C22	8.56±0.42^{c,h}	9.52±0.15^{d,h}	5.50±0.42^{e,h}	4.30±0.23^{f,h}
C24	5.40±0.33^{c,i}	3.76±0.32^{d,i}	4.65±0.34^{e,i}	4.12±0.24^{e,h}
C26	7.73±0.37^{c,h}	5.01±0.23^{d,j}	7.31±0.24^{c,j}	4.10±0.20^{e,h}
C28	12.42±0.58^{c,j}	12.90±0.27^{c,k}	13.27±0.64^{c,k}	12.31±0.40^{c,i}
C30	20.71±0.64^{c,k}	20.49±0.63^{c,l}	20.30±0.72^{c,l}	19.68±0.25^{c,j}
C32	15.41±0.21^{c,l}	13.62±0.28^{d,k}	14.43±0.73^{c,m}	15.80±0.34^{c,k}
C34	11.16±0.91^{c,j}	10.84±0.42^{c,m}	12.75±0.58^{d,k}	14.20±0.74^{e,l}
C36	7.56±0.43^{c,h}	8.51±0.51^{d,g}	9.25±0.29^{e,n}	11.24±0.95^{f,i}
C38	2.60±0.01^{c,m}	3.36±0.19^{d,i}	4.22±0.12^{e,i}	3.80±0.38^{e,h}
>C38	tr	tr	tr	tr
Total[b]	98.37±3.88^{c}	95.45±3.70^{c}	98.06±4.57^{c}	97.43±3.84^{c}

a: n=2 and values appear as Mean value ± SD. n.d.: not detected. tr: trace amount. b: The sum of each % is not 100 because there are some odd-numbered alcohol species and those with trace amounts. c-f: Values with different superscripts are significantly different in the same row ($p<0.05$). g-n: Values with different superscripts are significantly different in the same column ($p<0.05$). The graphical illustration of table 3.2 appears in Appendix 10.

Figure 3.12 shows the chromatograms of a sterol sample of WE from FF RBO, which was injected into the GC-MS for identification and GC-FID for quantification. In both chromatograms, the sterol sample generated similar peak patterns, and all of the sterol samples exhibited similar peak patterns when injected into the GC-FID. Nine and thirteen peaks appeared in the GC-MS and GC-FID analyses, respectively. Peak 1, 3, and 6 in the GC-MS chromatogram, which correspond to peak 3, 4 and 8 in the GC-FID chromatogram, had exactly the same molecular weights of trimethylsilyl (TMS)-derivatized campesterol, stigmasterol, and sitosterol, respectively, which have been studied by Gunawan et al. (2006). Therefore, other peaks in the GC-FID chromatogram were presumably identified by comparison with the results of that study. Peak 7, 8 and 9 in the GC-FID chromatogram, which correspond to peak 9, 12, and 13 in the GC-FID chromatogram, appear at the same positions with stigmastenol, cycloartenol,

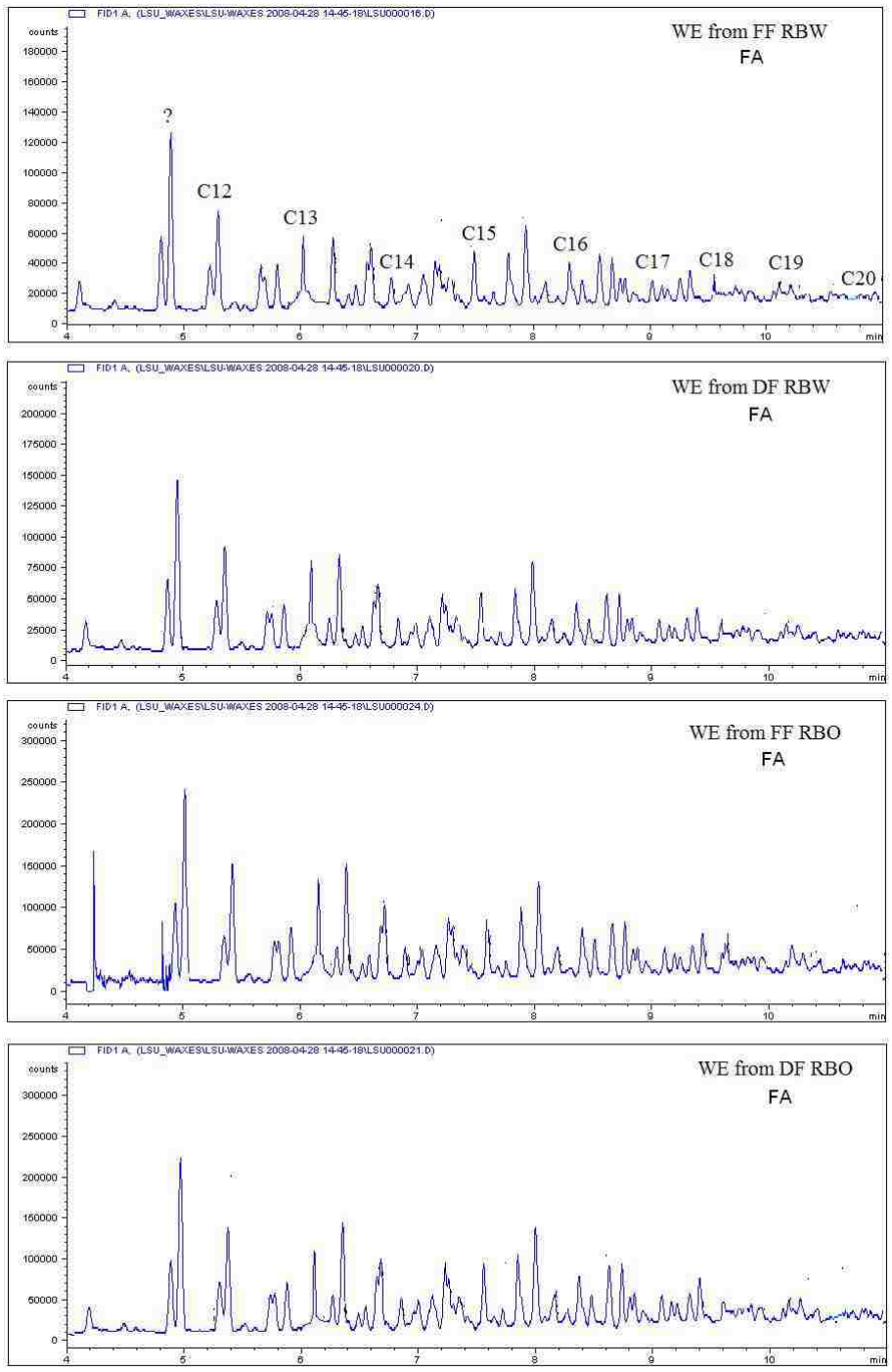


Figure 3.11. GC chromatograms of fatty acid fraction (FA) from WE. C12 – C20: Each number indicates the chain length of the saturated 1-fatty acid species. C22 (behenic acid) peak appears later.

and 24-Methylenecycloartanol in the chromatogram of Gunawan et al. (2006) although the molecular weights of 3 peak were not exactly the same as their study. Peak 1, 2, 5, 6, 10, and 11 in the GC-FID chromatogram did not appear in some samples (chromatograms not shown). It is not clear if peak 4 and 5 in the GC-MS chromatogram correspond to peak 5, 6, or 7 in the GC-FID chromatogram.

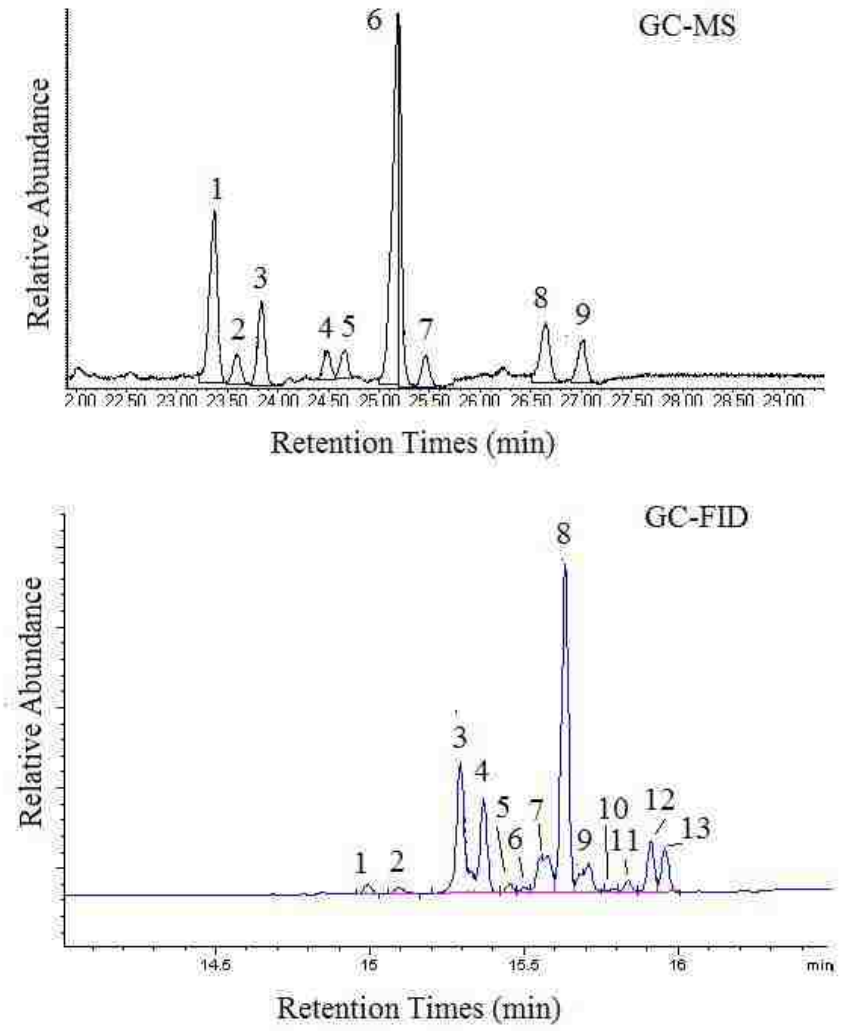


Figure 3.12. GC-MS and GC-FID chromatograms of a sterol sample. The retention time and molecular weight (M^+)of each peak was 1: 23.37 (472), 2: 23.59 (474), 3: 23.84 (484), 4: 24.49 (470), 5: 24.65 (472), 6: 25.20 (486), 7: 25.46 (488), 8: 26.65 (486), and 9: 27.02 (484). The M+ included the derivatizing (trimethylsilyl) group (M=72) (upper). See Appendix 11 for comparison of peak patterns in Gunawan et al. (2006).

