

SUPERCRITICAL FLUID EXTRACTION AND ANALYSIS OF PLANT OILS

A Thesis

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Abstract

Supercritical fluid extraction (SFE) of oxeye daisy (*Chrysanthemum leucanthemum* L.) seed, heartwood of Port-Orford cedar (POC) (*Chamaecyparis lawsoniana*), Alaska yellow cedar (AYC) (*Chamaecyparis nootkatensis*), and Eastern red cedar (ERC) (*Juniperus virginiana* L), and Chinese tallow (*Sapium sebiferum* L. Roxb.) seed was investigated in this study and compared with other extraction methods.

For the oxeye daisy seed extraction, Soxhlet extraction (SE) with hexane, microwave assisted extraction (MAE), and supercritical fluid extraction (SFE) were conducted. The results showed that as the extraction temperature decreased from 100 °C, the extraction rate increased until 30 °C using SFE. With an increase of extraction pressure from 100 bar to 300 bar, the extraction rate increased using SFE. Most ultraviolet waves did not pass through the oxeye daisy oils at a wavelength range 200 nm to 300 nm when the oil concentration was 0.4% (v/v). The MAE oil and SFE showed stronger DPPH radical-scavenging ability than SE oil at the same concentration.

For the three cedars extraction, samples were supercritical fluid extracted with CO₂ and Soxhlet extracted with hexane. The extracted oils were evaluated against two common wood decay fungi, brown-rot fungi (*Gloeophyllum trabeum*) and white-rot fungi (*Trametes versicolor*). The result showed that the SFE yield of ERC, AYC, and POC was 3.27%, 3.22%, and 3.29%, respectively. The SE yield of ERC, AYC, and POC was 0.80%, 0.71%, and 1.52%, respectively. The statistical analysis showed that SFE cedar oils had higher antifungal activities than SE cedar oils against both fungi. *In vitro* studies showed that AYC oil had the strongest antifungal activity, followed by POC oil and ERC oil.

For the Chinese tallow seed extraction, SFE and SE were conducted. 5% or 10% methanol (MeOH) was added to the supercritical CO₂ as a modifier. The results showed that the collecting time could significantly affect the extraction yield. The extraction yield with 10% MeOH (32.61%) is higher than with 5% MeOH (30.85%) or without MeOH (30.75%). More components could be extracted when using a modifier based on GC-MS analysis.

In sum, supercritical fluid extraction showed several advantages in the extraction of plant oils over Soxhlet extraction.

Chapter 1. Introduction

1.1 General Introduction

Traditional oil extraction often requires organic solvents, long extraction period and high temperature, such as distillation or Soxhlet extraction (SE), which may destroy some bioactive compounds during the processes. Recently developed extraction methods like supercritical fluid extraction (SFE) and microwave assisted extraction (MAE) have been used for oil extraction (Cravotto 2008; Bendahou 2008). SFE technology has been widely studied for seed oil extraction (Reverchon 1997; Machmudah et al. 2006; Valle et al. 2006; Westerman et al. 2006; Machmudah et al. 2007) and other natural products extraction (Lang and Wai 2001; Reverchon and De Marco 2006). SFE, often operating with low temperature and short extraction time, can minimize the influence on bioactive compounds and enhance the antibiotic property of the extracted oil. Several advantages of SFE were reviewed by Lang and Wai (2001) and are: (1) Supercritical fluids have relatively lower viscosity and higher diffusivity, which results in shorter extraction time; (2) Continuous fluid flowing through samples could provide quantitative or complete extraction; (3) The solvent power of the fluid can be manipulated by changing pressure and/or temperature; (4) Solutes dissolved in supercritical fluid can be easily separated by depressurization; (5) SFE is usually performed at low temperatures, which is good for studying thermally labile compounds; (6) Little sample quantity is needed; (7) Little or no organic solvent is needed, which is good for the environment. Based on these advantages, SFE is regarded as an environmentally friendly alternative to conventional industrial solvent extraction (Hauthal 2001).

Carbon dioxide (CO₂) is the most commonly used solvent for SFE, presenting very little toxicological or environmental hazards. In addition, carbon dioxide is non-reactive with most analytes, which serves as a good solvent. The phase diagram and density-pressure diagram of CO₂ are shown in Figure 1-1 and Figure 1-2, respectively. When above the critical point 73 atm and 31 °C (Figure 1-1), CO₂ is in its supercritical state, which has both gas-like and liquid-like properties. The density of supercritical CO₂ (SCC) is controllable by modifying the temperature and pressure of SCC. SCC offers the ability to solute many non-polar materials. Besides, the diffusivity of the SCC is higher than its liquid state. With higher diffusivity the SCC can penetrate into a sample matrix faster and thus the efficiency of extraction is higher than a corresponding liquid or gaseous extraction. Its critical temperature (31 °C) makes it an ideal solvent for extracting thermally labile materials and it dissipates from the extract after extraction. For instance, water's supercritical point is 374 °C and 218 atm. It is not suitable for extracting thermally labile materials. CO₂ is also non-toxic, non-flammable, environmentally acceptable and inexpensive (Kawahito et al. 2008). Based on different extraction purposes, some modifiers are used as a co-solvent to change to the polarity of CO₂. Because CO₂ is a non-polar solvent, if some polar components need to be extracted, the modifier such as methanol can change the polarity of the solvent (Joseph et al. 1993).

1.2 Objectives

The overall objective of this study was to conduct supercritical fluid extraction of some plant oils, analyze some properties of the extracted oils, find potential uses for the extracted oils, and compare the supercritical fluid extraction with other extraction methods. The study consisted of the following specific objectives.

1. Extract oxeye daisy seed oil with supercritical fluid extraction, microwave assisted extraction and Soxhlet extraction and compare these extraction methods. Then analyze the fatty acid profiles of the extracted oils with Gas Chromatography-Mass Spectrometry (GC-MS), and

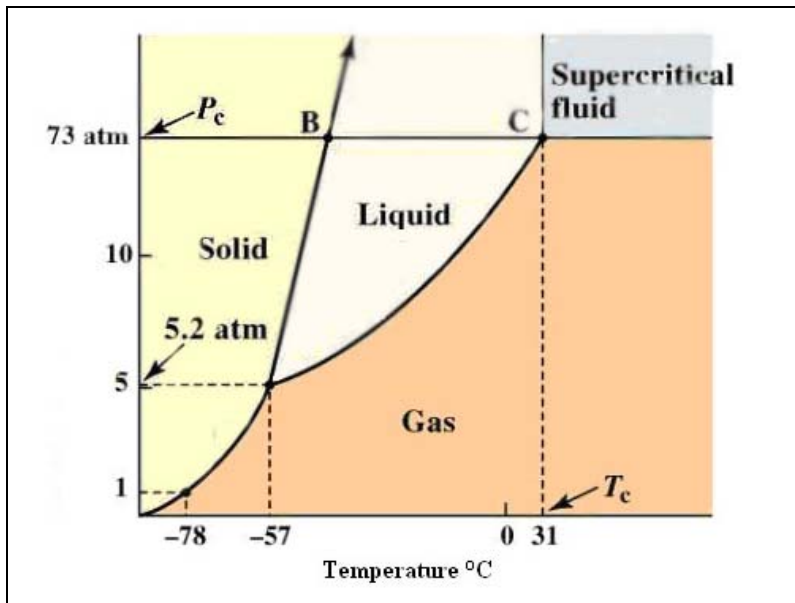


Figure 1-1 Phase diagram of carbon dioxide

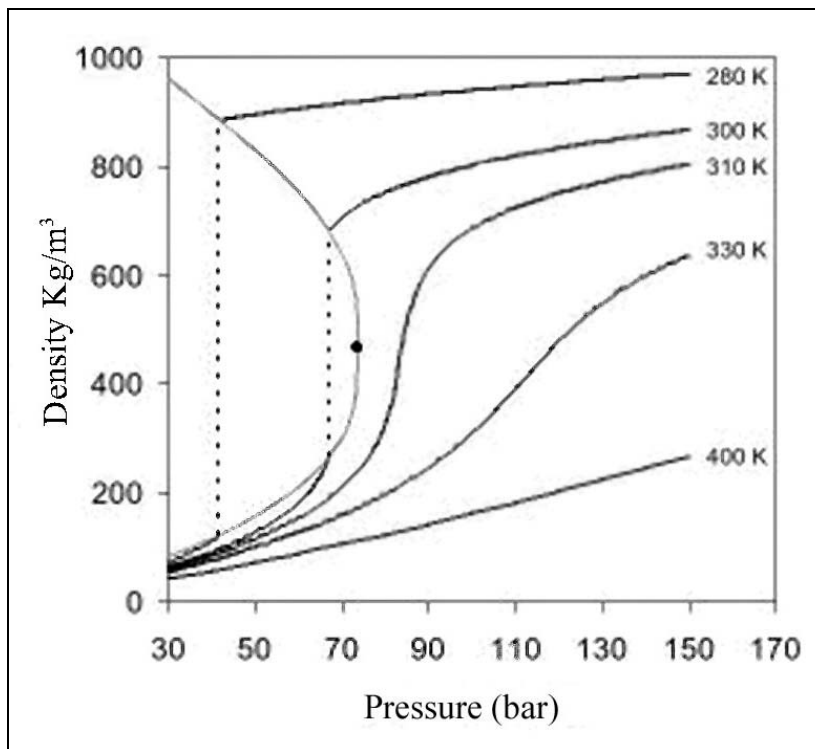


Figure 1-2 Density-pressure diagram of carbon dioxide

analyze the physical and chemical properties of the extracted oils (DPPH radical-scavenging assay, Ultraviolet/ Visible (UV/Vis) spectroscopy analysis).

2. Extract of Port-Orford cedar (*Chamaecyparis lawsoniana*), Alaska yellow cedar (*Chamaecyparis nootkatensis*), and Eastern red cedar (*Juniperus virginiana* L) oils with supercritical fluid extraction and Soxhlet extraction, compare the difference of these extraction methods, and analyze the main components of the extracted oils with Gas Chromatography-Mass Spectrometry (GC-MS), and analyze the antifungal activities of these oils.

3. Extract Chinese tallow tree seed oil with supercritical fluid extraction, analyze the main components of extracted oils with GC-MS, and operate supercritical fluid extraction with methanol as a modifier and analyze the modifier's effect on supercritical fluid extraction.

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Chapter 2. Oxeye Daisy (*Chrysanthemum leucanthemum* L.) Seed Oil Extraction and Analysis

2.1 Introduction and Literature Review

The common name, oxeye daisy (Figure 1-1), can refer to different kinds of plants such as *Chrysanthemum leucanthemum* L. (Syn *Leucanthemum vulgare*), *Callilepis laureola* and *Dimorphotheca tragus* (Daisy from B & T World Seeds 2008; Mitich 2000). The objective oxeye daisy in this research is *Chrysanthemum leucanthemum* L., which is known as ox-eye daisy and oxeye daisy. Oxeye daisy (*Chrysanthemum leucanthemum* L.) is listed as a noxious weed in the plants profile of the USDA (2008). It is considered to be a weed in Europe, Australia, and Canada (Howarth and Williams 1968). It spreads fast and is competitive to grasses. Most information regarding this plant pertains to distribution, growth and reproduction, history, and control (Olson and Wallander 1999; Holm et al. 1997, Cole 1998). There are few reports on its chemical properties and utilization.

The plant has been used for several uses such as an ornamental plant or treatment for ulcers. The leaves have also been used for human consumption in a salad (Mitich 2000). The effect of competition on growth of oxeye daisy was studied by Cole (1998) in order to find a good way to control this noxious weed. Plant competition was regarded as the key to managing or preventing an oxeye daisy problem in pastures or hay lands. Other literature review regarding taxonomy, growth and reproduction, distribution and habitat and control was also made by Cole (1998). Oxeye daisy seed can remain viable for a long time if conditions are not suitable for germination. It has resistance to many herbicides (Cole 1998). There is no published literature on the supercritical fluid extraction of oxeye daisy oil.

This study is intended to analyze the SFE oxeye daisy seed oil for the potential uses of this plant. If we can find an economical use, this noxious weed could become a useful species. A possible use for this plant is its seed oil. The reason why we are interested in its seed oil is that many plant seeds were found to have value-added use, such as Rosehip (*Cynosbaty pseudofructus*) (Szentmihalyi et al. 2002) and Physic nut (*Jatropha curcas* L.) in the pharmaceutical industry, cosmetics manufacturing, fuel or food (Machmudah et al. 2008). One of the encouraging samples is the utilization of meadowfoam (*Limnanthes alba*) which after discovering the special long chain fatty acids in its seed, the meadowfoam oil was increasingly studied and developed for commercial products. Meadowfoam has been successfully cultivated for commercial products (Meadowfoam 2008; Holser 2002; Adhvaryu, 2006).

There has been an increasing interest in researching plant oils as alternatives to petroleum oil (mineral oil) for lubricants and other industrial applications (Bondioli et al. 1998; Biresaw et al. 2003; Adhvaryu et al. 2004; Adhvaryu et al. 2006; Fox and Stachowiak 2007) because of the renewable and environmentally-friendly properties of plant oils. Another research area of seed oil is their application in cosmetics manufacturing.

Most seed oils are composed of triglycerides (TG) constituting of a complex mixture of fatty acids (FA) with varying length carbon chain and degrees of saturation. Some seed oils are also found as monoesters of long chain FA and fatty alcohols of varying chain length and degree of saturation (Adhvaryu et al. 2006). Most vegetable oils can be considered to be amphiphilic since they are composed of separated polar and nonpolar groups in the same molecule, which serves as a lubricating property. The polar groups constitute at least one ester functional group. The nonpolar groups are hydrocarbons of varying chain lengths, degrees of unsaturation, and stereochemistry (Biresaw et al. 2003). The triglyceride structure of vegetable oils provides

qualities desirable a lubricant, which is also the basis for the inherent disadvantages of vegetable oils as lubricants. Unsaturated double bonds in the fatty acids are active bonds for many reactions, including oxidation, lowering the oxidation stability of vegetable oils. Another concern is the susceptibility of the triglyceride ester to hydrolysis. The similarity in all vegetable oil structures is another disadvantage because only a narrow range of viscosities are available for their potential use as lubricants (Fox and Stachowiak 2007). So vegetable oils usually do not serve as direct lubricants. They serve as base oils or are used with some additives. Generally, the more saturated the fatty acid, the more stable the oil. The longer the carbon chain of a fatty acid, the higher the oil viscosity is. Thus, the chemical structure of oil components is very important to its application. The primary analysis method is GC-MS.

2.2 Materials and Methods

2.2.1 Materials and Specimen Preparation

The oxeye daisy seeds were purchased from Wildseed Farms (Fredericksburg, TX) and were mechanically harvested in May-June, 2007. The seed was stored in a Ziploc bag in a freezer (-4 °C). The seed was ground with a coffee grinder (20 g seed, ground for 1 min.) and sieved into two particle sizes, (1) 40-80 mesh and (2) finer than 80 mesh. Analytical grade n-Hexane was used for Soxhlet extraction and microwave assisted extraction (MAE). GC-MS grade methanol was used for UV/Vis analysis.

2.2.2 Soxhlet Extraction

Conventional SE was carried out with 200 mL of hexane on 10 g of oxeye daisy seeds (untreated, 40-80 mesh, and finer than 80 mesh) by Soxhlet apparatus. The extraction lasted for 24h at the boiling point of hexane. The extract was filtered to remove possible solid particles. Hexane solutions were then concentrated by rotary evaporation and the traces of solvent in residual oil were removed by nitrogen flushing. Yield was calculated based on Eqn. 1. The experiments were performed in triplicate.

$$\text{Yield} = \frac{m}{M} \times 100\% \quad (1)$$

Where m = weight of extracted oil; M = weight of sample (g).

2.2.3 Supercritical Fluid Extraction

Oxeye daisy seeds of different particle sizes (untreated seed, 40-80 mesh and finer than 80 mesh) were extracted under different temperatures (60 °C, 100 °C), different pressures (100 bar, 200 bar, and 300 bar) and different extraction times. Extractions were conducted with an Applied Separations Spe-ed SFE model 7070 apparatus (Applied Separations Inc., Allentown, PA) (Figure 2-1). About 3 g (0.0001g) of sample was weighed and added to a 10 mL stainless steel extraction vessel sealed with polypropylene wool at the top and the bottom of the extraction vessel. The extraction flow rate was 1.5L/min. (expanded gas) using industrial grade CO₂ and extracted oils were collected with 60 mL vials (neat collection). The extraction was performed by dynamic extraction until the vial weight no longer increased (about 90 min.). The vials were weighed before and after the extraction to get the weight of extracted oil, and SFE yields were calculated based on Eqn. 1. The vial was weighed every 5 min. Each extraction was replicated twice.