Table 3.3 contains the quantification data of each sterol samples. Sitosterol was the most abundant species that comprised 28-36% of the sterol content. The contents of seven major sterols reached 80-85%. WE from Soxhlet-extracted FF RBO contained slightly higher amounts of campesterol, stigmasterol, and sitosterol, while the contents of stigmastenol, cycloartenol, and 24-methylenecycloartanol were higher in WE from MAE-extracted FF RBO. WE from Soxhlet-extracted DF RBO contained more stigmastenol, and WE from MAE-extracted FF RBO had higher contents of stigmasterol and campesterol. A couple of sterol peaks did not appear in the GC-FID chromatograms of the sterol sample from MAE-extracted DF RBO.

So far the compositional difference between WE from RBW and RBO, between WE from FF RB and DF RB, and between WE from Soxhlet and MAE were evaluated. The results indicate that WE from RBW contains more long chain WE species and little or less sterol esters, and that saponified WE may be a good source of long chain alcohols and sterols. The extraction methods caused just a slight difference in the compositions of WE, alcohols, and sterols.

3.6. DISCUSSION

The saponification procedure used in this study followed the method of Vali et al. (2005). Even after saponification in 30% sodium hydroxide in isopropanol, the saponified products of WE from RBW did not possess any sterol peak in the HPLC chromatogram (fig. 3.4), which indicates that WE from RBW does not contain steryl esters or their content is too small to detect, or it could indicate that steryl esters are more resistant to alkali saponification than alkyl esters.. Several saponification or alcoholysis methods have been tried in wax studies. Gunawan et al. (2006) saponified 100 mg of steryl esters with 25 ml of 1 N potassium hydroxide in 90% ethanol, and the mixture was refluxed at 65°C under a nitrogen atmosphere until the reaction was

Table 3.3. Sterol compositions (%)[a] of WE from RBO.

	RB		FF RB		DF RB	
Peaks	RT (min)	Sterol[b]	SOXHLET	MAE (HEX, 80)	SOXHLET	MAE (HEX, 80)
1	14.99		1.82±0.08^{d,h}	2.18±0.04^{e,h}	3.34±0.25^{f,h}	1.53±0.15^{g,h}
2	15.09		1.38±0.12^{d,i}	1.61±0.03^{e,i}	1.71±0.17^{e,i}	1.18±0.13^{d,i}
3	15.29	Campesterol	18.70±0.50^{d,j}	16.19±0.97^{e,j}	16.96±0.87^{e,j}	19.31±1.02^{d,j}
4	15.36	Stigmasterol	11.25±0.80^{d,k}	11.19±0.48^{d,k}	11.06±0.61^{d,k}	10.34±0.48$^{d.k}$
5	15.45		1.46±0.09^{d,i}	1.92±0.22^{e,l}	1.75±0.42^{e,i}	0.95±0.12$^{f.l}$
6	15.50		0.75±0.02^{d,l}	0.86±0.05^{e,m}	0.95±0.07^{e,l}	n.d.
7	15.55	?	7.88±0.35^{d,m}	9.56±0.52^{e,n}	9.95±0.63^{e,m}	8.58±0.52^{e,m}
8	15.63	Sitosterol	34.92±1.72^{d,n}	32.35±1.57^{d,o}	28.43±2.11^{e,n}	35.97±1.92^{d,n}
9	15.70	Stigmastenol	6.50±0.40^{d,o}	8.03±0.67^{e,q}	9.23±0.27^{f,m}	5.49±0.38^{g,o}
10	15.78		0.54±0.04^{d,q}	1.12±0.09^{e,r}	0.58±0.04^{d,o}	n.d.
11	15.83		1.39±0.09^{d,i}	1.82±0.18^{e,l}	2.01±0.13^{e,i}	1.50±0.13^{d,h}
12	15.90	Cycloartenol	6.40±0.80^{d,o}	6.89±0.56^{d,s}	6.61±0.21^{d,q}	6.03±0.56^{d,q}
13	15.95	24-methylene-cycloartanol	4.75±0.35^{d,r}	6.24±0.24^{e,s}	6.32±0.45^{e,q}	6.00±0.24^{e,q}
Total[c]			97.09±1.07^{d}	97.50±2.65^{d}	96.94±2.78^{d}	96.87±1.27^{d}

a: n=2 and values appear as Mean value ± SD. n.d.: not detected. b: The identification of the peaks was presumably done by comparison with the study of Gunawan el al. (2006). c: The sum of each % is not 100 because there are some species with traceable amounts. d-g: Values with different superscripts are significantly different in the same row ($p<0.05$). h-s: Values with different superscripts are significantly different in the same column ($p<0.05$). The graphical illustration of table 3.3 appears in Appendix 12.

completed in 3–4 hr. Carelli et al. (2002) sapoinfied WE from sunflower seed oil with 2 N KOH for 6 hr until completion. Belavadi and Bhowmick (1988) used 10% potassium hydroxide in isopropanol for 4 hr, and found some unsaponifiable matter. Kanya et al. (2007) dissolved 0.25 g of WE in benzene: methanol (1:1, 10 ml), followed by addition of 95% ethanol (50 ml) and 60% aqueous potassium hydroxide (3 ml). The mixture was refluxed for 6 hr for the completion of saponification. Verleyen et al. (2002) saponified a vegetable oil sample (5 g) with 5 ml of 10 M aqueous potassium hydroxide and 45 ml of ethanol. The reaction lasted 30 min at 70 °C for completion. Except Belavadi and Bhowmick (1988), no study indicated any unsaponifiable steryl esters. Further research is necessary to explain this finding.

Mass analyzers have been mostly used to identify the structures and molecular weights of unknown substances. In this study, it was used to demonstrate the molecular weights of intact WE and the difference among WEs from different sources. However, the spectra only indicated the existence of esters and carbon chains. Considering the complexity of WE compositions, it is difficult to specify each WE component such as sterols or sterol esters. When the spectra were superimposed and the highest point of each peak was connected with a line, the compositional difference among WE samples from different sources became clear. An interesting result was that WE from FF RBW contained slightly more long chain species, which indicates that DF RB is already used for oil extraction, so even though the manufacturer tried to leave most of wax in the RB, small amounts of very long chain species could be extracted. That is also explained by the fact that mass spectra of WEs from DF RBW and DF RBO appeared similar.

In Appendix 8 and 9, the molecular weights of WE species are presented. However, it was almost impossible to find the exact molecular weights of any WE species in the mass spectra. The mass analyzer used in this study has the ionization method of electron impact, which is a

powerful method to reduce a large molecule into small fragments and the molecular weight of each ion is expressed in M+1 because the ion is hydrogenated. This does not allow the exact comparison between the molecular weights in Appendix 8 or 9 with those in the mass spectra.

Yoon and Rhee (1982) reported that RBW contains alcohols with chain lengths of C14-C30, and that the major alcohol species are C22, C24, C26, and C30. They found that especially in the hard wax C24 comprises more than half of the total alcohol content, which is different from this study. Belavadi & Bhowmick (1988) found that RBW contains long chain alcohols (C24-C32) and the dominant species are C30 (15%), C32 (12%), and C34 (14%), which is similar with the result of this study except the percentage. Garcia et al.(1996) published alcohol compositions (C22-24) of RBW, and they found that Soxhlet-extracted RBO contains more C30. In their study, the major alcohol species were C28(20%), C30(30%), and C32(20%). Vali et al. (2005) evaluated the compositions of RBW and reported that C22-C40 alcohols exist in RBW. Their results also indicated the presence of a small amount (<4%) of odd-numbered alcohols and branched isomers of species of C34, C36, and C38. In their study, more than 20% of C34 and 40% of C36 exist as branched isomer, and C38 (96%) is mostly branched isomers. In fig. 3.10, the upper 2 chromatograms show many peaks near C32 and C34. Considering very small amounts of odd-numbered alcohol, the peaks are probably branched forms of C32 and C34, which explains why the contents of C32 (25-35%) and C34 (23-33%) appear much higher than other alcohol species in this study.

The quantification of each fatty acid species was not accomplished in this research. Both the fatty acid samples after HPLC separation and the FA fractions before HPLC separation were injected into the GC-FID. They all exhibited almost identical peak patterns in the chromatograms. Both samples were also injected into GC-MS, and in some cases the peak

appearing at the same position with a standard was not identified as a fatty acid, which made the quantification process almost impossible. Until GC analysis, the fatty acid samples suffered two harsh conditions; saponification and acidification. Strong base and acid with heat could break down some fatty acids, especially unsaturated fatty acids. Gunawan et al. (2006) reported that more than 90% of fatty acids, which are contained in steryl esters, are unsaturated species (oleic and linoleic acids). They might be decomposed during those processes. One more critical condition, which may have caused numerous unwanted substances, can be the derivatization process. BCl-3 in methanol derivatizes a fatty acid into a methyl ester. If the fatty acid sample contains not only fatty acids but also other substances, it can cause unexpected chemical reactions. Several journal articles have been published on compositional analyses of RBW, but the papers do not contain GC chromatograms but only quantification data. The alcohol and sterol samples show nice chromatograms in this experiment. If there are alcohols or sterols, there must be fatty acids because alkyl esters and steryl esters contains fatty acids. This problem requires further study.

Belavadi & Bhowmick (1988), Garcia et al.(1996), and Vali et al. (2005) reported that fatty acids in RBW are C14-C24. Especially, Vali et al. (2005) indicated that the major components of fatty acids in RBW are C22 (~20%) and C24 (>60%). In this study, GC-MS analysis showed very small C22 peaks, which means that the concentrations of the fatty acids in the GC sample might be too low to be detected.