2.2.4 Microwave Assisted Extraction

Different particle sizes of oxeye daisy seeds (untreated seed, 40-80 mesh, and finer than 80 mesh) were extracted under different temperatures (60 °C, 90 °C) using a Milestone Ethos EX microwave extraction system (Shelton, CT) (Figure 2-2). Samples were weighed and put into an extraction vessel, adding 40 mL hexane and a stirring rod. The extraction time and the extraction

power were programmed by a computer. After the extraction ended, the extraction vessel was cooled in water and filtered through glass fiber filter paper and washed twice with 20 mL hexane. The hexane solution that was obtained was concentrated by rotary evaporation and the traces of solvent in residual oil were removed by nitrogen flushing. The weight of residual oil was calculated as neat yield. Yield is calculated based on Eqn. 1. Each extraction was replicated three times.



Figure 2-1 Applied separations Spe-ed SFE Model 7070 apparatus

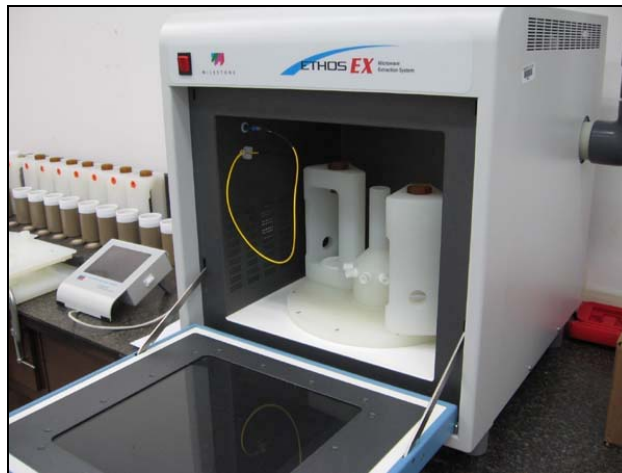


Figure 2-2 Milestone Ethos EX microwave extraction system

2.2.5 Microscope Analysis

Images of the oxeye daisy seeds were taken using a Leica Z16 microscope (Bannockburn, IL) coupled with a Nikon digital camera and a computer. Seeds were cut in order to see the inner structure of a seed. The Leica Z16 microscope was set at 2.0×1.0 and 8.0×1.0 magnification. The length of the sample and the length unit under different magnification were automatically calculated by a computer.

2.2.6 Gas Chromatography-Mass Spectrometry (GC-MS)

The fatty acid composition of oxeye daisy oil was determined by GC-MS as fatty acid methyl esters. The extracted oils were first methylated by BF_3 methylation method with slight

modification (Hwang, 2003). About 0.1g extracted oxeye daisy seed oil was weighed in a 50mL heart-shaped flask, then 5 mL 0.5 mol/L KOH/CH₃OH solution was added to initiate reaction. The sample was then refluxed at 90-100 °C for 13 min., followed by supplementing with 5 mL BF₃/CH₃OH solution and continuing to reflux for 2 min. Finally, 4 mL hexane was added to the mixture and further refluxed for another 1 min. The reaction was terminated by adding 10 mL saturated NaCl solution and cooled down to room temperature. The hexane layer was carefully collected and introduced into the GC-MS system. The GC-MS device was a Varian system with a DB-5 column. The carrier gas was helium. The initial oven temperature was maintained at 40 °C for 3 min. The temperature was then increased from 40 to 280 °C at a rate of 15 °C /min. The final oven temperature was 280 °C and was held for 21 min. The injector temperature was 250 °C, inject volume at 1 microliter. The MSD (mass selective detector) conditions were EI (electron impact ionization) of 70 eV and room temperature. The spectrum was compared with standard spectrum library (NIST) to get the most possible molecular structures and to determine the percentage contribution of individual elements.

2.2.7 Ultraviolet/ Visible (UV/Vis) Spectroscopy Analysis

The SE seed oil, MAE oil, and SFE oil were dissolved in GC grade methanol to make a series of solutions (0.4%, 0.08%, 0.008% (v/v)). The MAE oxeye daisy seed oil was extracted at 60 °C for 30 min. The SFE oxeye daisy seed oil was extracted at 60 °C, 200 bar. The solutions were analyzed with a Beckman Coulter DU 800 UV/Visible spectrophotometer (Fullerton, CA). Methanol was used as a blank.

2.2.8 DPPH Radical-Scavenging Assay

1, 1-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical-scavenging assay is an evaluation of antioxidant activities of the extracted oils. The purpose is to compare the antioxidant activities difference of the extracted oils. Radical-scavenging activity of extracts against stable DPPH was determined spectrophotometrically. A series of oil methanol solution at various concentrations were added to 5 mL of a methanol solution of DPPH (0.004%) to make a final oil concentration at 2%, 1%, 0.5% (v/v). The reaction mixture was vigorously shaken by hand and then kept in darkness for 30 min. at ambient conditions. The control was prepared as above without any extracted oil, and MeOH was used for baseline correction. The absorbance was measured at 517 nm, and the antioxidant capacity was expressed as percent inhibition, which was calculated using Eqn. 2

$$\% \text{ radical scavenging activity} = (\text{control OD} - \text{sample OD}) / \text{control OD} \times 100\% \quad (2)$$

OD is an abbreviation of optical density. Higher antioxidant ability results in higher DPPH percent inhibition.

2.3 Results and Discussion

2.3.1 Soxhlet Extract Yields

Figure 2-3 shows the Soxhlet extraction yield of the different particle sizes seed. The oil yield of finer than 80 mesh oxeye seed was 22.22%, slightly higher than that of 40-80 mesh oxeye seed 20.54%, while that of untreated seed was 0.85%. The ground seed oil yields (40-80 mesh, finer than 80 mesh) were dramatically higher than that of untreated seed. This is because the untreated seed has a hard shell which solvent could hardly penetrate. After the seed is ground, the shell breaks, the inner oily material is directly exposed to solvent. This was also supported by a microscopic view of the seed, which we will discuss later (2.3.4). The extraction yield of finer than 80 mesh seed was slightly higher than that of 40-80 mesh seed. This is because small particle sample size provides high surface area. In addition, the smaller size sample contains less seed shell.

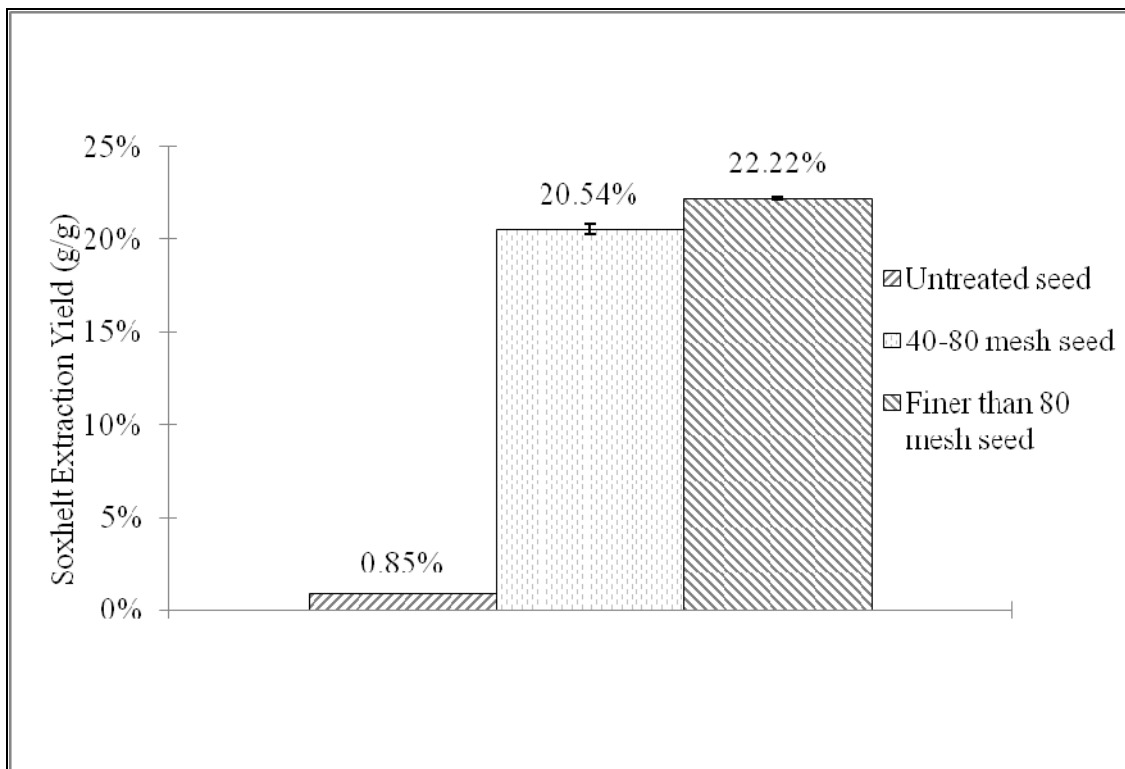


Figure 2-3 Soxhlet extraction yields of different sample particle sizes

2.3.2 Supercritical Fluid Extraction

Different particle size seeds were first extracted at (100 °C, 300 bar) to see which particle size is the best for SFE. The SFE yield of untreated seed, 40-80 mesh seed, and finer than 80 mesh seed was 1.66%, 19.69, and 22.12%, respectively. So finer than 80 mesh seed is selected as samples for later on SFE experiments, which is compatible with the result of Soxhlet extraction.

Figure 2-4 shows the extraction pressure effect on SFE yield. Finer than 80 mesh oxeye daisy seed was extracted at 60 °C at different pressures (100 bar, 200 bar, 250 bar and 300 bar). Almost nothing was extracted at 100 bar. This is probably because at the low pressure the solvent density and extraction power was too weak. 60 °C 100 bar is almost out of supercritical phase from the phase diagram (Figure 1-1) and it tends to be gas. It was found that with the increase of extraction pressure the extraction rate increases. The slope of extraction at 300 bar was sharpest in the first 10 min. Generally, higher extraction pressure results in more extraction power. But with the higher extraction pressure, the more sophisticated and expensive equipments are required.

The extraction temperature effect on SFE yield is shown in Figure 2-5. Finer than 80 mesh oxeye daisy seed was extracted at 200 bar with different temperatures (100 °C, 60 °C, 40 °C, 30 °C and 25 °C). Extraction at 100 °C showed the lowest extraction rate. As the extraction temperature decreased, the extraction rate increased until 30 °C. 30 °C showed the highest extraction rate. When the extraction temperature decreased to 25 °C, the extraction rate was a little lower than 30 °C. This is because carbon dioxide at 25 °C 200 bar was no longer in its supercritical state but rather in a liquid state according to the phase diagram (Figure 1-1). Although, the density of liquid was high, its diffusivity was low at this time.

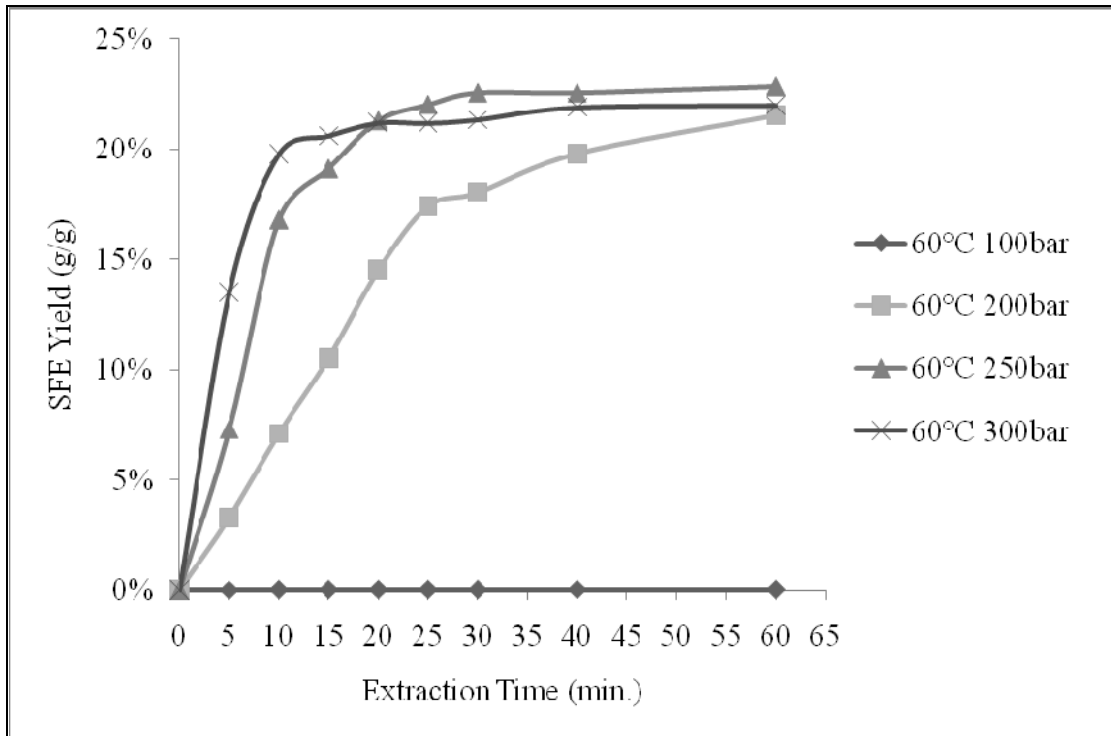


Figure 2-4 Extraction pressure effect on supercritical fluid extraction yield

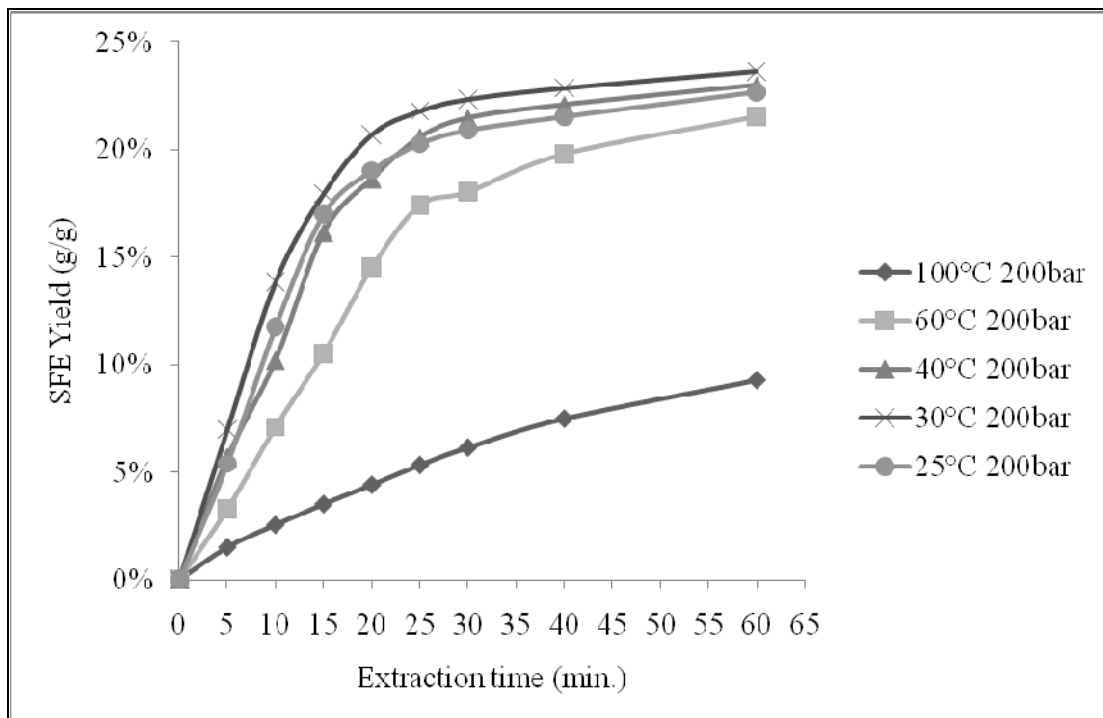


Figure 2-5 Extraction temperature effect on supercritical fluid extraction yield

Finer than 80 mesh oxeye daisy seeds were extracted at different pressures (200 bar, 250 bar, 300 bar) with different temperatures (60 °C, 40 °C)(Table 2-1). Because SFE is a very efficient extraction method, there was not much yield difference when extraction was performed for 60 min. All yields were above 20% at 60 min. However, there was much difference between yields of different extraction condition for the first 5 min. Extraction yields of 300 bar were above 10%, however those of 250 bar and 200 bar were not. This indicates that extraction pressure significantly affects the extraction rate. Besides, at the same pressure, extraction yield at 40 °C was constantly higher than at 60 °C for the first 5 min. This indicates extraction temperature can affect the extraction rate too. Generally SFE at high pressure and low temperature gives high extraction rate.

Table 2-1 Supercritical fluid extraction yields of oxeye daisy seed under different temperatures and different pressures

Supercritical fluid extraction yields at different extraction time									
P ¹ /bar	T ² /°C	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.	40 min.	60 min.
200	60	3.30%	7.11%	10.53%	14.51%	17.41%	18.02%	19.79%	21.53%
200	60	3.97%	6.94%	10.61%	15.19%	17.45%	19.26%	20.27%	22.08%
250	60	7.30%	16.81%	19.12%	21.30%	22.02%	22.56%	22.57%	22.85%
250	60	7.43%	15.35%	19.82%	21.54%	22.23%	22.51%	23.28%	23.45%
300	60	13.52%	19.78%	20.61%	21.19%	21.18%	21.34%	21.90%	22.00%
300	60	11.14%	19.41%	21.11%	21.80%	21.79%	22.26%	22.21%	21.98%
200	40	5.70%	10.22%	16.16%	18.65%	20.57%	21.46%	22.08%	22.99%
200	40	5.75%	11.35%	16.59%	19.25%	20.11%	21.11%	22.02%	23.47%
250	40	8.44%	17.07%	21.01%	22.39%	22.43%	22.92%	23.55%	23.65%
250	40	8.17%	16.67%	20.39%	21.70%	22.31%	22.70%	22.93%	23.22%
300	40	11.59%	20.93%	22.49%	22.70%	23.17%	23.40%	23.08%	23.46%
300	40	10.77%	19.97%	21.92%	22.43%	22.74%	22.90%	23.25%	23.35%

¹ Supercritical fluid extraction pressures.

² Supercritical fluid extraction temperatures.

2.3.3 Microwave Assisted Extraction

3g different particle size oxeye daisy seeds (untreated seed, 40-80 mesh seed, and finer than 80 mesh seed) were extracted with 40 mL hexane at 60 °C for 30 min. in the microwave extraction system (Figure 2-6). The MAE yield of untreated seed was still very low (0.78%). The result is compatible with SE. Also, finer than 80 mesh seed was selected for subsequent MAE extraction experiments.

Finer than 80 mesh oxeye daisy seed was extracted with 40 mL hexane for 10 min. at different temperatures (60 °C, 100 °C) (Figure 2-7). There was no much extraction yield difference between these two extraction temperatures because these oxeye daisy seed oil were almost fully extracted.

In addition, different sample weight to solvent volume ratios (1.5g/40 mL, 3g/40 mL, 6g/40 mL) were tested at 60 °C for 10 min. (Figure 2-8). There was not much difference between these extraction yields too. Because MAE is a very efficient extraction method, all yields were above 20%. If the Soxhlet extraction yield (22.22%) (Figure 2-3) is regarded as total recovery. Then almost all the oil in the finer than 80 mesh seed was extracted.

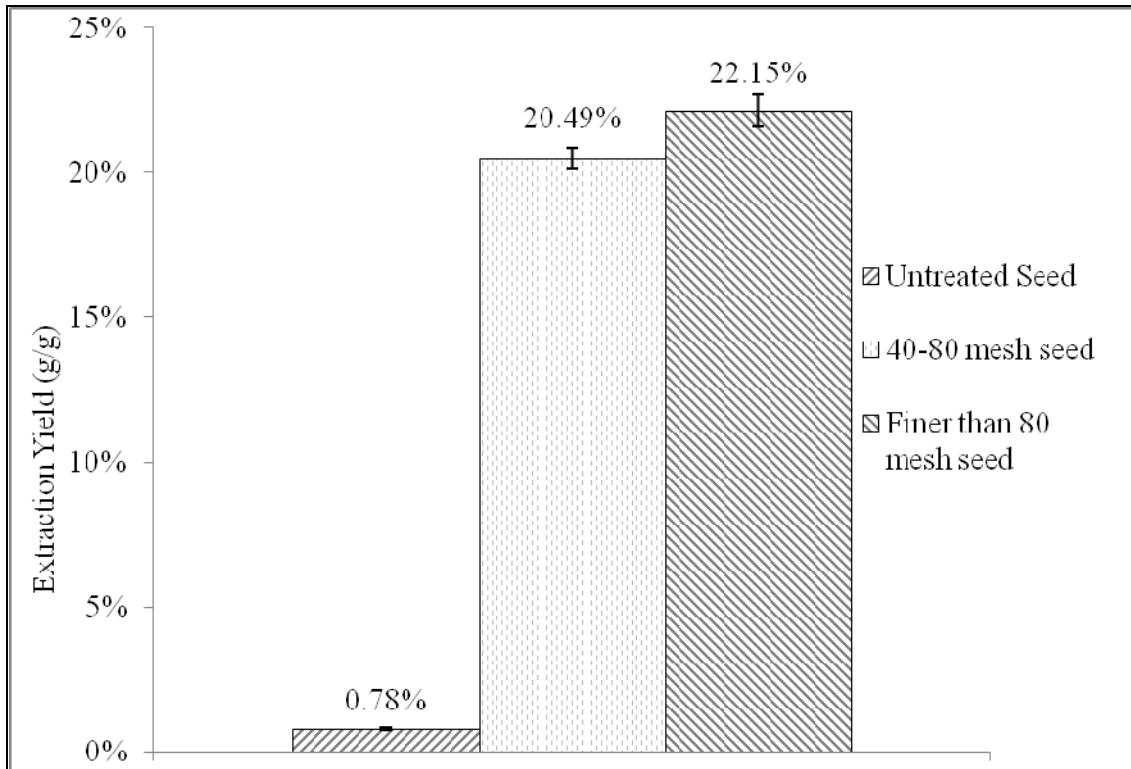


Figure 2-6 Microwave assisted extraction yields of different particle size oxeeye daisy seeds

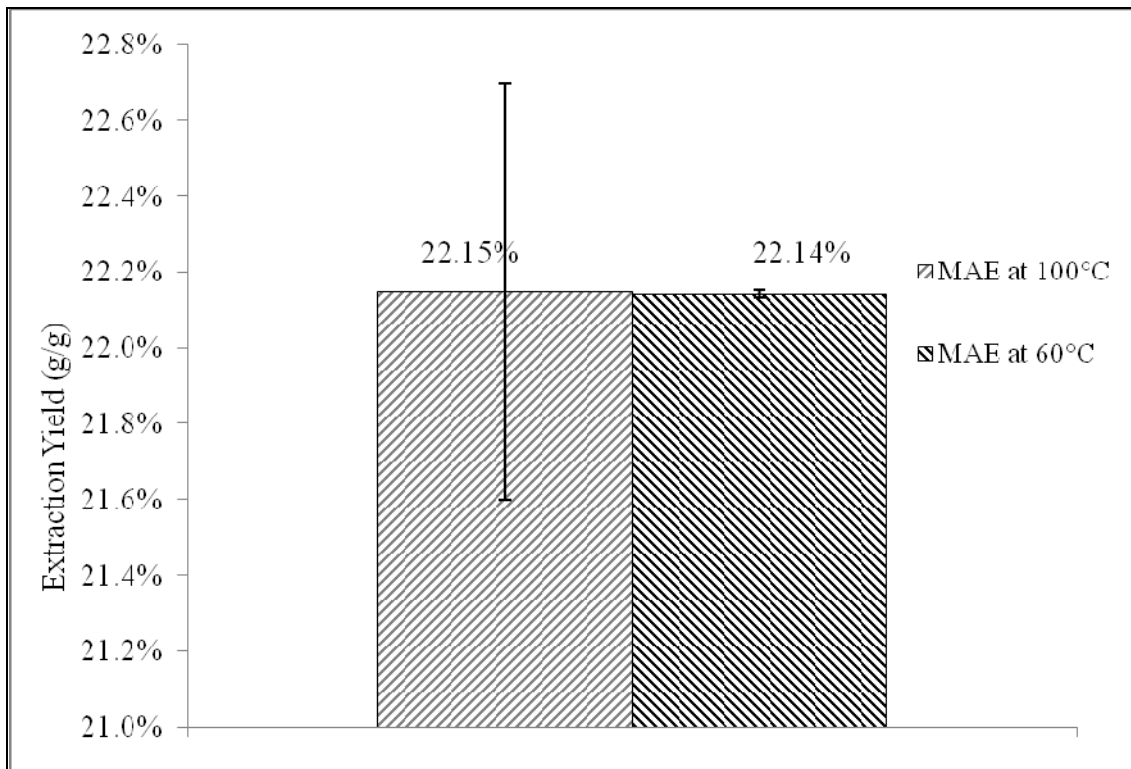


Figure 2-7 Microwave assisted extraction yields of different extraction temperatures

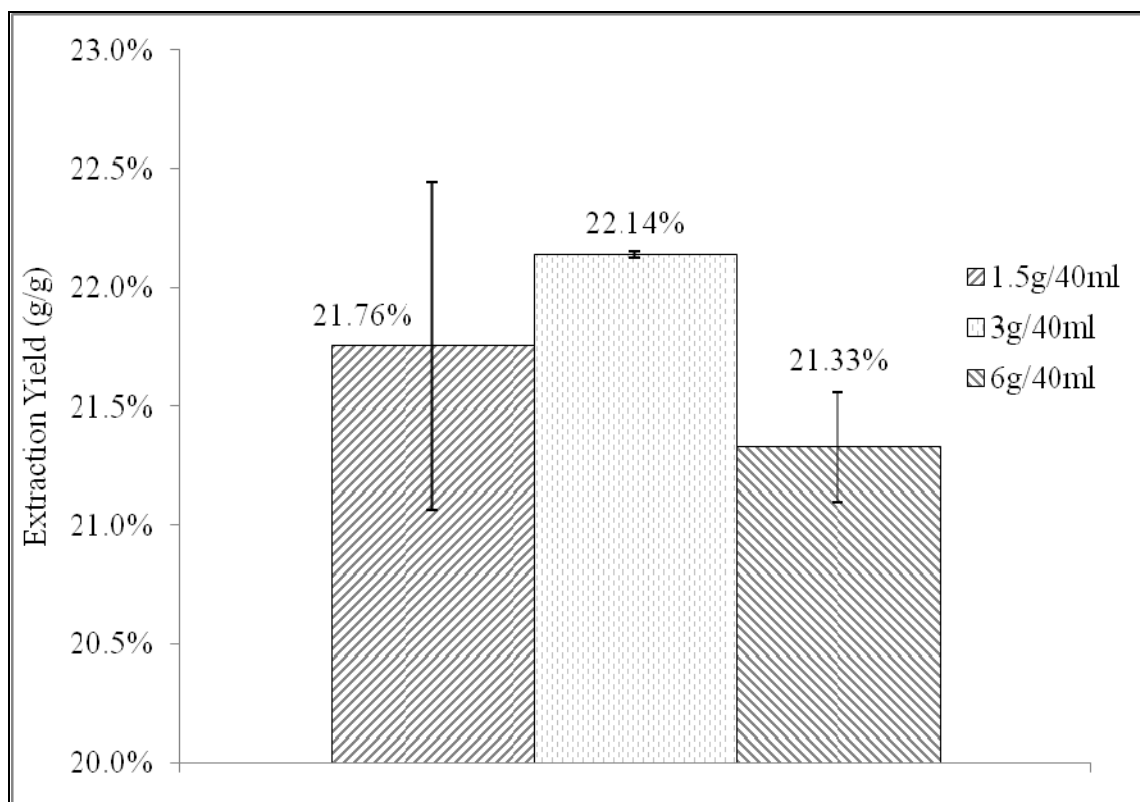


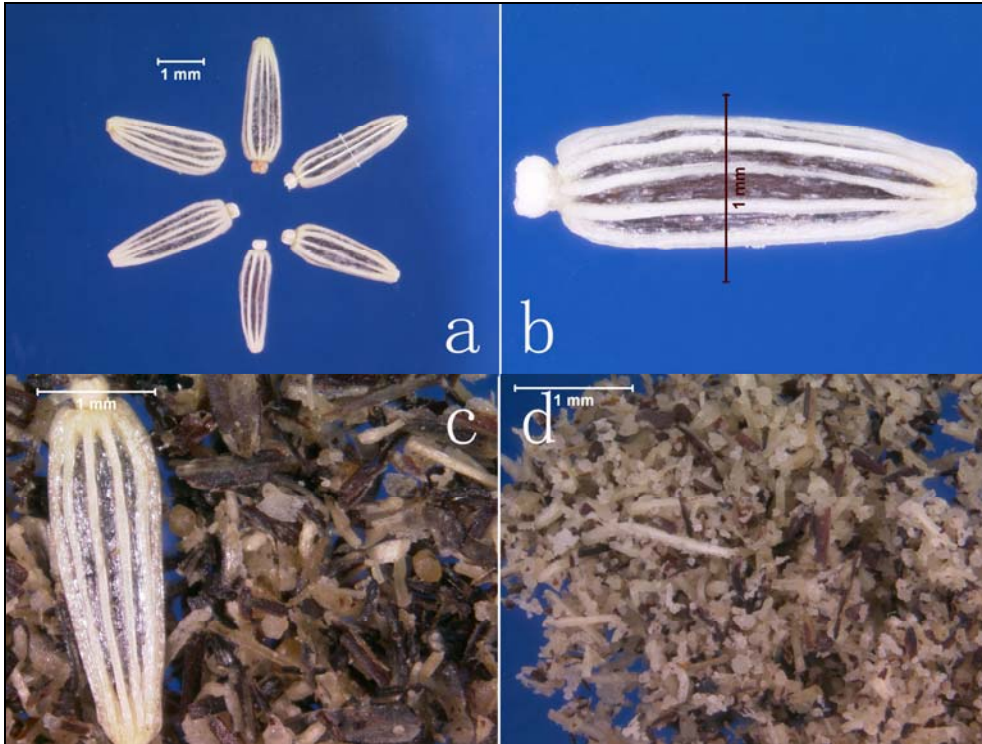
Figure 2-8 Microwave assisted extraction yields of different sample weight to solvent volume ratios

These comparisons were not able to show the temperature and sample weight to solvent volume ratio's effect on MAE extraction yield.

2.3.4 Microscope Analysis

Figure 2-9 shows the images of oxeye daisy seeds and ground oxeye daisy seed under a light microscope. There are several white ridges on the seed coat. The average diameter of an individual seed is about 0.7 mm (Figure 2-9 c). The length of an individual seed is about 3 mm. Figure 2-9 (c) shows the image of ground seed materials between 40 and 80 mesh, while Figure 2-9 (d) shows the image of ground seed materials passing through the 80 mesh screen. The small particle size gives more surface area, which supports the previous extraction data that smaller particle size gives better extraction yield.

Figure 2-10 shows the inner structure of oxeye daisy seeds. Figure 2-10 (b), Figure 2-10 (c) and Figure 2-10 (d) are magnified parts of Figure 2-10 (a). Inside the woody hard shell, there is a large amount of white waxy material, which contains oil. The thickness of the seed shell shown in Figure 2-10 (b) is 54 μm . The inner diameter and length of the seed shown in (a) is 0.5 mm and 1.9 mm, respectively. From the section view, the inner part is protected by the woody shell, which pressurized hexane and CO_2 could barely penetrate. This is supported by the previous extraction data (2.3.1, 2.3.2, 2.3.3). This probably accounts for why oxeye daisy seed is regarded as a noxious weed from a competitiveness perspective. The oxeye daisy seed may not be able to be digested by birds or other animals because the seed is protected by the hard shell. In addition, oxeye daisy seed can remain viable for a long time if conditions are not suitable for germination.