The identification of each peak in GC chromatograms of sterol samples was done presumptively. Among 6 peaks that were identified in the GC-MS chromatogram, only 3 presented the exact molecular weights. The others were identified by comparison with the results of Gunawan et al. (2006) even though the molecular weight did not match. The peak pattern in

their chromatogram was very similar with those of this study, which might be used for peak identification (Appendix 11). However, to identify all the peaks in the GC-FID chromatogram, there must be further studies.

Based on the results from this research, although Soxhlet extraction and MAE made slight differences in the compositions of alcohols and sterols, in consideration of relatively short extraction time and less solvent, MAE may be a more suitable candidate for RBO extractions or RBW researches.

3.7. CONCLUSION

Although the analysis of fatty acid composition could not be done, the alcohol and sterol compositions of WE from RBW and RBO were evaluated and compared. The HPLC methods for separation of alcohols and sterols from saponified WE, which can be used in biological tests, were established. General aspects of RBWE composition after saponification were evaluated by Mass analysis and GC analysis. DF RB itself or DF RBO itself can be used as a good source of WE because the mass spectra of WE from DF RBW and DF RBO appeared similar, which indicates that WE compositions of DF RBW and DF RBO are also similar. The results opened new insight into WE from RBW and RBO, and the comparison between the compositions of WE from RBW and RBO provided general information on the contributions of chain lengths to wax crystallization. Of particular interest was the fact that alcohols from RBW had C32 and C34 as the most abundant esters, whereas alcohols from RBO had C28 and C30 as the predominate esters. This difference could be significant with regard to effects on cholesterol metabolism of the different fractions. Thus, biological testing of these two materials would provide new insight into the possible role of carbon chain length on cholesterol metabolism.

3.8. REFERENCES

Adhikari P, Hwang KT, Park JN, and Kim CK. Policosanol Content and Composition in Perilla Seeds. J. Agric. Food Chem. 2006, 54:5359-5362.

Belavadi VK and Bhowmick DN. An investigation of rice bran oil tank settlings. JAOCS. 1988, 65(2):241-245.

Bianchi G, Tava A, Vlahov G, and Pozzi N. Chemical structure of long chain esters from Sansa olive oil. JAOCS. 1994. 71(4):365-369.

Busson-Breysse J, Farines M and Soulier J. Jojoba wax: its esters and some of its minor components. JAOCS. 1994. 71(9):999-1002.

Carelli AA, Frizzera LM, Forbito PR, and Crapiste GH. Wax Composition of Sunflower Seed Oils. JAOCS. 2002. 79(8):763-768.

Garcia A, De Lucas A, Rincon J, Alvarez A, Gracia I, and Garcia MA. Supercritical carbon dioxide extraction of fatty and waxy material from rice bran. JAOCS,1996, 73(9):1127-1131.

Grob K and Lanfranchi M. Determination of free and esterified sterols and of wax esters in oils and fats by coupled liquid chromatography-gas chromatography. J. Chromatogr. 1989. 471:397-405.

Gunawan S, Vali SR, and Ju Y-H. Purification and Identification of Rice Bran Oil Fatty Acid Steryl and Wax Esters. JAOCS, 2006, 83(5):449-456.

Hu X, Daun JK, and Scarth R. Characterization of wax sediments in refined canola oil. JAOCS. 1993. 70(5):535-537.

Hwang KT, Cuppett SL, Weller CL, Hanna MA. Properties, Composition, and Analysis of Grain Sorghum Wax. JAOCS, 2002, 79(6):521-527.

Ito S, Suzuki T, and Fujino Y. Wax lipid in rice bran. Cereal Chemistry, 1983, 60(3):252-253.

Kanya TCS, Rao LJ, and Sastry MCS. Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries. Food Chemistry. 2007. 101:1552–1557

Lagarda MJ, Garcia-Llatas G, and Farre R. Analysis of phytosterols in foods. J. Pharmaceutical and Biomedical Analysis. 2006. 41:1486–1496.

Misra S and Ghosh A. Analysis of epicuticular waxes. In: Linskens HF and Jackson JF, editors, Essential oils and waxes. Modern Methods of Plant analysis, vol 12. 1991, Springer-Verlag, Berlin, Germany. pp206-229

Nota G, Naviglio D, Romano R, Sabia V, Musso SS, Improta C. Determination of the wax ester content in olive oils. Improvement in the method proposed by EEC regulation 183/93. J Agric Food Chem. 1999. 47(1):202-5.

Personal correspondence with Casey Grimm and Steve Lloyd, USDA, New Orleans. 2008.

Reiter B and Lorbeer E. Analysis of the wax ester fraction of olive oil and sunflower oil by gas chromatography and gas chromatography-Mass spectrometry. JAOCS. 2001. 78(9):881-888.

Vali SR, Ju Y, Kaimal TNB, and Chern Y. A Process for the Preparation of Food-Grade Rice Bran Wax and the Determination of Its Composition. JAOCS, 2005, 82(1):57-64

Verleyen T, Forcades M, Verhe R, Dewettinck K, Huyghebaert A, and De Greyt W. Analysis of free and esterified sterols in vegetable oils. JAOCS. 2002, 79(2):117-122.

Yoon SH and Rhee JS. Composition of waxes from crude rice bran oil. JAOCS, 1982. 59:561-563.

CHAPTER 4.
SUMMARY AND CONCLUSIONS

This research was originally designed to develop efficient methods for extraction of oil and wax from rice bran, to establish separation or preparation methods for wax esters from rice bran wax, to obtain more accurate analytical methods for wax esters using HPLC and GC, and to test wax components on cells or animals for their possible health-promoting effects.

In Chapter 1, various extraction methods were tested for higher RBO and RBW yields, and WE in RBW and RBO samples were quantified by HPLC analysis. The resulting data indicates that hexane is more efficient for wax extraction than isopropanol, and that MAE with hexane rather than conventional Soxhlet extraction method achieved higher WE contents in the processed RBO and in the refined RBW. HS and HWS methods for crude RBO recovery influence the crude RBO yields. The higher extraction temperature of MAE with isopropanol increased the yields of DF RBO, but it did not improve the FF RBO and RBW yields. Of particular significance was that DF RB contained high levels of RBW. Even though the oil yield from DF RB was much less than FF RB, the refined RBW contents were not significantly different from FF RB. The HPLC analysis proved that not all RBW is WE and not all RBWE is crystallizable. The study also established an efficient procedure of RBW preparation for GC analysis; extraction with MAE, winterization, solvent fractionation, and HPLC separation. This method can be used not only for RBW analysis but also analysis of WE from other edible oils. MAE with hexane was found to be the most efficient method for generation of higher amounts of RBW as well as higher WE contents. MAE required less time and less solvent than Soxhlet extraction.

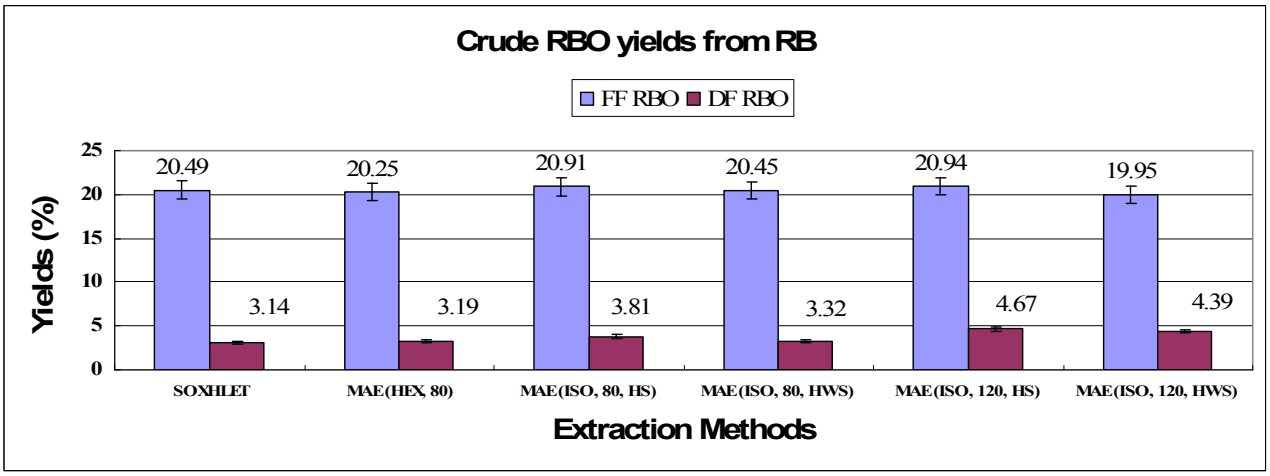
In Chapter 2, several HPLC methods for analysis and separation of WE from RBO were developed and tested with standards and RBO samples. WE was not separated with one simple

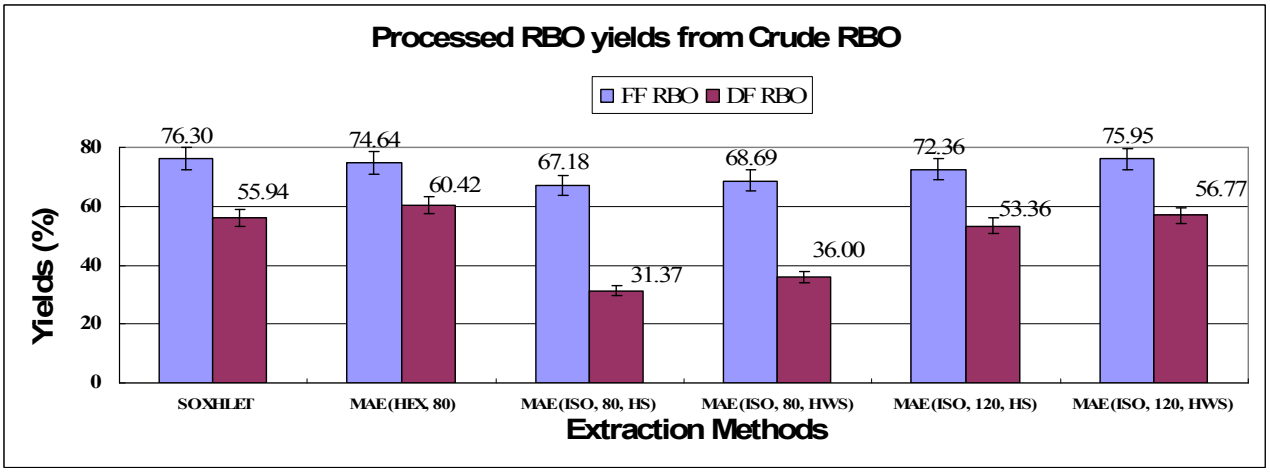
solvent system, and 2 or more solvents were required to achieve proper resolution. The 2SI method, originally designed to separate fatty acid methyl esters from steryl esters, failed to separate them, but was useful for the measurement of total WE in a sample as well as WE separation. Preparative or analytical C18 columns showed several advantages over silica columns, but C18 methods could not elute the WE in a short time period or as one peak, and were not appropriate for the measurement or separation of total WEs in an oil sample. The prep C18 method and all other analytical C18 methods may have potential for preparation of WEs with specific chain lengths or structures.