(a) and (b) whole oxeye daisy seeds; (c) 40-80 mesh seed; and (d) finer than 80 mesh seed.
 Figure 2-9 Images of oxeye daisy seeds

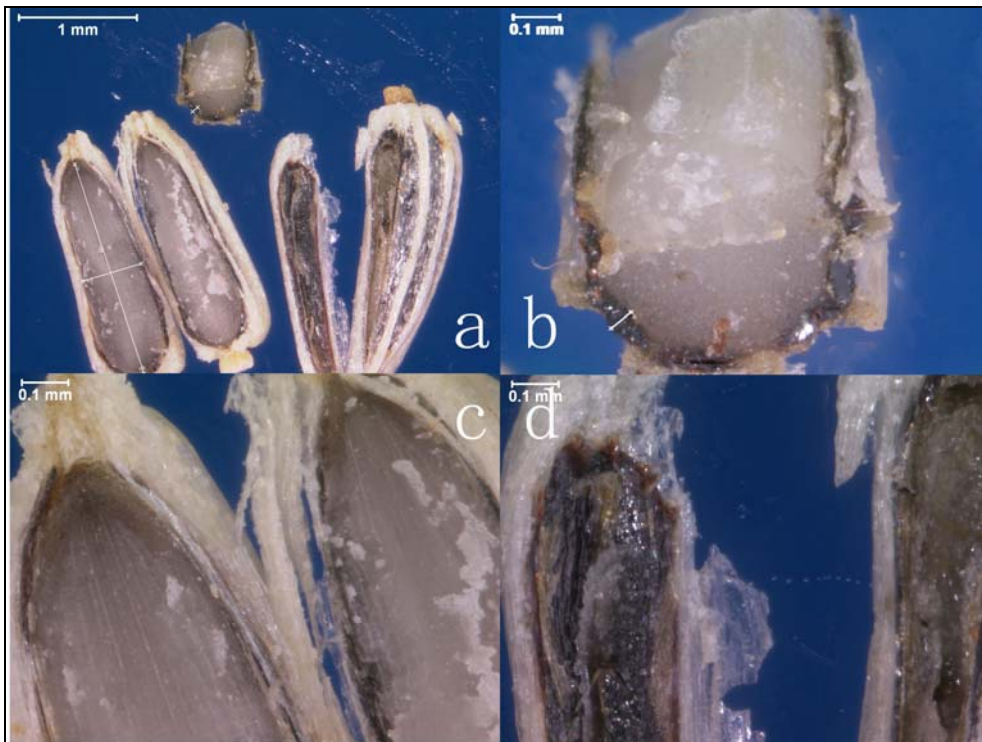
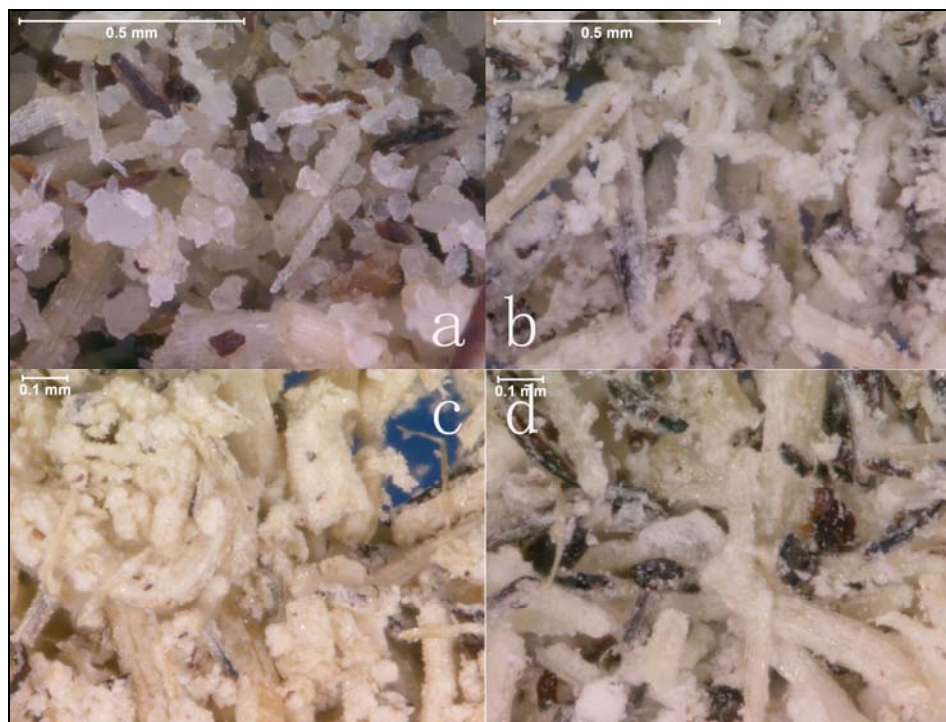


Figure 2-10 Inner structure of oxeye daisy seeds

Figure 2-11 shows comparative images of finer than 80 mesh oxeye daisy seed before and after extractions. The particles shrank and lost their brightness and oily outlook, especially Figure 2-11 (b). Also, from Figure 2-11 (c), the residue gained brown color, which could also be seen with the naked eye. This is probably the result of the long Soxhlet extraction period. Some components may be oxidized during long extraction time (24 h) at high temperature.



(a) Before extraction; (b) After supercritical fluid extraction (60 °C, 200 bar); (c) After Soxhlet extraction; (d) After microwave assisted extraction (3g/40 mL, 60 °C).

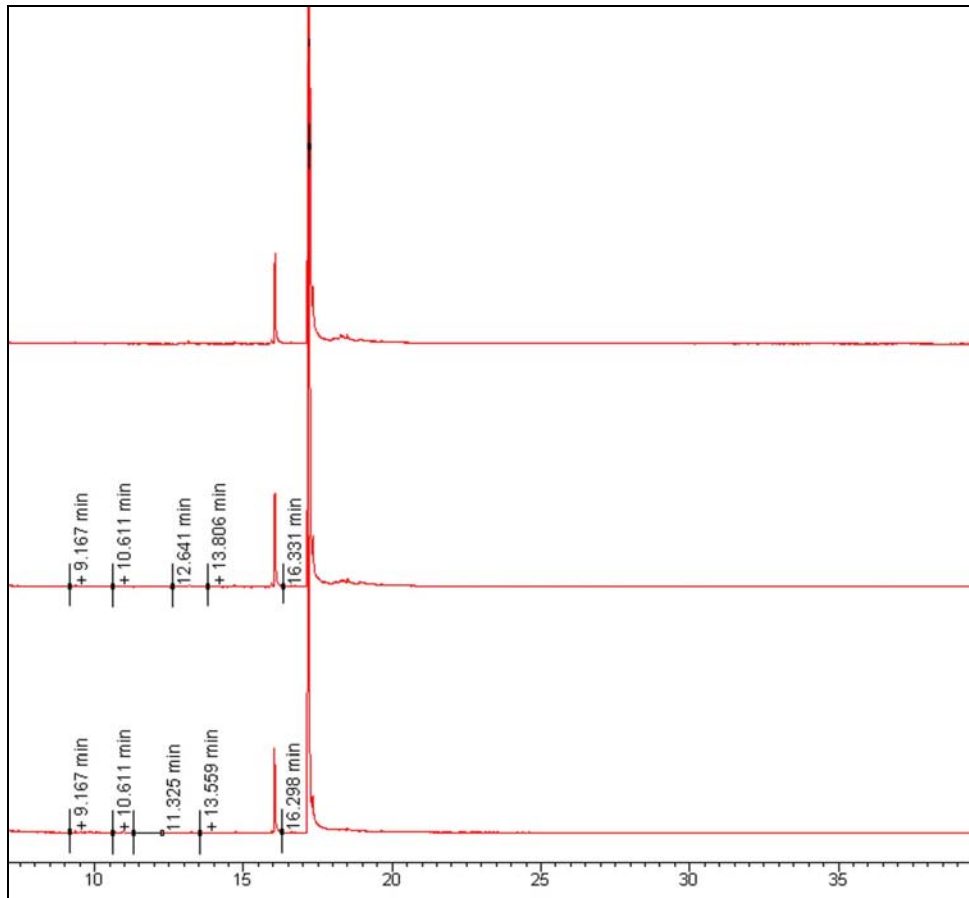
Figure 2-11 Comparison of the seed structure before and after extractions

2.3.5 Gas Chromatography-Mass Spectrometry

The fatty acid methyl esters from SE oil, MAE (60 °C) oil, and SFE (60 °C, 300 bar) oil were tested for GC-MS analysis. There are two significant peaks in the GC spectrums of the samples (Figure 2-12). The spectrums of the three extraction methods for oxeye daisy seed oils are very similar to each other. This means the main components of the three extracted oils are similar. Identification of the peaks was made by retention time and a National Institute of Standards and Technology (NIST) library search.

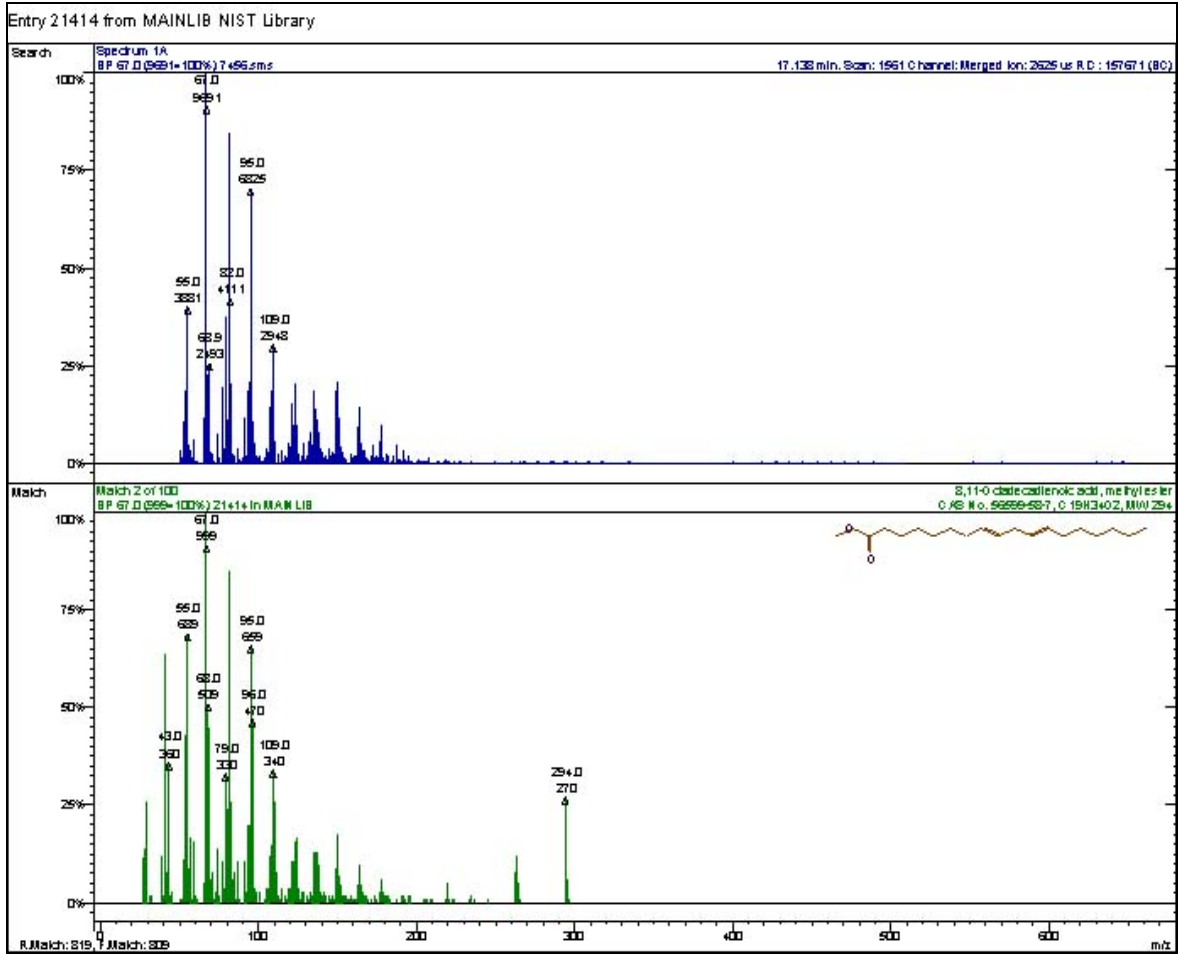
The sharp peak around 17 min. was identified as 8, 11-Octadecadienoic acid, methyl ester. The mass spectrum of the sample at 17 min. and standard mass spectrum of 8, 11-Octadecadienoic acid, methyl ester in the NIST library are shown in Figure 2-13.

The smaller peak around 16 min. was identified as Hexadecanoic acid, methyl ester. The mass spectrum of the sample at 16 min. and standard mass spectrum of Hexadecanoic acid, methyl ester in the NIST library are shown in Figure 2-14. Because the oxeye daisy seed was methylated before the GC-MS test, the fatty acid profile of triglyceride of the oxeye daisy seed was mainly composed of unsaturated C₁₈ and saturated C₁₀ compounds. Because the oil contains long C₁₈ carbon chains, it might be used as an additive to lubricant oil.



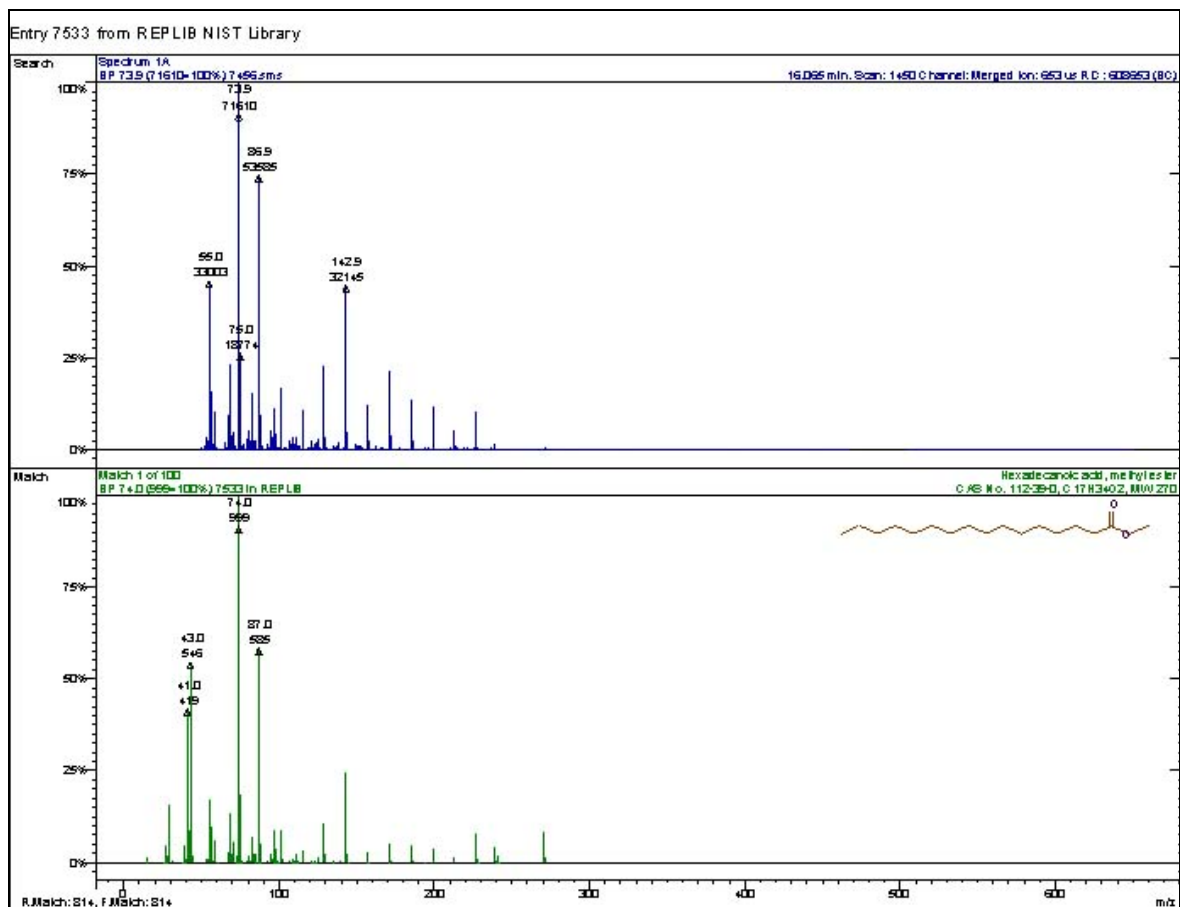
Top is the supercritical fluid extract oil spectrum; Middle is the microwave extracted oil spectrum; Bottom is the Soxhlet extraction oil spectrum.

Figure 2-12 GC spectrums of fatty acid methyl esters from extracted oils



Top is the mass spectrum of the sample at 17 min.; Bottom is the standard mass spectrum of 8, 11-Octadecadienoic acid, methyl ester.

Figure 2-13 Mass spectrum of sample at 17 min. and standard mass spectrum of 8, 11-Octadecadienoic acid, methyl ester



Top is the mass spectrum of the sample at 16 min.; Bottom is the standard mass spectrum of Hexadecanoic acid, methyl ester.

Figure 2-14 Mass spectrum of sample at 16 min. and standard mass spectrum of Hexadecanoic acid, methyl ester

2.3.6 Ultraviolet/ Visible (UV/Vis) Spectroscopy Analysis

Figure 2-15 shows the UV/Vis spectrums of three SE, MAE and SFE oils at the concentration of 0.008% (v/v). Three featured peaks for all these oils are at 203 nm, 230 nm and 270 nm. This represents absorption of the double bonds of fatty acid in the oils as shown in the GC-MS analysis 2.3.5.

Figure 2-16 shows the UV/Vis spectrums of three SE, MAE, and SFE oils at the concentration of 0.4% (v/v). At the elevated concentration, most ultraviolet wave did not pass through the oxeye daisy oils at wavelength range 200 nm to 300 nm when oil concentration was 0.4% (v/v). This feature gives the oil a potential to be sun blocker additive to block ultraviolet wave. In addition the oxeye daisy oil has a naturally pleasing scent. These are good characteristics for sun blocker additives. The green line Figure 2-16 c (SFE oil) has the lowest transmittance which indicates that it blocked more ultraviolet wave. This implies that the SFE oil has superior ultraviolet block ability.

2.3.7 DPPH Radical-Scavenging Assay

Figure 2-17 shows the DPPH radical-scavenging assay result of three different extracted oils of different concentrations. DPPH radical- scavenging ability of these oils is

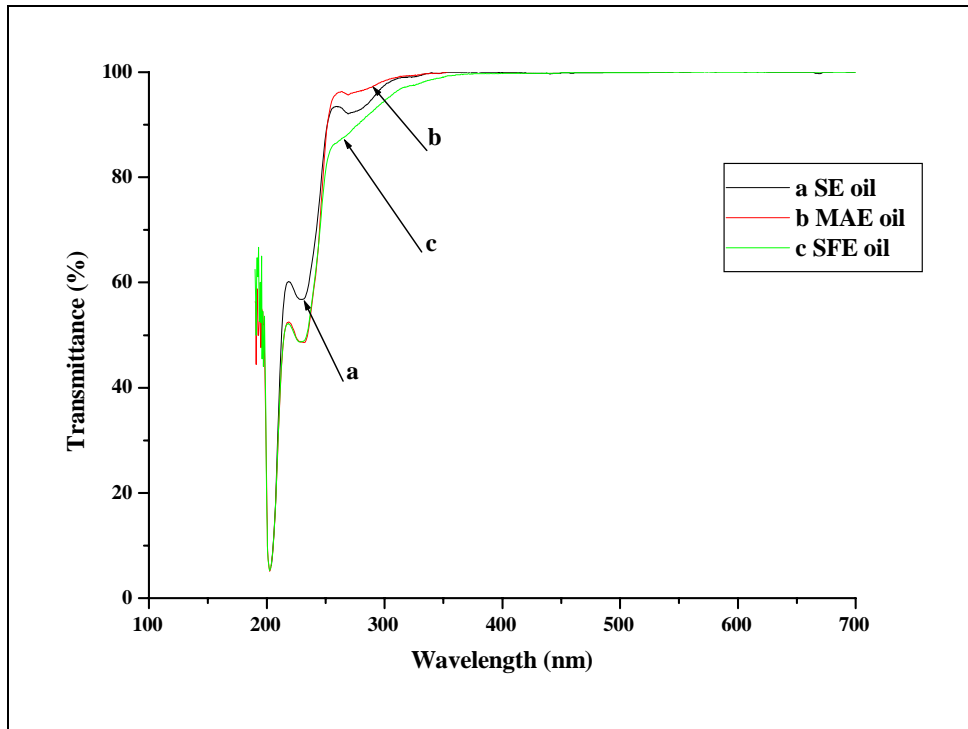


Figure 2-15 UV/Vis spectrums of 0.008% (v/v) extracted oxeye daisy seed oils

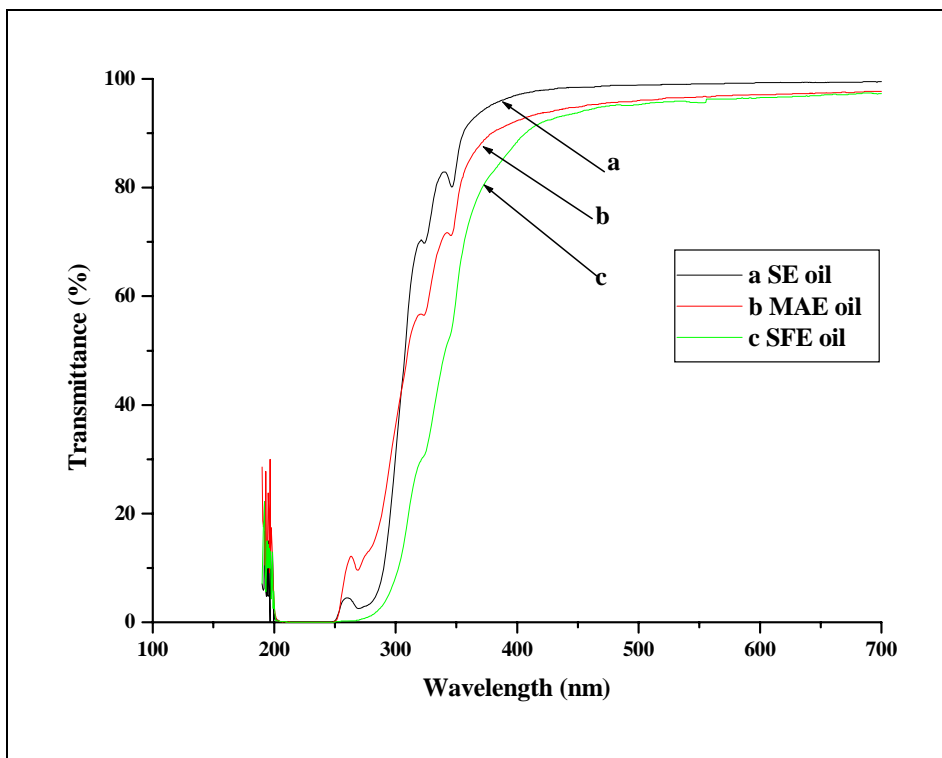


Figure 2-16 UV/Vis spectrums of 0.4% (v/v) extracted oxeye daisy seed oils

concentration-dependent. The MAE oil (60 °C) and SFE (60 °C, 300 bar) show stronger DPPH radical-scavenging ability than SE oil at the same concentration. This indicates the antioxidant ability of SE oils is weaker than the other two oils. In another words, the SE oil was oxidized stronger than the other two oils during the extraction process. SFE and MAE can minimize the oxidation of extracted natural products.

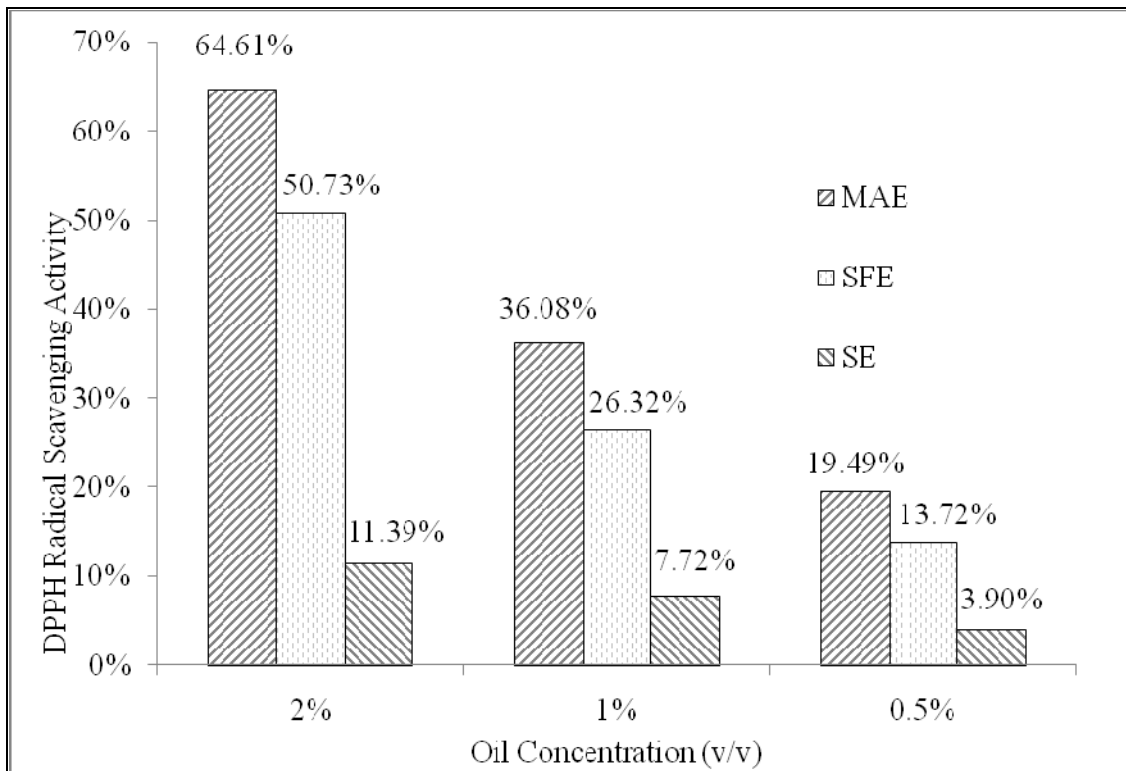


Figure 2-17 DPPH radical-scavenging assays of extracted oils

2.4 Summary

Oxeye daisy seed can be effectively extracted by supercritical carbon dioxide. The sample particle size could affect the extraction yields. Finer than 80 mesh seed showed the highest extraction yields for all the Soxhlet extraction (22.22%), microwave assisted extraction (22.15%), and supercritical fluid extraction (22.12%). The extraction temperature and extraction pressure significantly affect the extraction rate of SFE. The GC-MS spectrums of the three extraction methods for oxeye daisy seed oils were very similar to each other. The fatty acid profile of oxeye daisy oil was composed of unsaturated C₁₈ carbon chain and saturated C₁₀ carbon chain. Most ultraviolet waves did not pass through the oxeye daisy oils at a wavelength range of 200 nm to 300 nm when oil concentration was 0.4% (v/v). The MAE oil (60 °C) and SFE (60 °C, 300 bar) showed stronger DPPH radical-scavenging ability than SE oil at the same concentration. The antioxidant property and ultraviolet wave block property give the oxeye daisy seed oil a potential to be sun blocker additive oil.

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Chapter 3. Cedar Oils Extraction and Related Analysis

3.1 Introduction and Literature Review

The relationship between chemical composition and durability in wood was first reported by Hawley et al. (1924). Some heartwood has the inherent ability to resist biological degradation, often referred to as “natural durability” or “decay resistance” (Eaton and Hale 1993). Three North American important commercial wood species, Port-Orford cedar (POC) (*Chamaecyparis lawsoniana*), Alaska yellow cedar (AYC) (*Chamaecyparis nootkatensis*) and Eastern red cedar (ERC) (*Juniperus virginiana*), are known to have significant natural durability.

Cedar species have been reported to have special bioactivity against termites and wood decay fungi (Liu 2004; Gao et al. 2008). Evaluations on antifungal properties (Gao et al. 2008), biocidal application (Dolan 2007), and termiticidal activities (Liu 2004) of POC extracts have been reported. A chemical ecological study of the components of the essential oil of ERC from different habitats was performed by Setzer et al. (1992). Volatile oil from ERC, consisting primarily of cedrene (a terpene) and cedral has been used in perfumery (Semen and Hiziroglu 2005) and as an insect repellent. ERC oil has been widely used in a very broad range of products due to its unique properties, such as odor and repellency or toxicity to many pests. In addition, the antibiotic activities of AYC have been studied extensively. For example, antimicrobial activity of essential oil from AYC has been tested against anaerobic bacteria and yeast (Johnston 2001). Heartwood extractives from AYC have been tested for resistance to termites and fungi (Taylor et al. 2006). The composition of the leaf oil from AYC has also been reported (Andersen and Syrdal 1970; Cheng and Von 1970).

In most of these studies, conventional SE methods were used, which were often time consuming and involved organic solvents and high temperatures. Some bioactive components in cedar oils may be affected during these extraction processes. Supercritical CO₂ (SCC) has several advantages in extracting non-polar chemicals especially for natural products (Eller and King 2000). The low viscosity and high diffusivity of SCC can result in higher extraction efficiencies and CO₂ can be easily removed from the extract, leaving an extract that is uncontaminated by any solvent residue. However, SFE of these three cedar oils has been rarely reported (Eller and King 2000). Comparison of the antifungal activities of these SCC extracted oils has not been reported.

The objectives of this research were to compare the extraction efficiency between hexane SE and SCC extraction, identify the main chemical components by GC-MS, compare the chemical composition difference between Soxhlet extracts and SCC extracts, and evaluate the antifungal activities of the SCC extracts of the three cedar woods.

3.2 Materials and Methods

3.2.1 Materials and Specimen Preparation

Brown-rot fungi (*Gloeophyllum trabeum*) and white-rot fungi (*Trametes versicolor*) were cultured from existing laboratory stock. The air dried heartwoods of Port-Orford cedar (POC), Alaska yellow cedar (AYC), and Eastern red cedar (ERC) were received from a saw mill in Oregon. The heartwoods of three kinds of wood were initially air-dried and then cut into small strips with a razor blade. The strips were reduced to 20-40 mesh in a Wiley mill and then stored at -4 °C until extraction.

3.2.2 Soxhlet Extraction

The ground heartwood of POC, AYC, and ERC were Soxhlet extracted (SE) using n-hexane. Extraction was conducted using 200 mL of n-hexane for 2 g of ground heartwood. The

extractions were processed for 24 h at the boiling point of hexane. Siphoning of solvent occurred about every 1 hour. The extracted hexane solution was filtered to remove possible solid particles. Hexane solutions were then concentrated by rotary evaporation, and the traces of solvent in residual oil were removed by nitrogen flushing. The Soxhlet extraction residue (SER) was air dried and stored for further supercritical fluid extraction. The weight of extracted oil was measured and calculated to determine Soxhlet extraction yield in accordance with Eqn. 3. The experiments were performed in triplicate.

$$\text{Yield} = \frac{m}{M} \times 100\% \quad (3)$$

Where m = weight of extracted oil (g); M = weight of cedar heartwood sample (g).

3.2.3 Supercritical Fluid Extraction

SFE was conducted with an Applied Separations Spe-ed SFE model 7070 apparatus (Applied Separations Inc., Allentown, PA), with a restrictor of 80 °C. About 2 g (0.0001g) samples were weighed and added to a 10 mL stainless steel extraction vessel sealed with polypropylene wool at the top and bottom of the extraction vessel. The extraction flow rate was set as 1.5L/min. (expanded gas) using industrial grade CO₂ and extracted oils were collected with 60 mL vials (neat collection). The extraction was performed by dynamic extraction until the vial weight no longer increased (about 60 min.). Ground heartwood of POC, AYC, and ERC were extracted at 60 °C and 2,900 psi (200 bar). The Soxhlet extraction residue (SER) of POC, AYC, and ERC from a previous Soxhlet extraction was also extracted at 60 °C and 2,900 psi (200 bar). The moderate SCC pressure of 2,900 psi (200 bar) is a concern for industrial applications. Generally, higher pressure results in more extraction power. But with the higher extraction pressure, more sophisticated and expensive equipment is required. A moderate SCC extraction temperature was set at 60 °C because if the temperature was set too low, the temperature difference may become a main effect between SCC and SE (hexane boiling point is 69 °C at room condition.). On the other hand, if the temperature was set too high, the SCC extraction power will greatly drop at relatively low extraction pressure. The vials were weighed before and after the extraction to get the weight of extracted oil, and SCC extraction yields were calculated based on Eqn. 3. Each extraction was replicated twice.

3.2.4 Gas Chromatography-Mass Spectrometry (GC-MS)

The SCC extracted oils were tested with a Varian GC-MS system with a DB-5 column. The carrier gas was helium. The initial oven temperature was maintained at 40 °C for 3 min. The temperature was then increased from 40 to 280 °C at a rate of 15 °C/min. The final oven temperature was 280 °C and was held for 21 min. The injector temperature was 250 °C, inject volume at 1 microliter. The MSD (mass selective detector) conditions were EI (electron impact ionization) of 70 eV and room temperature. The spectrum was compared with a standard spectrum library (NIST) to obtain most possible molecular structures and to determine the percentage contribution of individual elements. Relative percentages were obtained by integration and summation of peak areas.