In Chapter 3, although the analysis of fatty acid composition could not be done, the alcohol and sterol compositions of WE from RBW and RBO were evaluated and compared. The HPLC methods for separation of alcohols and sterols from saponified WE, which could then be used in biological tests, were established. General aspects of RBWE composition after saponification were evaluated by Mass analysis and GC analysis. DF RB itself or DF RBO itself can be used as a good source of WE because the mass spectra of WE from DF RBW and DF RBO appeared similar, which indicates that WE compositions of DF RBW and DF RBO were also similar. The results opened a new insight into WE from RBW and RBO, and the comparison between the compositions of WE from RBW and RBO provided general information on the chain lengths of crystallizable and noncrystallizable wax. Of particular interest was the fact that alcohols from RBW had C32 and C34 as the most abundant esters, whereas alcohols from RBO had C28 and C30 as the predominate esters. This difference could be significant with regard to effects on cholesterol metabolism of the different fractions. Thus, biological testing of these two materials would provide new insight into the possible role of carbon chain length on cholesterol metabolism.

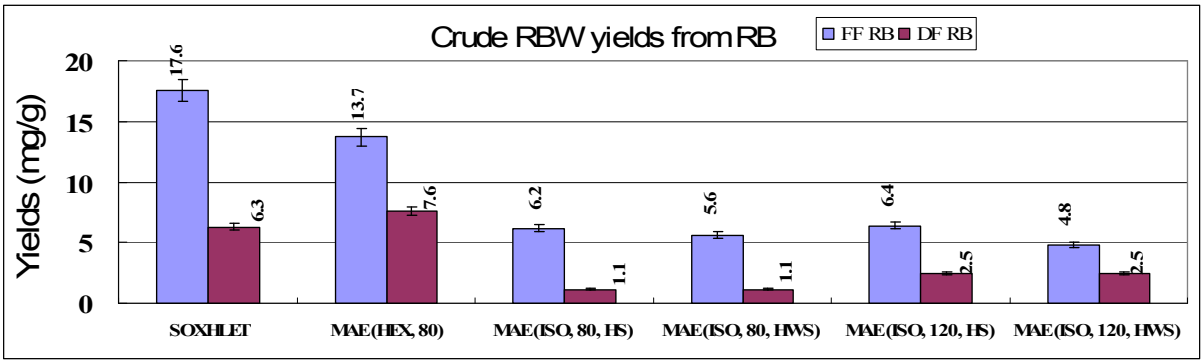
The results of this research established an efficient procedure for separation of long chain alcohols and sterols from RBW; a microwave extraction procedure with hexane, a winterization process, solvent fractionation, HPLC separation of WE, a saponification process, and HPLC separation of alcohols and sterols. In addition, WE analysis methods were also designed: including both quantification of WE in RBW or RBO using HPLC with 2SI column and ELSD detection and saponification prior to GC analysis using a high temperature GC column. The data and methods developed in this research can be used for further biological experiments or WE analysis.

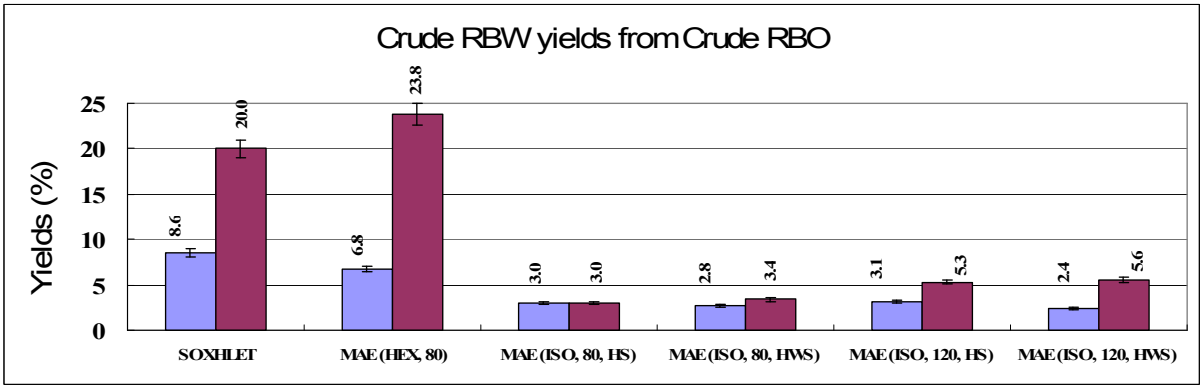
APPENDIX 1: GRAPHICAL ILLUSTRATION OF TABLE 1.1

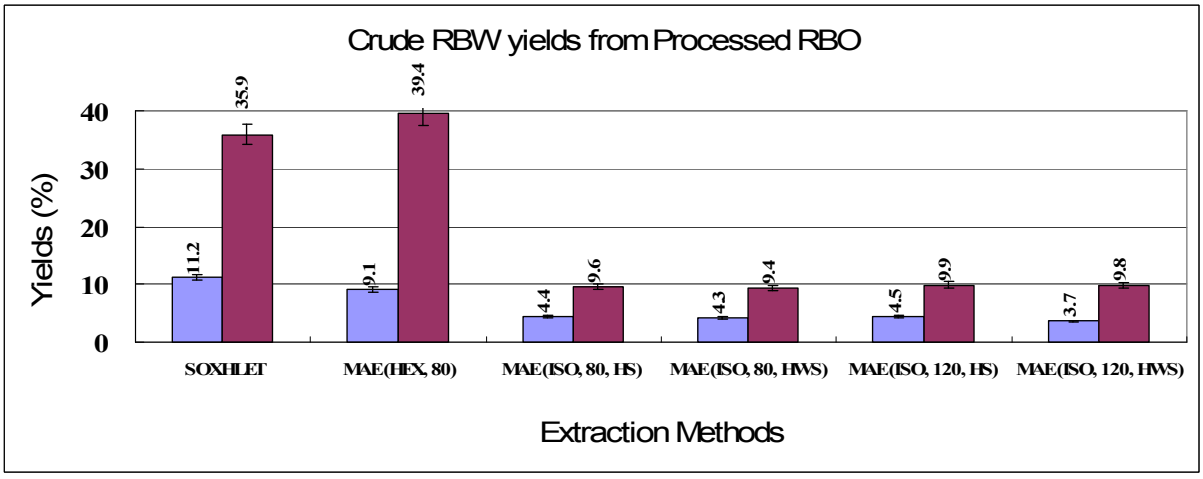




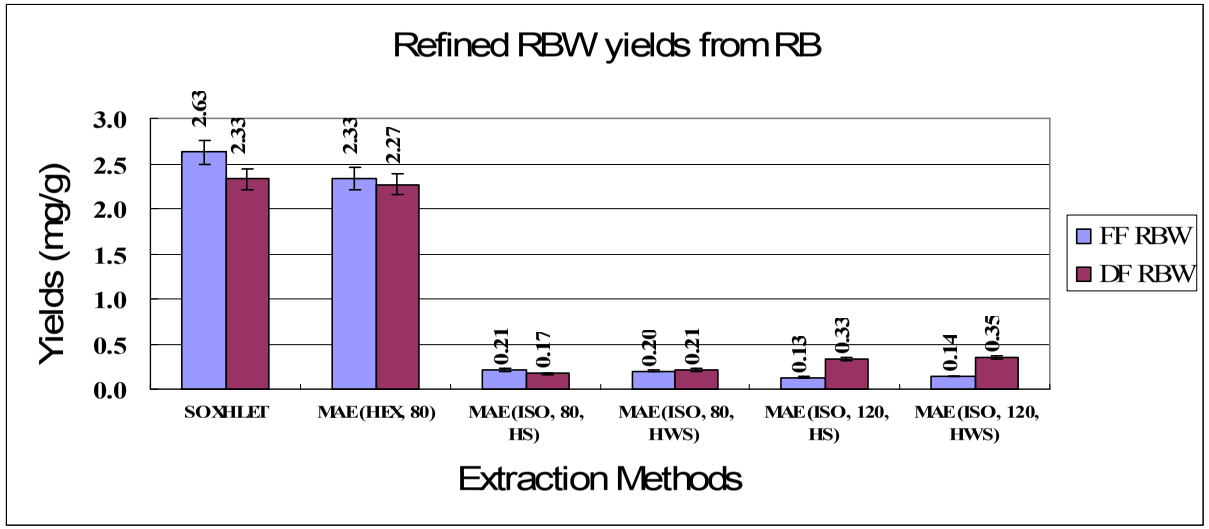
APPENDIX 2: GRAPHICAL ILLUSTRATION OF TABLE 1.2