3.2.5 Comparison of Antifungal Activity Test against White-rot and Brown-rot Fungi

The antifungal activities were evaluated according to literature (Gao et al. 2008) with some modifications. Medium were prepared using 2% malt extract, 1.5% agar, and 0.005% yeast extract and sterilized for 20 min. The extracted oils were first dissolved in acetone to make a series of acetone solutions with different concentration. Two mL acetone solutions were mixed with 98 mL culture medium to make final concentrations of 0.015, 0.03, 0.06, 0.13, 0.25, 0.50, and 1.00 mg/mL (oils weight to medium volume) and then transferred into Petri dishes (100 mm

× 15 mm). Control Petri dishes were treated with 2 mL of acetone and 98 mL of medium. Either brown-rot fungi (*Gloeophyllum trabeum*) or white-rot fungi (*Trametes versicolor*) was inoculated in the center of the Petri dishes and incubated at room condition. When the control Petri dishes fungi grew to the edges, the diameter of the fungi was measured and the antifungal index (AI) was expressed as % inhibition, which was calculated by the Eqn. 4. This estimation of antifungal activities was carried out in triplicate and the results were averaged. IC₅₀ is considered as the oil concentration in medium when AI is 50%.

$$AI = \frac{D_2 - D_1}{D_1} \times 100\% \quad (4)$$

Where D₂ = diameter growth in the control dishes (mm); and D₁ = diameter growth in the experimental Petri dishes with extracts (mm).

3.2.6 Statistical Analysis

The data were analyzed using SAS 9.0 software (SAS 2008). Analysis of variance (ANOVA) and Duncan multiple comparisons tests were performed. Comparisons were conducted at alpha=0.05. Yield differences and antifungal activities difference were analyzed.

3.3 Results and Discussion

3.3.1 Effect of Extraction Methods on Extraction Yield

The SCC extraction yield for POC, AYC, and ERC oils was 3.29±0.08%, 3.22±0.50%, and 3.27±0.35%, respectively (Figure 3-1). The SE yield for POC, AYC, and ERC oils was 1.52±0.05%, 0.80±0.03%, and 0.71±0.04%, respectively. The SCC extraction of SER (SCC SER) yields of POC, AYC, and ERC was 1.61±0.06%, 1.66±0.11%, and 1.68±0.11%, respectively.

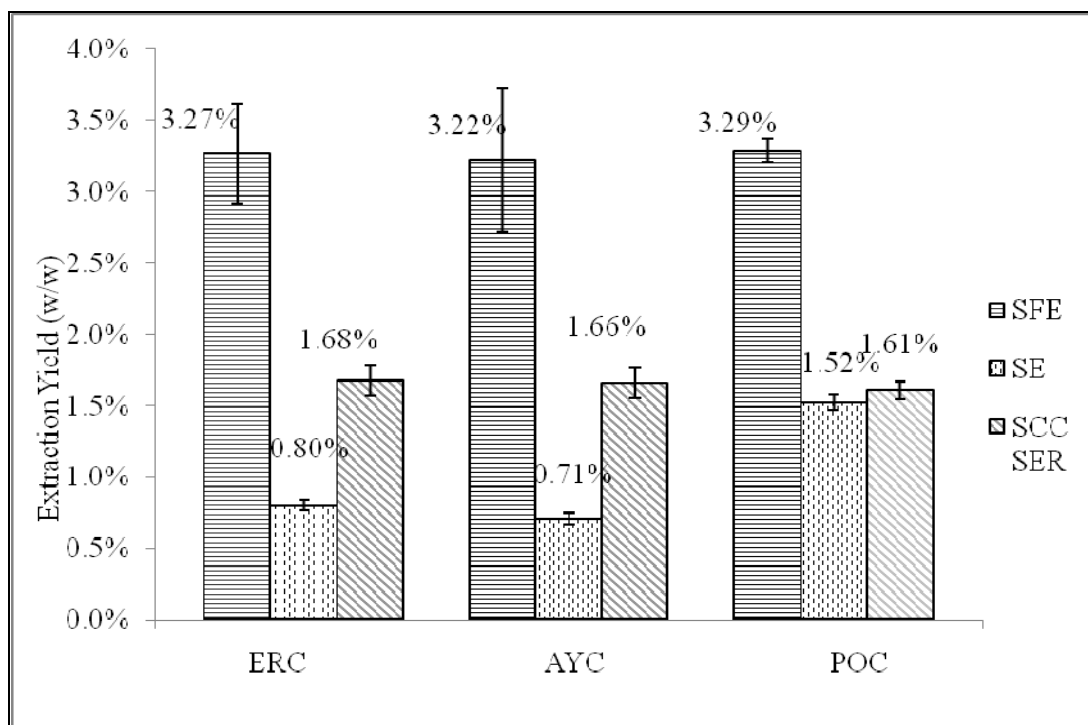


Figure 3-1 Supercritical fluid extraction and Soxhlet extraction yields of three cedar oils

The statistical analysis (Table 3-1) showed there was a significant yield difference between SFE (group A) and SE (group B) extraction ($p < 0.05$). There was no significant yield

difference between cedar species (group A). On the other hand, after the SE extraction, the extracted residue can be further extracted by SCC. The SCC SER yields were greater than the SE extraction yields. This suggests that the SCC extraction method is more effective than the SE extraction method. In addition, SCC extraction normally lasts for 1 hour while Soxhlet extraction requires almost 1 day. This is because SCC has high diffusivity and low viscosity and low surface tension and thus enhances mass transfer inside the solid matrix. In addition, the sum of SE yield and SCC SER yield is less than the SFE yield of each cedar species. This implies that some volatile compounds may be lost during the Soxhlet extraction. Besides, some compounds may be further lost during the rotary evaporation and nitrogen flushing process.

Table 3-1 Summary of Duncan's multiple range test of the effect of extraction methods and species on yields

		Number of sample	Mean of yield	Duncan grouping*
Methods	Supercritical fluid extraction	6	0.032690	A
	Soxhlet	6	0.010167	B
Species	Alaska yellow cedar	4	0.020160	A
	Port-Orford cedar	4	0.024108	A
	Eastern red cedar	4	0.020018	A

*Means with the same letter are not significantly different.

3.3.2 Gas Chromatography-Mass Spectrometry (GC-MS)

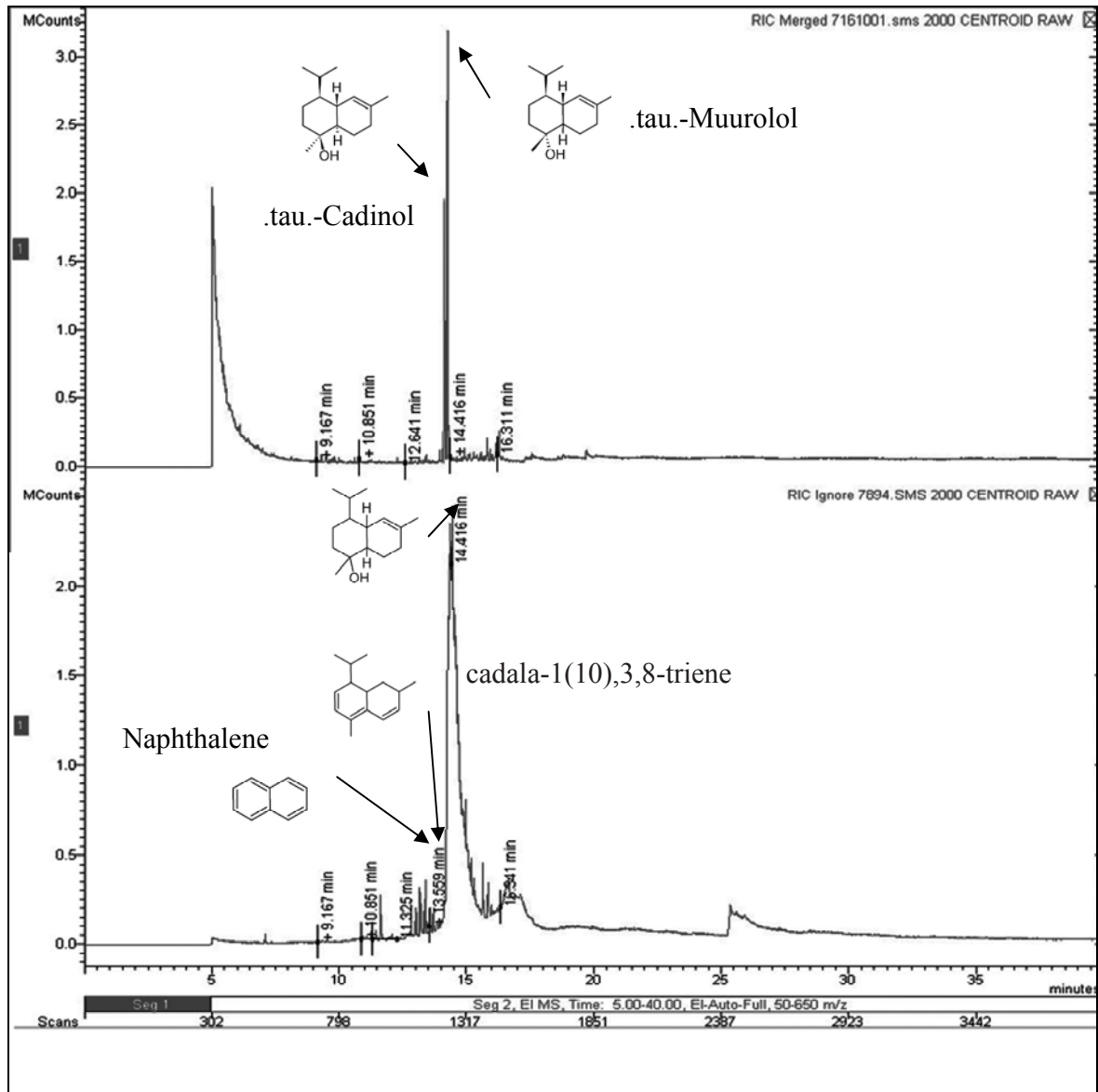
The GC-MS spectrum of SFE POC oil (Figure 3-2 top) shows two main peaks, which indicates that there are two main components in SFE POC oil. The two main components were determined to be .tau.-Cadinol (14.213 min., 40.85%) and .tau.-Muurolol (14.318 min., 42.15%). The peak areas of SE and SFE POC oils spectrums are summarized in Table 3-2.

Table 3-2 Summary of uncorrected peak areas of GC-MS of Port-Orford cedar oils

	Retention time (min.)	Component	Peak area (%)	Molecular formula
Supercritical fluid extraction	14.213	.tau.-Cadinol	40.85%	C ₁₅ H ₂₆ O
	14.255	1-Naphthalenol	5.54%	C ₁₅ H ₂₆ O
	14.318	.tau.-Muurolol	42.15%	C ₁₅ H ₂₆ O
Soxhlet extraction	11.619	Bicyclo[4.1.0]heptane,3,7,7-trimethyl	0.86%	C ₁₀ H ₁₈
	13.209	Naphthalene	0.63%	C ₁₀ H ₈
	13.406	cadala-1(10),3,8-triene	0.75%	C ₁₅ H ₂₂
	14.011-15.032	broad peak could not be isolated	91.11%	

In contrary, the GC-MS spectrum of SE POC oil (Figure 3-2 bottom) showed more small peaks than that of SFE POC oil. This implied that SE POC extract was relatively more complicated compared to SFE POC extract. A broad peak (from 14.011 min. to 15.032 min.) was thought to be a combination of Cadinol, Muurolol, and their derivatives because its retention

time is similar to that of these compounds. In the SE POC oil, there might be more molecules with the formula $C_{15}H_{26}O$ as a result of a chemical bond shift or rotation due to the longer time and higher temperature. The mixture of molecules with similar structure led to a broad peak which was difficult to separate.



*Top is the GC-MS spectrum of supercritical carbon dioxide extracted oil of Port-Orford cedar;
Bottom is the GC-MS spectrum of Soxhlet extracted oil of Port-Orford cedar.*

Figure 3-2 GC-MS spectrums of supercritical fluid extraction and Soxhlet extraction oils of Port-Orford cedar

Two more peaks were identified at less retention times (13.406 min. (cadala-1(10),3,8-triene) and 13.209 min. (Naphthalene)). Compared to .tau.-Cadinol ($C_{15}H_{26}O$) and .tau.-Muurolol ($C_{15}H_{26}O$) in SFE POC oil, cadala-1(10),3,8-triene ($C_{15}H_{22}$) is a result of

losing -OH and -H bonds of C₁₅H₂₆O structure, and Naphthalene (C₁₀H₈) is a result of further decomposition of these molecules. It indicates that the high temperature and long extraction time of SE could cause the decomposition of some of oil components (Pickett et al. 1975; Eller and King 2000).

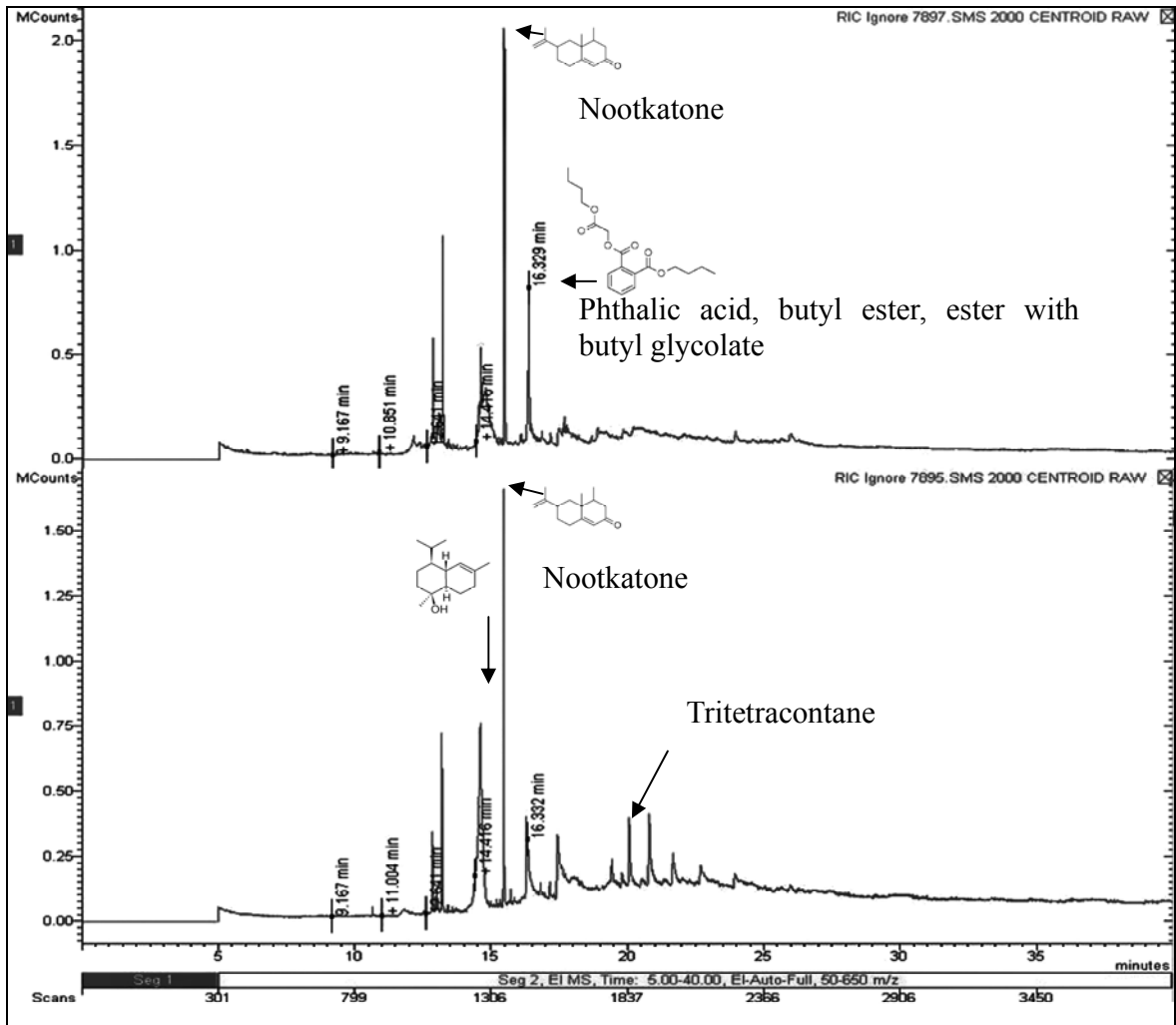
The peak areas of SE and SCC AYC oils spectrums are summarized in Table 3-3. The GC-MS spectrum of SFE AYC oil (Figure 3-3 top) shows the highest peak was at 15.441 min. (36.90%). This peak was indentified to be Nootkatone (C₁₅H₂₂O), which is thought to be the main antifungal component (Manter et al. 2006). The GC-MS spectrum of SE AYC oil (Figure 3-3 bottom) also had the Nootkatone peak at 15.465 min. (16.51%). Figure 3-3 shows that the retention time of the main peaks were similar to that of SFE AYC oils except that there were two more peaks in SE AYC oil spectrum. This implies that SE AYC oil contains more components such as C₄₃H₈₈. Nootkatone content in SE AYC oil was comparatively lower than in SFE AYC oil.

Table 3-3 Summary of uncorrected peak areas of GC-MS of Alaska yellow cedar oils

	Retention time (min.)	Component	Peak area (%)	Molecular formula
Supercritical fluid extraction	12.842	Isolongifolene	8.14%	C ₁₅ H ₂₆ O
	13.19	4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptan	17.07%	CH ₁₅ H ₂₂
	14.591	.gamma.-Gurjunenepoxide-(2)	5.18%	C ₁₅ H ₂₄ O
	15.441	Nootkatone	36.90%	C ₁₅ H ₂₂ O
	16.33	Phthalic acid, butyl ester, ester with butyl glycolate	17.29%	C ₁₈ H ₂₄ O ₆
Soxhlet extraction	13.211	4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptan	5.22%	CH ₁₅ H ₂₂
	14.611	.tau.-Cadinol	33.95%	C ₁₅ H ₂₆ O
	15.465	Nootkatone	16.51%	C ₁₅ H ₂₂ O
	16.307	Hexadecanoic acid	4.35%	C ₃₅ H ₆₈ O ₅
	17.45	Oleic Acid	6.99%	C ₁₈ H ₃₄ O ₆
	19.439	Dotriacontane	1.59%	C ₃₂ H ₆₆
	20.073	Tritetracontane	5.74%	C ₄₃ H ₈₈
	20.809	Tritetracontane	7.89%	C ₄₃ H ₈₈

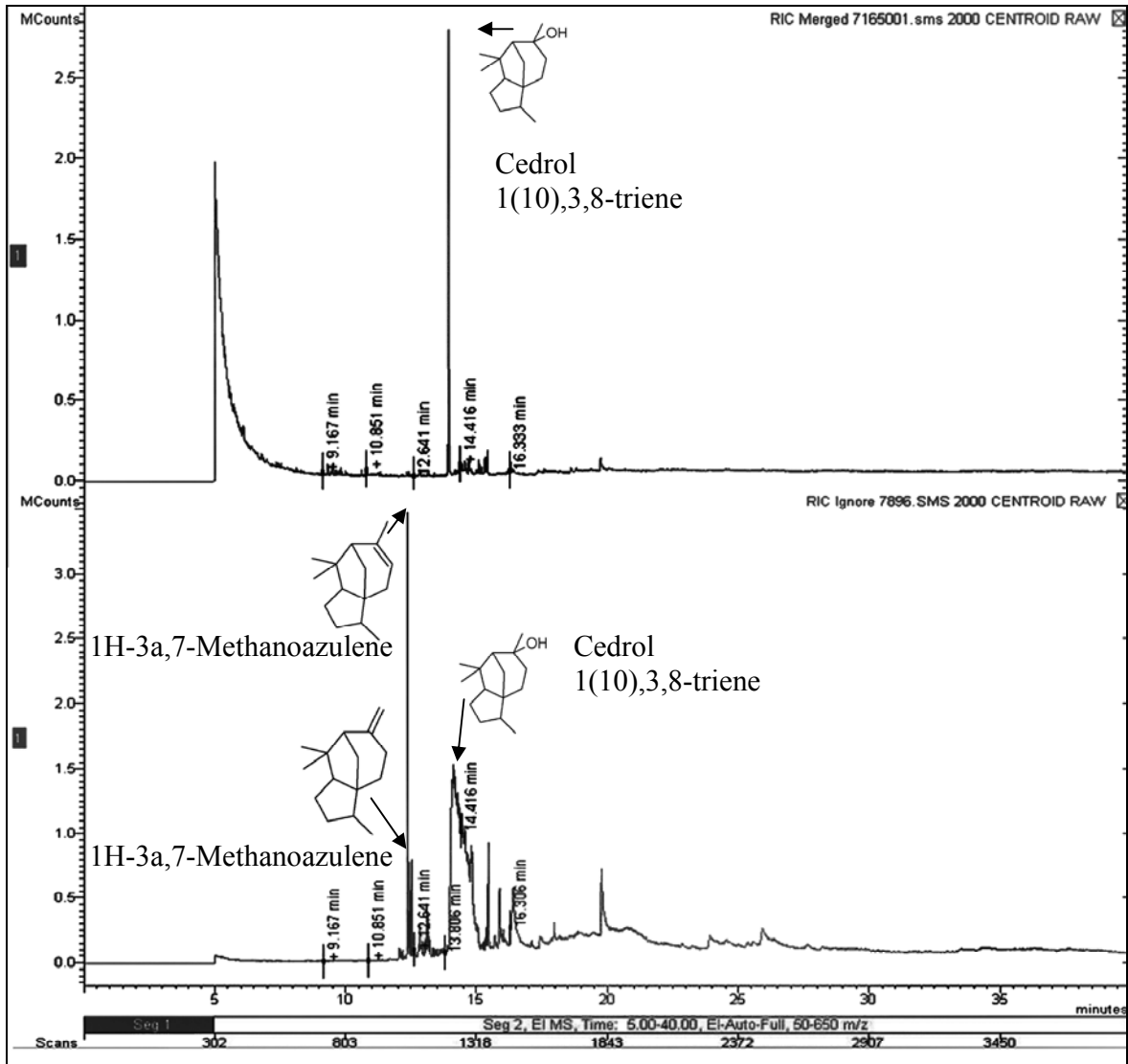
The GC-MS spectrum of SFE ERC oil (Figure 3-4 top) shows one main peak. The main component was identified as cedrol (13.992 min, 78.60%). The GC-MS spectrum of SE ERC oil (Figure 3-4 bottom) shows a broad peak around 13.992 min., which was difficult to separate. The broad peak was thought to be a combination of cedrol and its derivatives because of their similar retention time to that of cedrol.

As stated before, it is possible that the higher temperature and longer extraction time of SE causes transformation of cedrol to its derivatives. In addition, there were two more peaks which were thought to be 1H-3a,7-Methanoazulene at 12.361 min. (7.18%) and 1H-3a,7-Methanoazulene at 12.442 min. (1.57%). Figure 3-4 shows that



Top is the GC-MS spectrum of supercritical carbon dioxide extracted oil of Alaska yellow cedar;
 Bottom is the GC-MS spectrum of Soxhlet extracted oil of Alaska yellow cedar.

Figure 3-3 GC-MS spectrums of supercritical fluid extraction and Soxhlet extraction oils of Alaska yellow cedar



Top is the GC-MS spectrum of supercritical carbon dioxide extracted oil of Eastern red cedar;
 Bottom is the GC-MS spectrum of Soxhlet extracted oil of Eastern red cedar.

Figure 3-4 GC-MS spectrums of supercritical fluid extraction and Soxhlet extraction oils of Eastern red cedar

1H-3a,7-Methanoazulenes (C₁₀H₈) is a result of losing -H and -OH bonds of cedrol. It is possible that the SE conditions caused the cedrol to lose H₂O to generate 1H-3a,7-Methanoazulenes. The peak areas of SE and SCC ERC oils spectrums are summarized in Table 3-4.

Table 3-4 Summary of uncorrected peak areas of GC-MS of Eastern red cedar oils

	Retention time (min.)	Component	Peak area (%)	Molecule formula
Supercritical fluid extraction	13.992	Cedrol	78.60%	C ₁₅ H ₂₆ O
	15.476	2(3H)-Naphthalenone	6.21%	C ₁₅ H ₂₂ O
Soxhlet extraction	12.361	1H-3a,7-Methanoazulene	7.18%	C ₁₀ H ₈
	12.442	1H-3a,7-Methanoazulene	1.57%	C ₁₀ H ₈
	12.513	Thujopsene	1.50%	C ₁₅ H ₂₄
	14.109-14.824	broad peak could not be isolated	63.38%	C ₁₅ H ₂₆ O
	15.449	2(3H)-Naphthalenone	2.43%	C ₁₅ H ₂₂ O
	19.786	1,2-Benzenedicarboxylicacid,diisooctyl ester	2.45%	C ₂₄ H ₃₈ O ₄

3.3.3 Antifungal Activity Test against White-rot Fungi

Figure 3-5 shows the antifungal effects of different cedar oils against white-rot fungi. An image of the antifungal test using white-rot fungi is shown in Figure 3-6. All of these oils showed different degrees of inhibition on the growth of white-rot fungi. One week was required for the white-rot fungi growth to reach the edges of the control dishes.

The Duncan's multiple range comparisons for species and methods indicated that the AI of SFE extracted oils was significantly higher than that of SE extracted oils (Table 3-5), suggesting that SFE extracted cedar oils (group A) has better antifungal activities than that of SE extracted oils (group B) against white-rot fungi. One reason is that SE decomposes some bioactive components, which is supported by previous GC-MS analysis. SFE AYC oil (group A) showed the strongest white-rot fungi resistance, followed by SFE POC oil (group B), and SFE ERC oil (group C). AYC oils have Nootkatone, which has been reported to have strong antifungal activity (Manter et al. 2006). Nootkatone content in SFE AYC oil was higher than SE AYC oil, leading to stronger SFE AYC oil antifungal activities. One of the most interesting results of the study was the extremely effective antifungal activities of AYC and POC as compared to that of ERC, particularly AYC. As shown in the evaluation of AI, a characteristic of the antifungal activities is most AI was attained below the concentration of 0.25 mg/mL. The AI data within the ranges of 0.25 mg/mL were therefore rectified to use the natural logarithm instead of the actual raw data to test by regression analysis using Eqn. 5.

$$AI = a \cdot \ln(c) + b \quad (5)$$

The $\ln(c)$ is the natural logarithm of the concentration.

Table 3-6 is a summary of linear regression of AI and natural logarithm of concentration. Based on the regression line, IC₅₀ is calculated when AI is 50%. The IC₅₀ of SFE POC and SFE AYC oils were estimated to be 0.1648 mg/mL and 0.0704 mg/mL, respectively. The IC₅₀ of SE POC and SE AYC were estimated to be 0.2034 mg/mL and 0.1514 mg/mL, respectively. Because antifungal properties of ERC oils are weak, the SE oil concentration was out of the

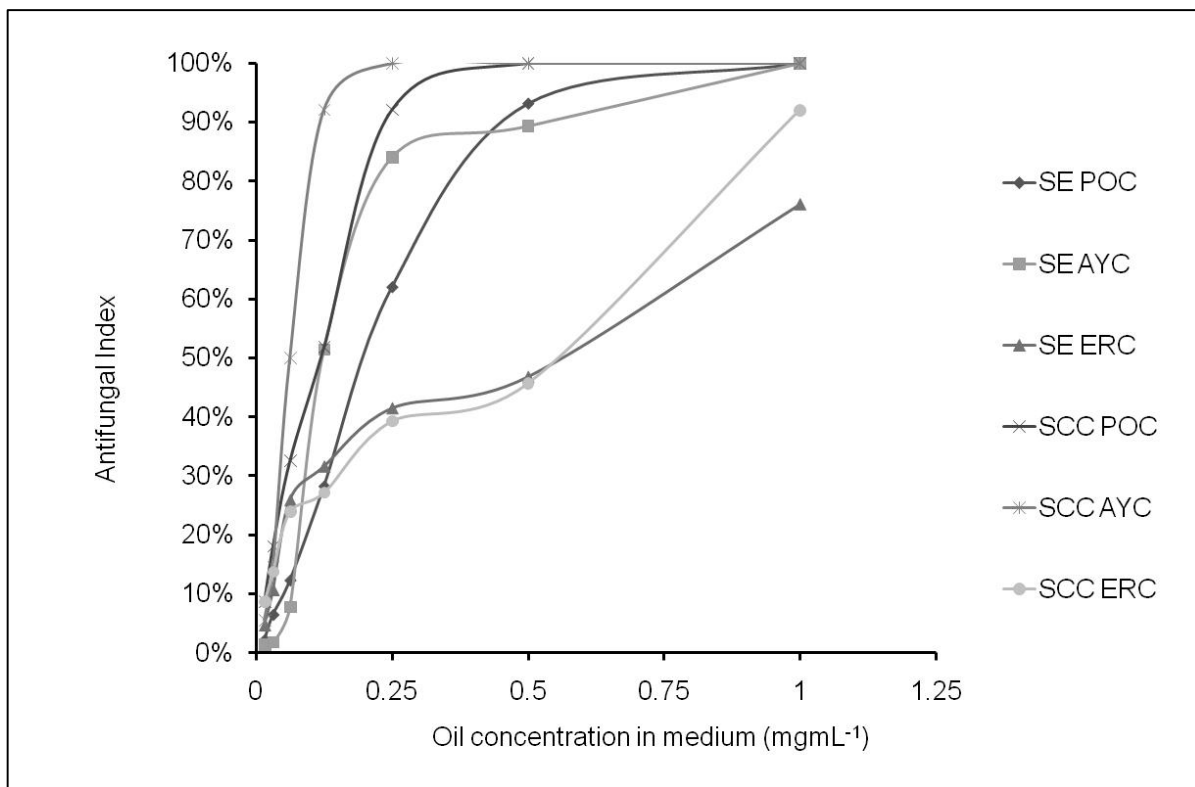
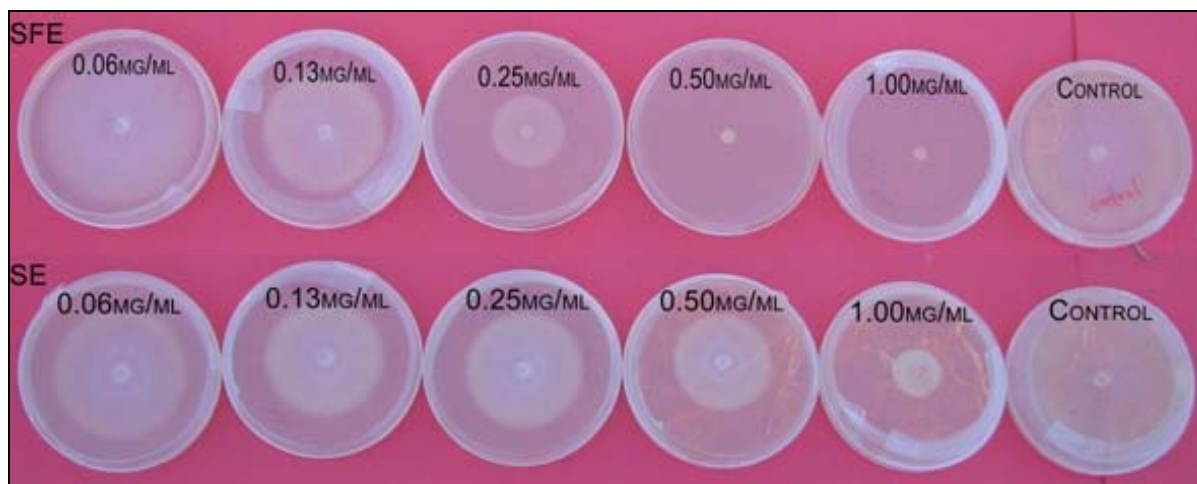


Figure 3-5 Antifungal indexes of three cedar oils against white-rot fungi



The top row is SFE extracted samples, and the bottom row is SE extracted samples
 Figure 3-6 Sample image of antifungal test against white-rot fungi

Table 3-5 Summary of Duncan's multiple range test of the effect of extraction methods and species on the antifungal index for white-rot fungi

		Number of sample	Mean of antifungal index	Duncan grouping*
Methods	Supercritical fluid extraction	45	0.67830	A
	Soxhlet extraction	45	0.56669	B
Species	Alaska yellow cedar	30	0.77453	A
	Port-Orford cedar	30	0.66036	B
	Eastern red cedar	30	0.43260	C

**Means with the same letter are not significantly different at alpha=0.05*

Table 3-6 Summary of linear regression for white-rot fungi

Group	Equation	R-square	IC₅₀
Supercritical fluid extraction of Alaska yellow cedar	$y = 8.129x - 0.0730$	0.9865	0.0704
Soxhlet extraction of Alaska yellow cedar	$y = 3.8081x - 0.0767$	0.9582	0.1514
Supercritical fluid extraction of Port-Orford cedar	$y = 3.4604x + 0.0704$	0.9923	0.1648
Soxhlet extraction of Port-Orford cedar	$y = 2.5528x - 0.0253$	0.998	0.2034
Supercritical fluid extraction of Eastern red cedar	$y = 1.2015x + 0.1088$	0.9073	
Soxhlet extraction of Eastern red cedar	$y = 1.4507x + 0.0883$	0.8385	

regression range when the AI is 50%. IC_{50} of ERC oils could not be determined from the regression.