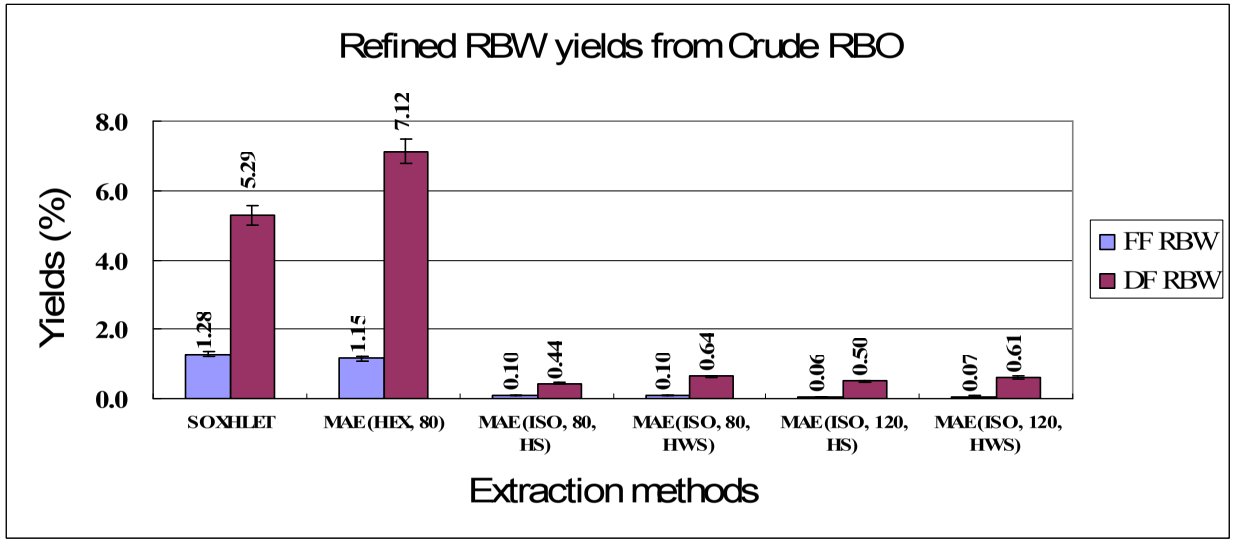


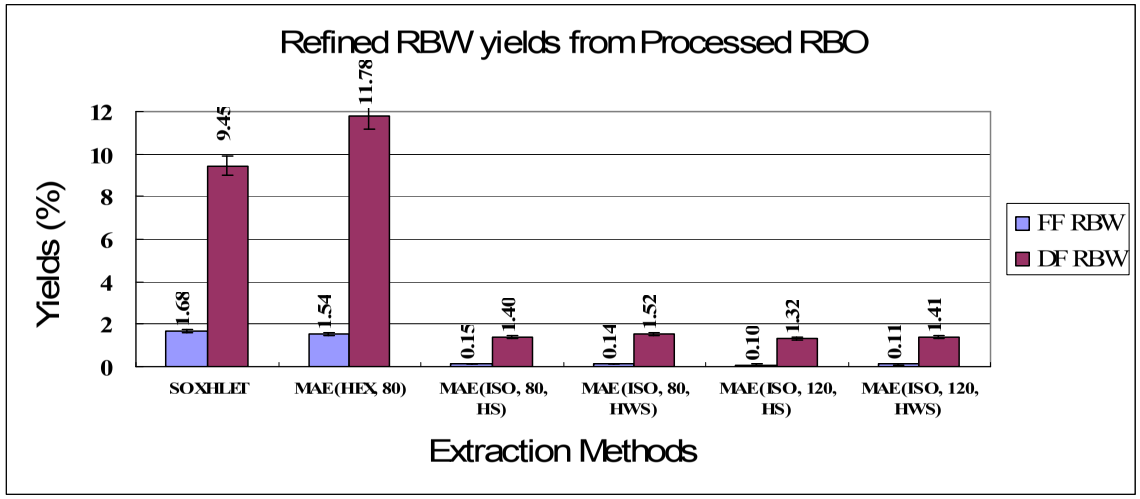


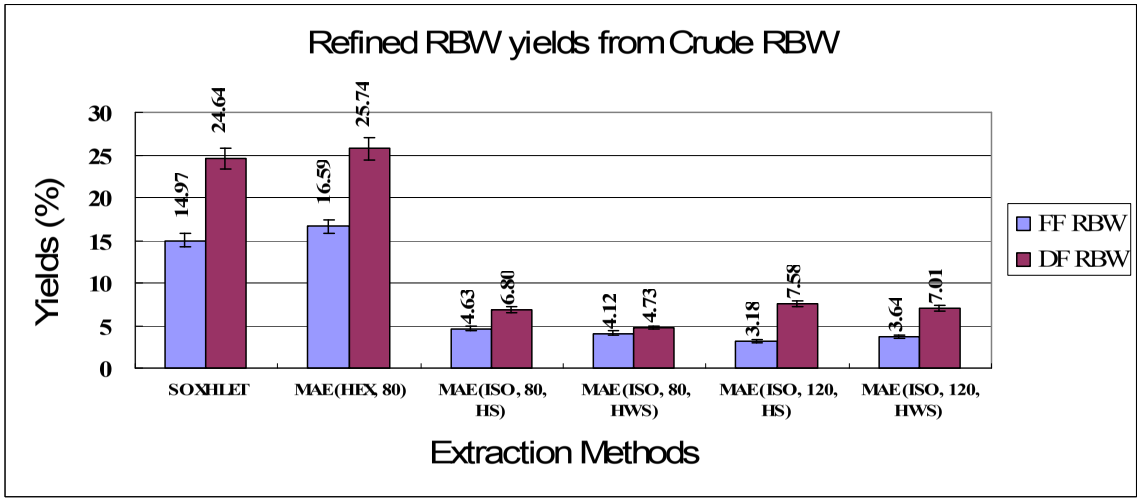


APPENDIX 3: GRAPHICAL ILLUSTRATION OF TABLE 1.3

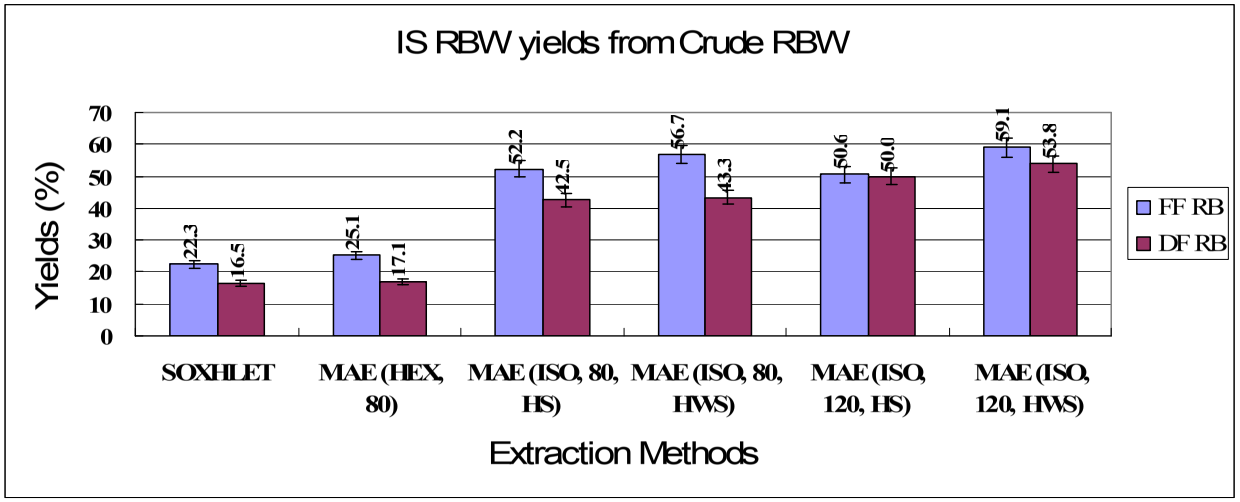


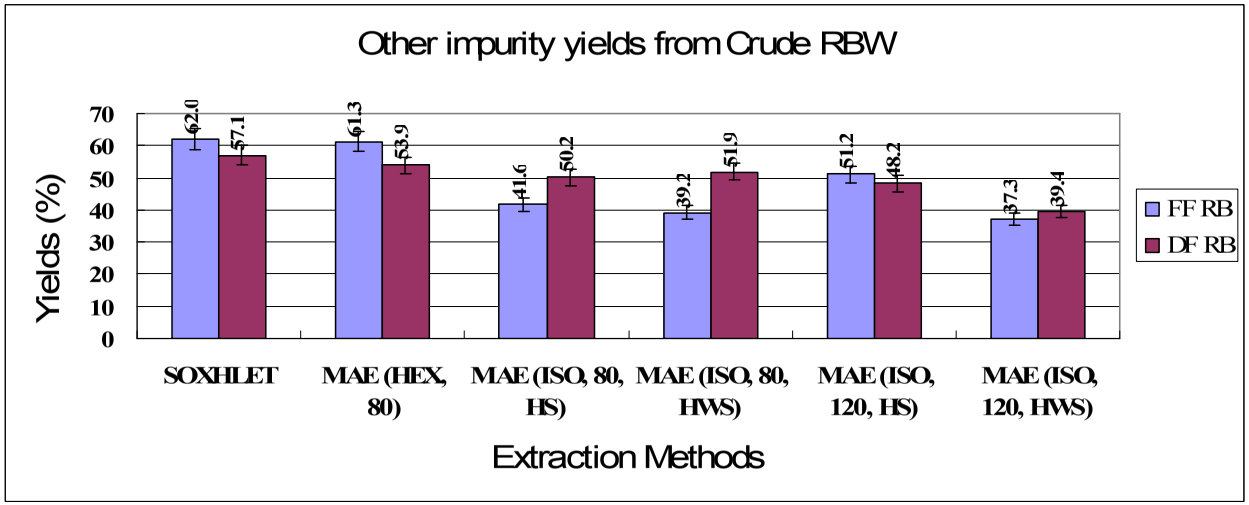



Refined RBW yields from Processed RBO
Yields (%)
12
10
8
6
4
2
0
1.68
9.45
1.54
11.78
0.15
1.40
0.14
1.52
0.10
1.32
0.11
1.41
SOXHLET
MAE(HEX, 80)
MAE(ISO, 80, HS)
MAE(ISO, 80, HWS)
MAE(ISO, 120, HS)
MAE(ISO, 120, HWS)
Extraction Methods
FF RBW
DF RBW

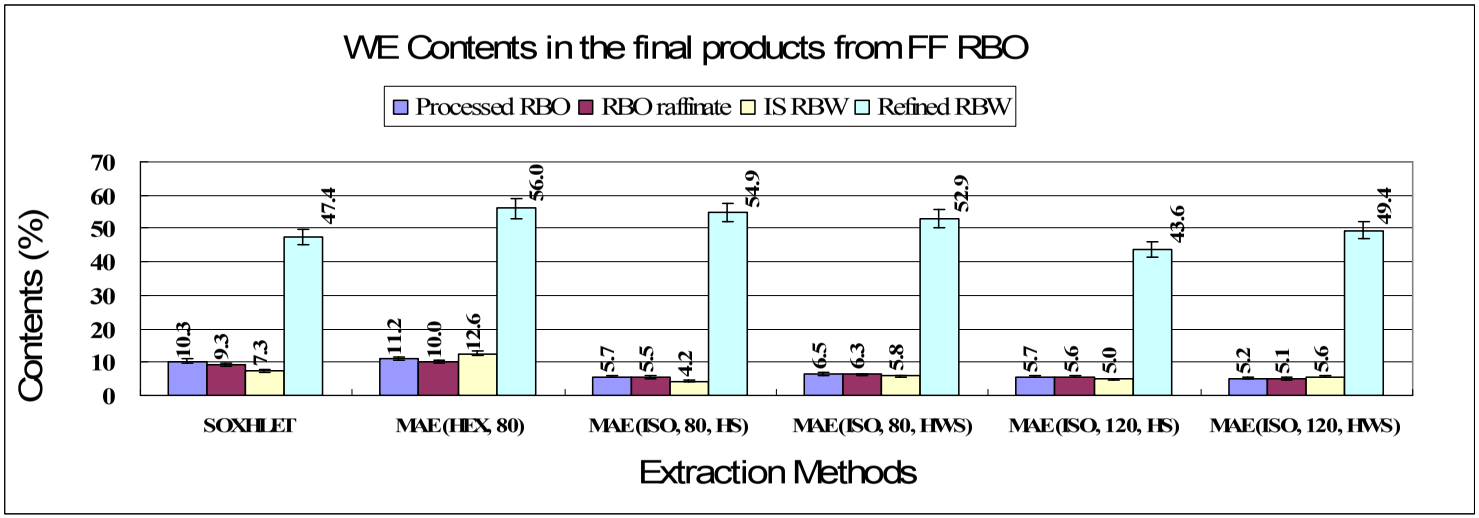

Refined RBW yields from Crude RBW
Yields (%)
30
25
20
15
10
5
0
14.97
24.64
16.59
25.74
4.63
6.80
4.12
4.73
3.18
7.58
3.64
7.01
SOXHLET
MAE(HEX, 80)
MAE(ISO, 80, HS)
MAE(ISO, 80, HWS)
MAE(ISO, 120, HS)
MAE(ISO, 120, HWS)
Extraction Methods
FF RBW
DF RBW

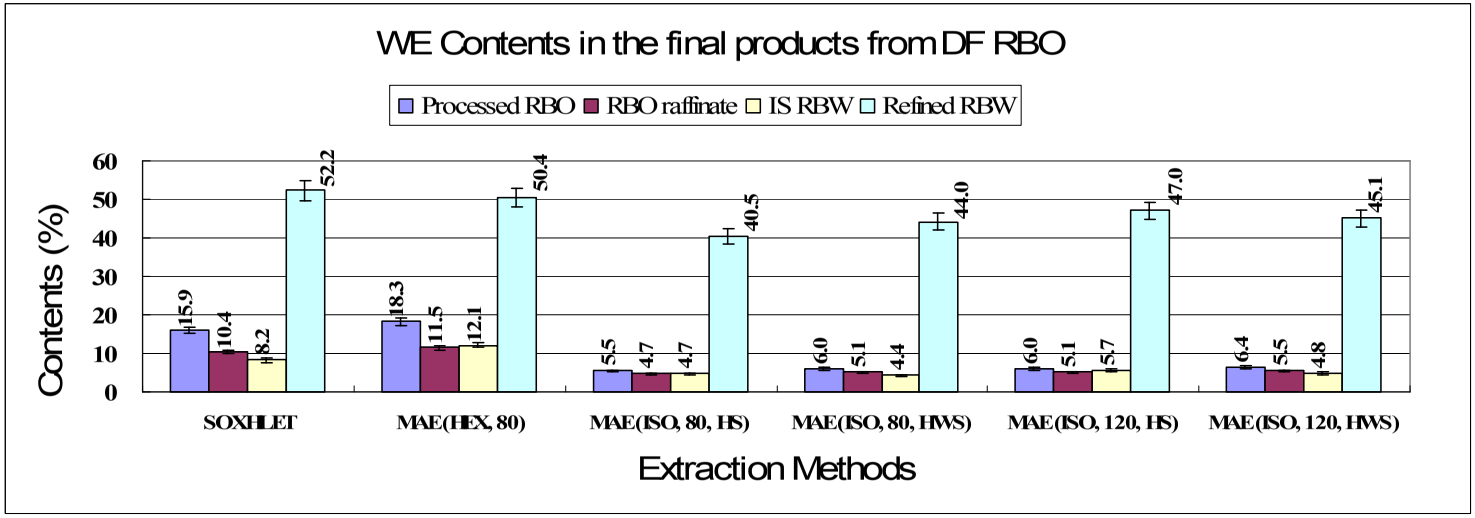
APPENDIX 4: GRAPHICAL ILLUSTRATION OF TABLE 1.4



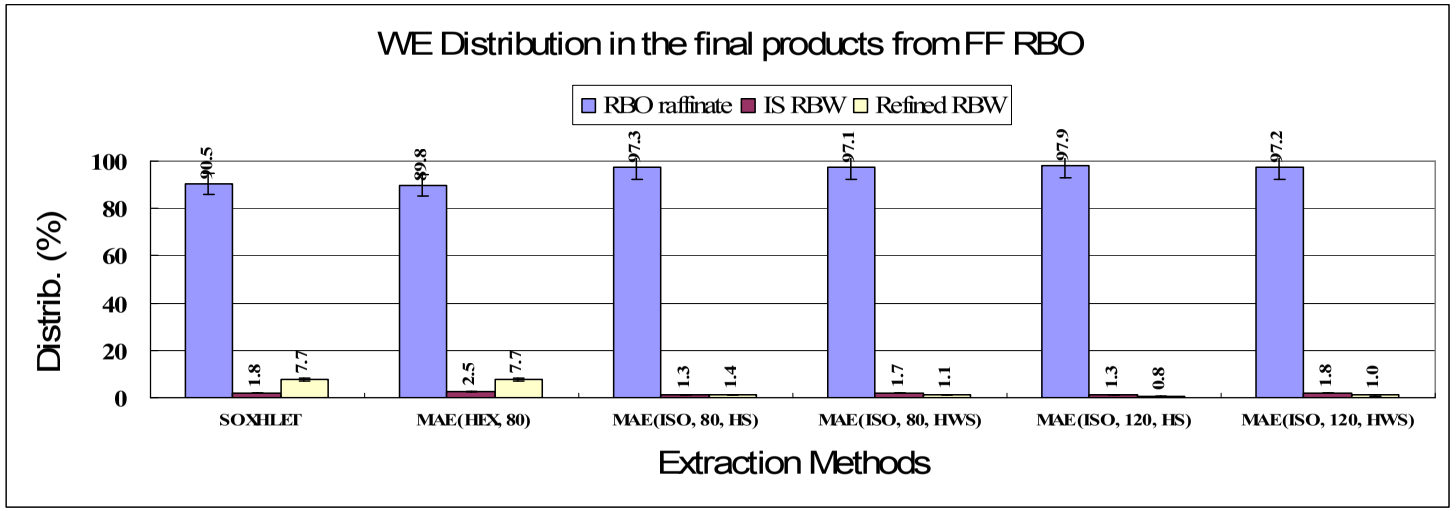


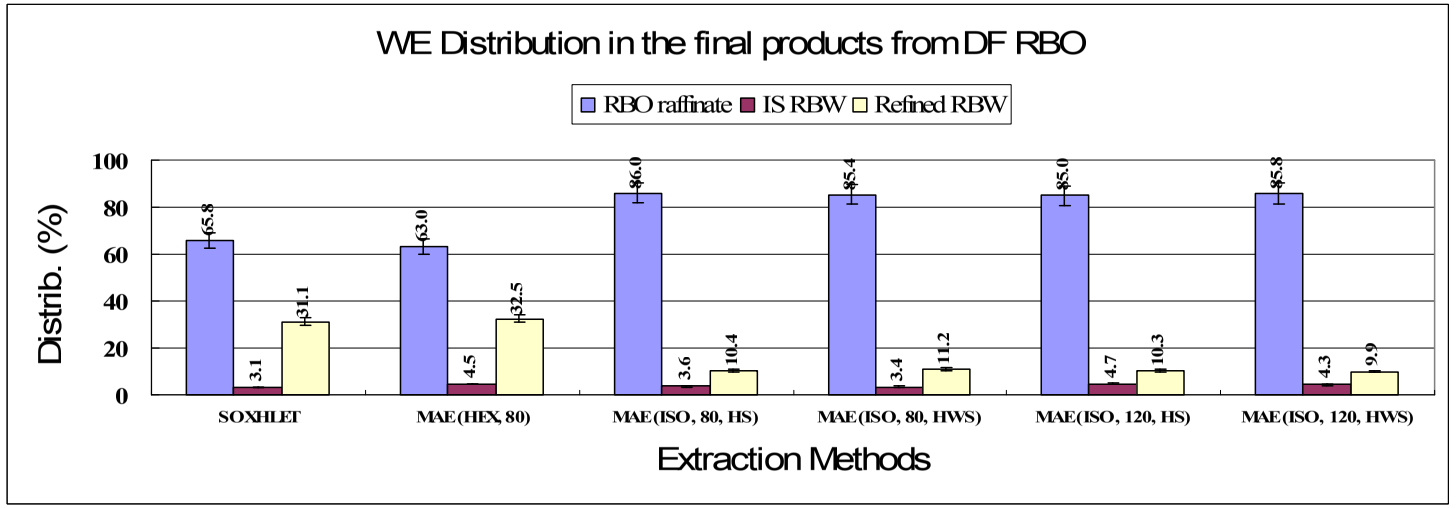
APPENDIX 5: GRAPHICAL ILLUSTRATION OF TABLE 1.5





APPENDIX 6: GRAPHICAL ILLUSTRATION OF TABLE 1.6





APPENDIX 7: SOLVENT PROPERTY CHART*

Solvent	Formula	Boiling Point (^{o}C)	Density (g/mL)	Relative polarity
Cyclohexane	C_6H_{12}	80.7	0.779	0.006
Pentane	C_5H_{12}	36.1	0.626	0.009
Hexane	C_6H_{14}	69	0.655	0.009
Heptane	C_7H_{16}	98	0.684	0.012
Carbon tetrachloride	CCl_4	76.7	1.594	0.052
p-xylene	C_8H_{10}	138.3	0.861	0.074
Toluene	C_7H_8	110.6	0.867	0.099
Benzene	C_6H_6	80.1	0.879	0.111
Diethyl ether	$C_4H_{10}O$	34.6	0.713	0.117
Methyl *t*-butyl ether	$C_5H_{12}O$	55.2	0.741	0.148
Dioxane	$C_4H_8O_2$	101.1	1.033	0.164
Tetrahydrofuran (THF)	C_4H_8O	66	0.886	0.207
Ethyl acetate	$C_4H_8O_2$	77	0.894	0.228
Chloroform	$CHCl_3$	61.2	1.498	0.259
Methylene chloride	CH_2Cl_2	39.8	1.326	0.309
2-butanone	C_4H_8O	79.6	0.805	0.327
Acetone	C_3H_6O	56.2	0.786	0.355
t-butyl alcohol	$C_4H_{10}O$	82.2	0.786	0.389
Dimethyl sulfoxide (DMSO)	C_2H_6OS	189	1.092	0.444
Acetonitrile	C_2H_3N	81.6	0.786	0.460
2-propanol	C_3H_8O	82.4	0.785	0.546
1-butanol	$C_4H_{10}O$	117.6	0.810	0.602
1-propanol	C_3H_8O	97	0.803	0.617
Acetic acid	$C_2H_4O_2$	118	1.049	0.648
Ethanol	C_2H_6O	78.5	0.789	0.654
Methanol	CH_4O	64.6	0.791	0.762
ethylene glycol	$C_2H_6O_2$	197	1.115	0.790
Water	H_2O	100	0.998	1.000

*This table is originally from the website of Division of Organic Chemistry, American Chemical Society (http://organicdivision.org/organic_solvents.html), and modified to fit into the page.

APPENDIX 8: MOLECULAR WEIGHT* TABLE OF ALKYL ESTERS

ALCOHOLS	FATTY ACIDS						
Name	Myristic acid	Palmitic acid	Stearic acid	Arachidic acid	Docosa-noic acid	Tetracosa-noic acid	Hexacosa-noic acid
Chain length	C14	C16	C18	C20	C22	C24	C26
MW*	228	256	284	312	340	368	396
Hexadecanol C16 242	452	480	508	536	564	592	620
Octadecanol C18 270	480	508	536	564	592	620	648
Eicosanol C20 298	508	536	564	592	620	648	676
Docosanol C22 326	536	564	592	620	648	676	704
Tetracosanol C24 354	564	592	620	648	676	704	732
Hexacosanol C26 382	592	620	648	676	704	732	760
Octacosanol C28 410	620	648	676	704	732	760	788
Triacontanol C30 438	648	676	704	732	760	788	816

* The molecular weight of each compound was obtained from NIST CHEMISTRY WEBBOOK (http://webbook.nist.gov/chemistry/) and the website of Sigma-Aldrich company (http://www.sigmaaldrich.com/Area_of_Interest/The_Americas/United_States.html).

* The molecular weight of each alkyl ester was calculated by the following equation;
MW of Alkyl esters = MW of Alcohol + MW of Fatty acid – MW of Water
(MW of Water = 18)

ALCOHOLS	FATTY ACIDS						
Name	Myristic acid	Palmitic acid	Stearic acid	Arachidic acid	Docosa-noic acid	Tetracosa-noic acid	Hexacosa-noic acid
Chain length	C14	C16	C18	C20	C22	C24	C26
MW*	228	256	284	312	340	368	396
Dotriacontanol C32 466	676	704	732	760	788	816	844
Tetratriacontanol C34 494	704	732	760	788	816	844	872
Hexatriacontanol C36 522	732	760	788	816	844	872	900
Octatriacontanol C38 550	760	788	816	844	872	900	928
Tetracontanol C40 578	788	816	844	872	900	928	956
Dotetracontanol C42 606	816	844	872	900	928	956	984
Tetrateteracontanol C44 634	844	872	900	928	956	984	1012

* The molecular weight of each compound was obtained from NIST CHEMISTRY WEBBOOK (http://webbook.nist.gov/chemistry/) and the website of Sigma-Aldrich company (http://www.sigmaaldrich.com/Area_of_Interest/The_Americas/United_States.html).

* The molecular weight of each alkyl ester was calculated by the following equation;
MW of Alkyl esters = MW of Alcohol + MW of Fatty acid – MW of Water
(MW of Water = 18)

APPENDIX 9: MOLECULAR WEIGHT* TABLE OF STERYL ESTERS

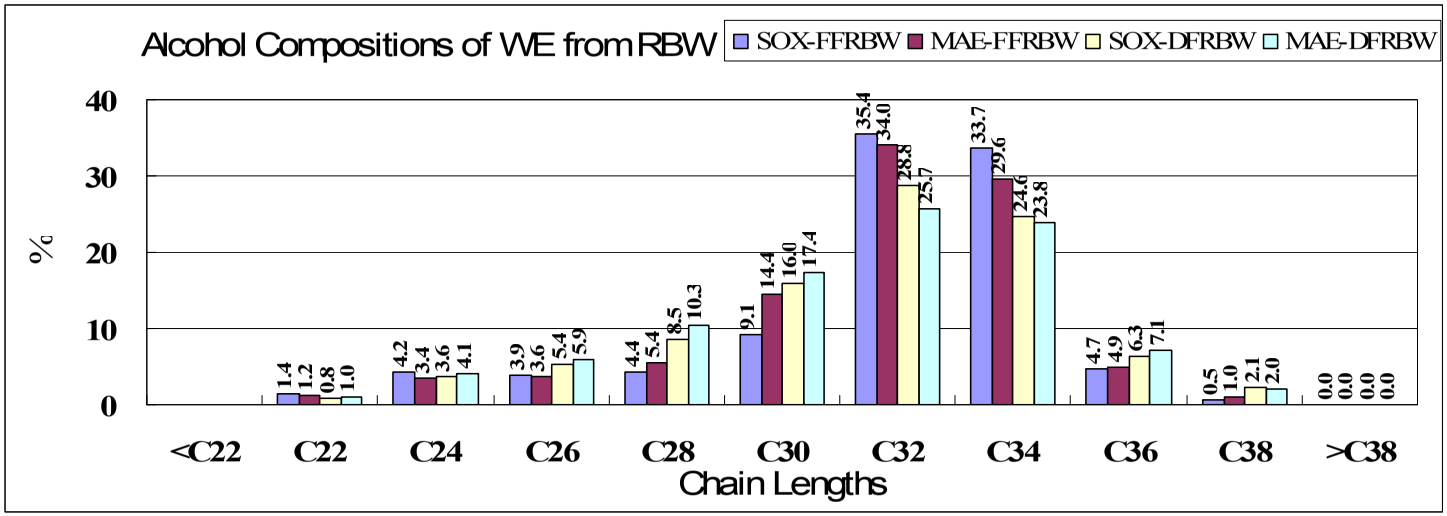
FATTY ACIDS	STEROLS						
Name	Campe-sterol	Stigma-sterol	Sito-sterol	Stigma-stenol	Citrosta-dienol	Cyclo-artenol	24-Methylene-cycloartanol
MW	400	412	414	414	426	426	440
Myristic acid C14, 228	610	622	624	624	636	636	650
Palmitic acid C16, 256	638	650	652	652	664	664	678
Stearic acid C18, 284	666	678	680	680	692	692	706
Oleic acid C18:1, 282	664	676	678	678	690	690	704
Linoleic acid C18:2, 280	662	674	676	676	688	688	702
Linolenic acid C18:3, 278	660	672	674	674	686	686	700
Arachidic acid C20, 312	694	706	708	708	720	720	734
Docosanoic acid C22, 340	722	734	736	736	748	748	762
Tetracosanoic acid C24, 368	750	762	764	764	776	776	790
Hexacosanoic acid C26, 396	778	790	792	792	804	804	818

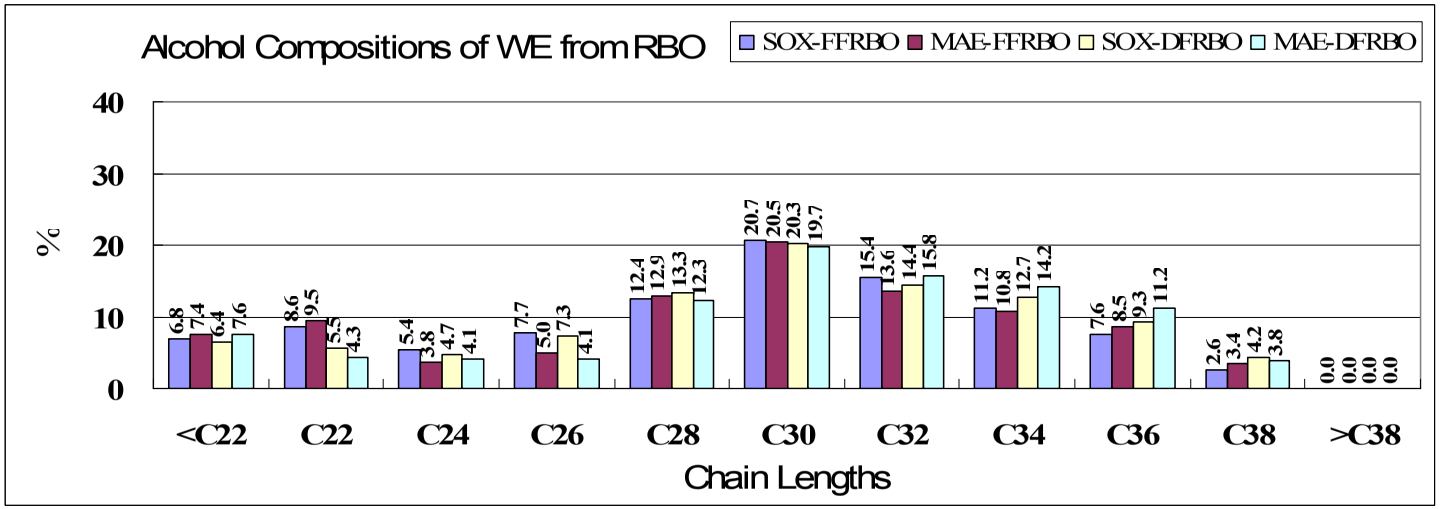
* The molecular weight of each fatty acid was obtained from NIST CHEMISTRY WEBBOOK (http://webbook.nist.gov/chemistry/) and the website of Sigma-Aldrich company (http://www.sigmaaldrich.com/Area_of_Interest/The_Americas/United_States.html).

* The molecular weight of each sterol was referred from Gunawan et al. (2006, JAOCS, 83(5):449-456).

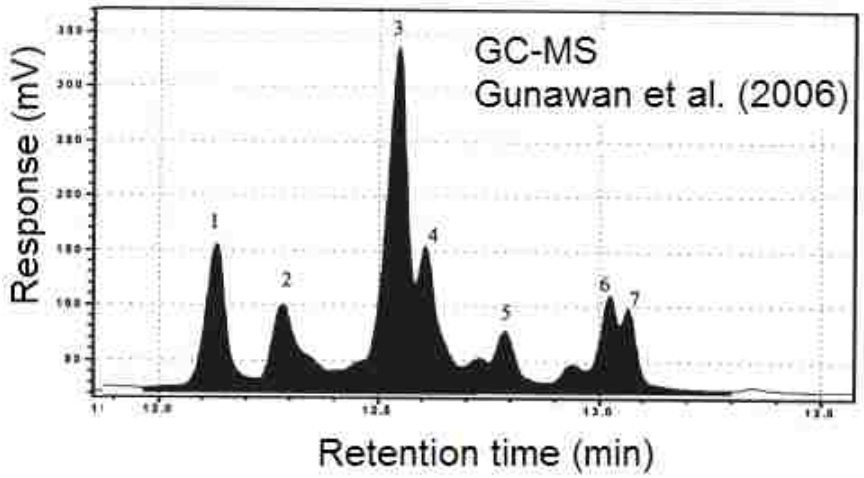
* The molecular weight of each steryl ester was calculated by the following equation;
MW of steryl esters = MW of Sterol + MW of Fatty acid – MW of Water
(MW of Water = 18)

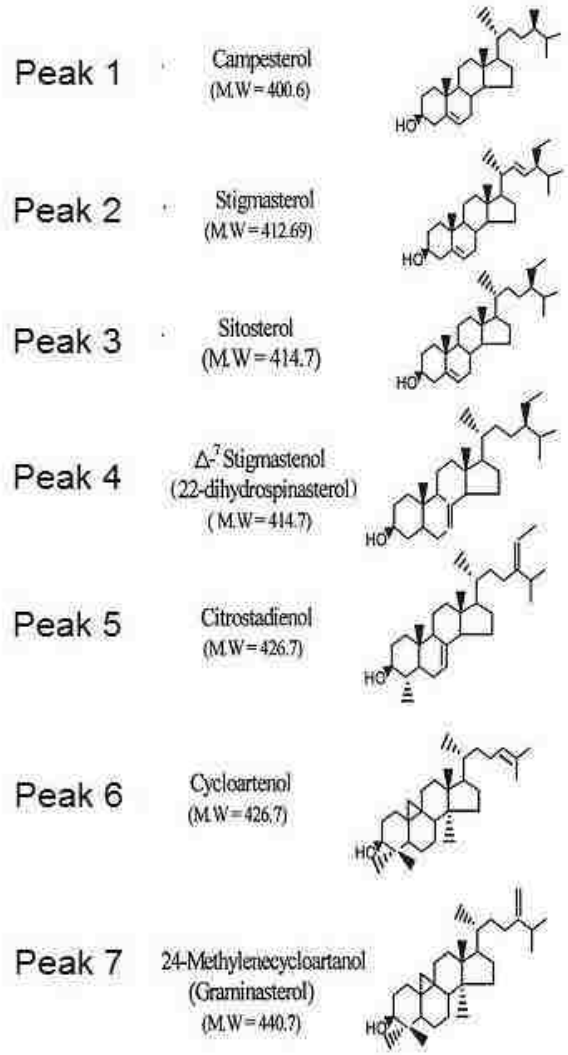
APPENDIX 10: GRAPHICAL ILLUSTRATION OF TABLE 3.1 AND 3.2.





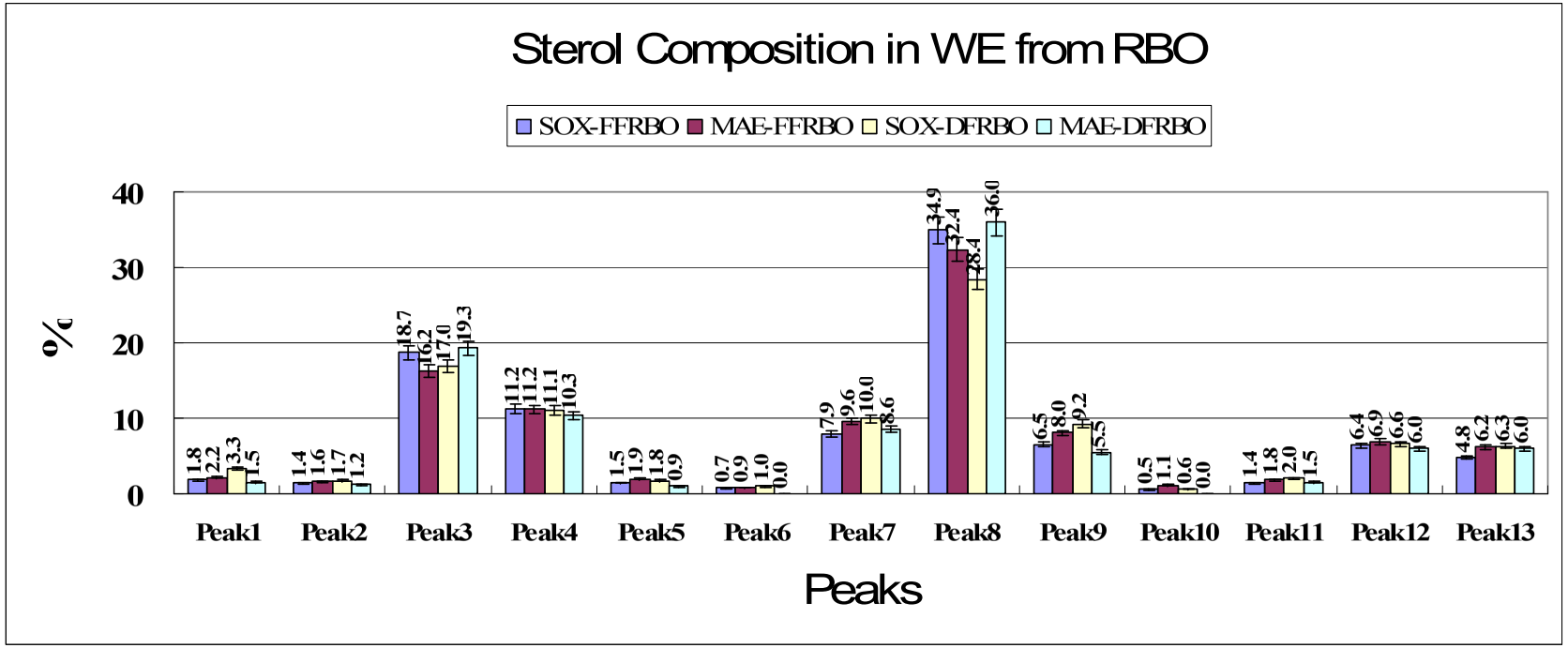
APPENDIX 11: GC-MS CHROMATOGRAM OF STEROLS IN GUNAWAN et al. (2006)





These pictures were originally from Gunawan et al. (2006, JAOCS, 83(5):449-456), and slightly modified.

APPENDIX 12: GRAPHICAL ILLUSTRATION OF TABLE 3.3



VITA

Junghong Kim was born in August 1972, in Pusan, Republic of Korea. He enrolled in Pukyong National University, Pusan, Republic of Korea, in March 1991, majoring in chemistry. In September 1992, he joined the Korean Army and served as a private until December 1994, which was obligatory to all Korean young men. In 1996, for one year, he studied English language at Language Connection International, Toronto, Canada. He continued studying chemistry in March 1997, and earned a Bachelor of Science degree in February 1999. After graduation, he taught English and science to high school students at several private schools. He entered the graduate program at Pusan National University in March, 2000, and received a master's degree in biology in February, 2002. In January 2002, he accepted full-time employment as a laboratory manager with Laboratory of Enzymology and Molecular Biology, Department of Nutrition and Life Science, Pukyong National University. In August 2004, he was accepted as a graduate student in the Food Science Department, Louisiana State University, Baton Rouge, Louisiana. His research focused on rice bran oil and wax, and he obtained his doctorate in food science in the Summer semester of 2008.