3.3.4 Antifungal Activity Test against Brown-rot Fungi

Figure 3-7 shows the antifungal effect of different cedar oils against brown-rot fungi. An image of antifungal test using brown-rot fungi is shown in Figure 3-8. All these oils also showed a different degree of inhibition to the growth of brown-rot fungi. It took 2 weeks for the brown-rot fungi growth to reach the edges of the control dishes. The statistical analysis (Table 3-7) also showed that AYC (group A) had the highest AI against brown-rot fungi, followed by POC (B), and then ERC (group C). The Duncan's multiple comparisons also indicated that the mean AI of SFE oils was significantly higher than hexane SE oils, suggesting SFE cedar oils had better antifungal activities than SE oils against brown-rot fungi. Again, one possible reason for this is the differences in SE and SFE. Table 3-8 is a summary of linear regression of AI and natural logarithm of concentration for brown-rot fungi. Based on the regression line, IC_{50} was calculated when AI is 50%. The IC_{50} of SFE POC and SFE AYC were estimated to be 0.2443 and 0.0613 $mgmL^{-1}$, respectively. The IC_{50} of AYC was estimated to be 0.1018 $mgmL^{-1}$. Because the inhibition of SFE ERC oil, SE ERC oil and SE POC oil was too weak, the concentrations of these oils were out of the regression range when AI was 50%. The IC_{50} of SFE ERC oil, SE ERC oil, and SE POC oil could not be determined from the regression.

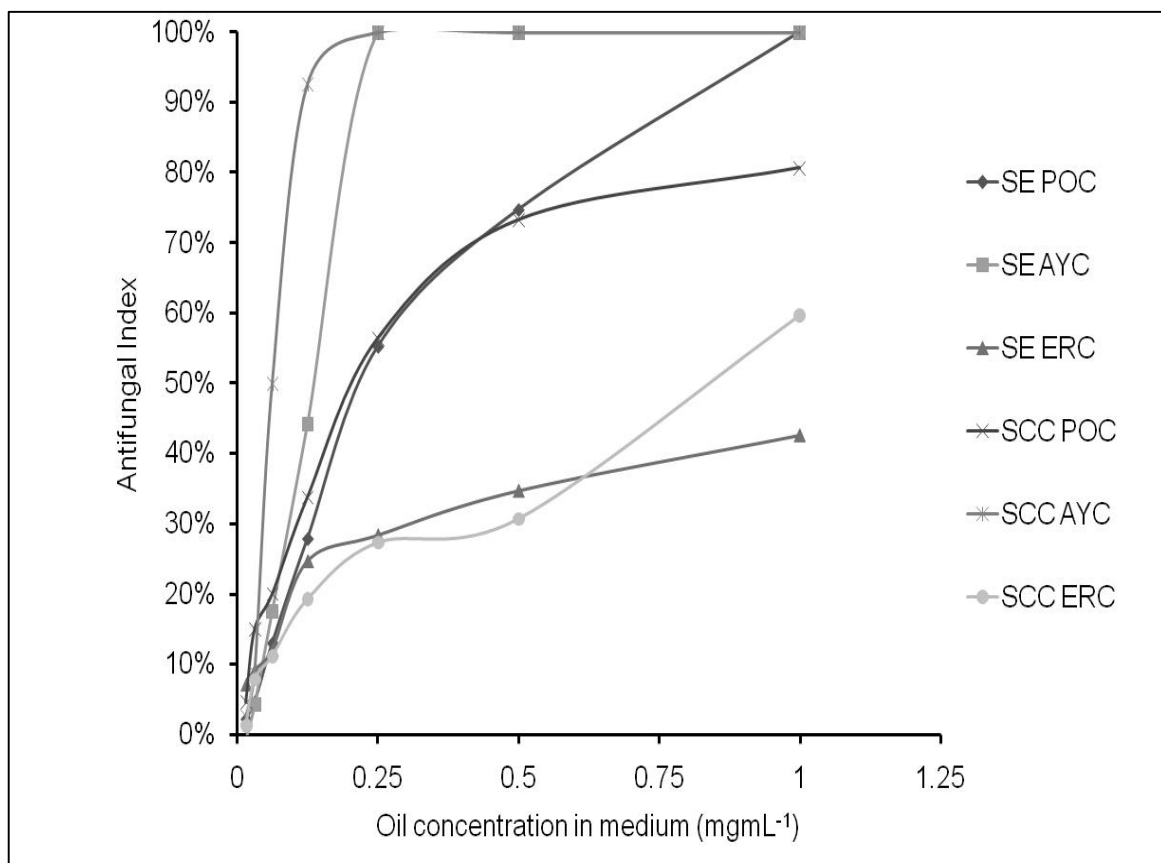
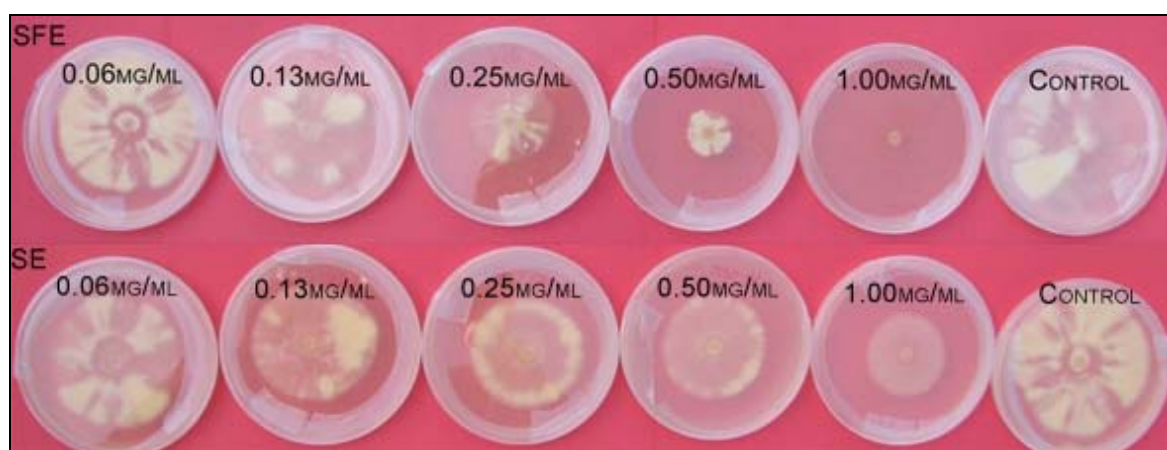


Figure 3-7 Antifungal indexes of three cedar oils against brown-rot fungi



The top row is SFE extracted samples, and the bottom row is SE extracted samples
 Figure 3-8 Sample image of antifungal test against brown-rot fungi

Table 3-7 Summary of Duncan's multiple range test of the effect of extraction methods and species on antifungal index for brown-rot fungi

		Number of samples	Mean of antifungal index	Duncan grouping*
Methods	Supercritical fluid extraction	41	0.62344	A
	Soxhlet extraction	45	0.49722	B
Species	Alaska yellow cedar	30	0.81921	A
	Port-Orford cedar	30	0.51781	B
	Eastern red cedar	26	0.30100	C

*Means with the same letter are not significantly different at $\alpha=0.05$

Table 3-8 Summary of linear regression for brown-rot fungi

Group	Equation	R-square	IC ₅₀ *
Supercritical fluid extraction of Alaska yellow cedar	$y = 0.4021x + 1.6226$	0.9400	0.0613
Soxhlet extraction of Alaska yellow cedar	$y = 0.3485x + 1.2961$	0.8547	0.1018
Supercritical fluid extraction of Port-Orford cedar	$y = 0.1764x + 0.7486$	0.9362	0.2443
Soxhlet extraction of Port-Orford cedar	$y = 0.1867x + 0.7228$	0.8782	
Supercritical fluid extraction of Eastern red cedar	$y = 0.0917x + 0.3882$	0.9814	
Soxhlet extraction of Eastern red cedar	$y = 0.0836x + 0.3942$	0.9112	

*IC₅₀ is considered as the oil concentration in medium when AI is 50%

3.4 Summary

It was found that supercritical fluid extraction was more efficient than Soxhlet extraction for yield and extraction time. The SFE extracts composition was similar to SE extracts but contained fewer minor components. SE extraction can cause the decomposition of cedar oil components. The SFE cedar oils showed stronger antifungal activity than SE cedar oils against both white-rot and brown-rot fungi. Supercritical carbon dioxide extracted POC oil and supercritical carbon dioxide extracted AYC oil showed strong antifungal activities *in vitro*, which is encouraging for further study. The wood-derived oils that were evaluated in this study have potential for developing environment-friendly wood preservatives.

3.5 References

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Chapter 4. Chinese Tallow Tree Seed Oil Extraction and Related Analysis

4.1 Introduction and Literature Review

Chinese tallow tree (*Sapium sebiferum* (L.) Roxb. syn. *Triadica sebifera* (L.) Small) is an invasive tree species in the southeastern United States. Previous research of Chinese tallow tree has focused on the recovery of the wax/oil for the manufacture of products such as soap and candles (Xu et al. 1991). More recently, various compounds, some with biological activity, have been isolated from the roots, bark, and leaves (Liu et al. 1988; Yang and Kinghorn 1985). Besides being a source of chemicals, this fast-growing tree has also been evaluated as a biomass resource. Some studies showed Chinese tallow tree can produce four times as much biomass as fast-growing poplars grown in northern climates (Scheld and Cowles 1981). The potential of Chinese tallow tree (wood/bark, leaves, and seeds) as a raw material for bio-based chemical and energy production using hydrothermal conversion was studied by Shupe and Catallo (2006), which found that the energy values of the tallow seeds are much higher than those typically reported for hardwood stemwood. Spectrophotometric and chromatographic investigations of Chinese tallow tree seed oil was studied by Huang et al. (1949). And the optical rotation property was found (Huang et al. 1949). The isolation and identification of the optically active compound was studied later (Sprecher et al. 1965).

The fatty acids component of the oil was studied by Narang and Sadgopal 1958. However, the glycerides structure of Chinese tallow tree seed oil was not indicated in this paper. This was done by Christie (1969). Large amount of unsaturated fatty acid was found in the seed. There were considerable amounts of linoleic and linolenic acids which were in greatest abundance in the 2- and 3-positions, respectively. (Christie 1969). Recent research showed that Chinese tallow tree seed has high content of oil/wax (43.1%) (Eberhardt et al. 2007). There is no published report on supercritical fluid extraction of Chinese tallow tree oil. The objectives of this research were to study the application of supercritical fluid extraction in tree seed oil and the modifier's effect on the supercritical carbon dioxide extraction.

4.2 Materials and Methods

4.2.1 Materials and Specimen Preparation

The tallow tree seeds were collected from Pineville, LA of date on (1) Sep 25th and date (2) Aug 8th. The seeds were freeze dried and then separately ground with a coffee grinder. The ground seeds were stored in zip-lock bags in a freezer (-4 °C) until extraction. GC grade methanol (MeOH) was use as the modifier.

4.2.2 Supercritical Fluid Extraction

SFE were conducted with an Applied Separations Spe-ed SFE model 7070 apparatus (Applied Separations Inc., Allentown, PA). About 2 g (0.0001g) samples were weighed and added to a 10 mL stainless steel extraction vessel sealed with polypropylene wool at the top and bottom of the extraction vessel. The extraction flow rate was set as 1.5 L/min. (expanded gas) with a restrictor of 100 °C using industrial grade CO₂. The extracted oils were collected with 60 mL vials (neat collection). The extraction was performed by dynamic extraction until the vial weight no longer increased (about 60 min.). Chinese tallow seeds of different collecting dates were extracted at 40 °C, 300 bar.

Additionally, 5% and 10% methanol were added to the supercritical CO₂ as modifier to change its polarity for tallow seed of date (1). The vials were weighed before and after the extraction to get the weight of extracted oil, and SCC extraction yields were calculated based on Eqn. 1. Each extraction was replicated three times.

4.2.3 Gas Chromatography-Mass Spectrometry (GC-MS)

The extracted oils were tested by a Varian GC-MS. The sample was dissolved in hexane and introduced into the ion trap mass spectrometer. The GC-MS device was a Varian system with a DB-5 column. The carrier gas was helium. The initial oven temperature was maintained at 40 °C for 3 min. The temperature was then increased from 40 °C to 280 °C at a rate of 15 °C/min. The final oven temperature was 280 °C and was held for 21 min. The injector temperature was 250 °C, inject volume at 1 microliter. The MSD (mass selective detector) conditions were EI (electron impact ionization) of 70 eV and room temperature. The spectrum was compared with standard spectrum library (NIST) to get possible molecular structures.

4.3 Results and Discussion

4.3.1 Different Collecting Date's Effect on SFE Yield

The extraction yield of tallow seed of date (1) was 30.74%. However, the extraction yield of tallow seed of date (2) was 12.60% (Figure 4-1). This is because the tallow seed of date (2), Aug 8th, was not ripe and the seed was still growing. The oil in the seed was not fully developed. This indicates that the seed collecting time significantly affects the oil content in tallow seed. Although, the extraction yield of tallow seed of date (1) was higher, it may not be the best collection time to get the maximum extraction yield. Higher content of oil/wax (43.1%) of Chinese tallow seed has been obtained. (Eberhardt et al. 2007). The purpose of this experiment was to show the effect of collecting time on extraction yield.

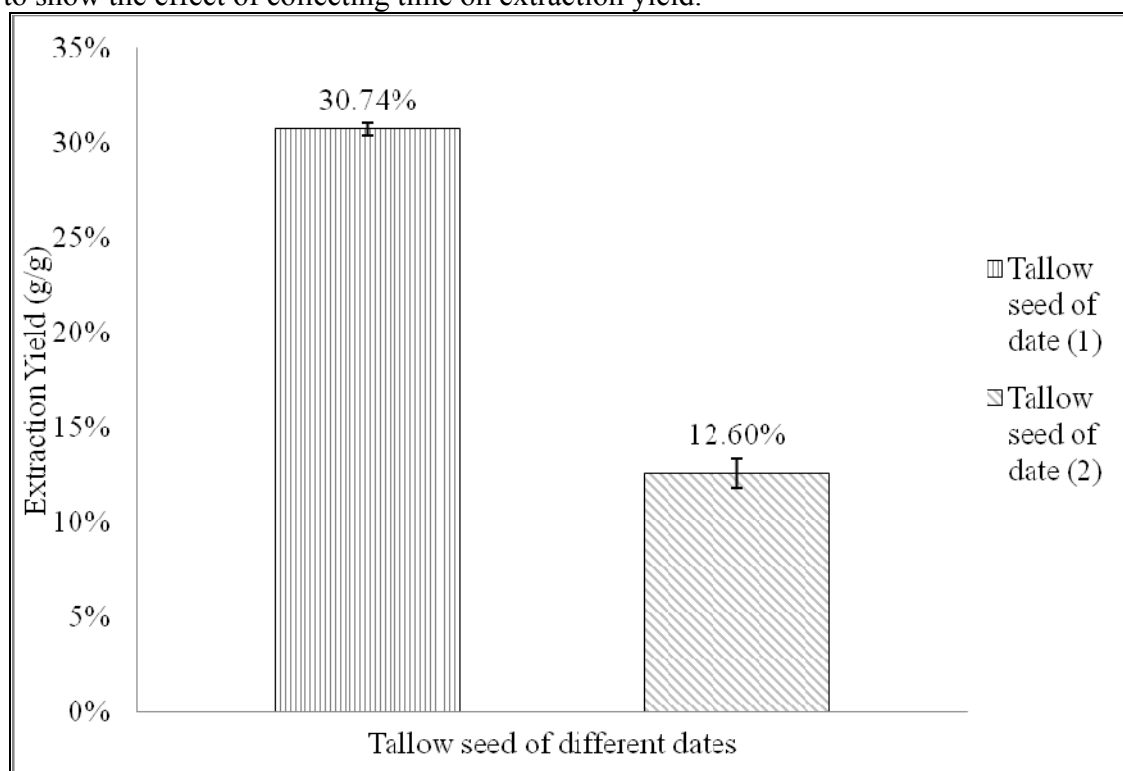


Figure 4-1 Extraction yields of Chinese tallow seeds of different dates

4.3.2 Modifier's Effect on SFE Yield

Figure (4-2) shows the modifier's effect on extraction yield of tallow seed of date (1). The extraction yield with 10% MeOH was higher than those with 5% MeOH or without MeOH. With the modifier, the polarity of supercritical carbon dioxide is modified. When MeOH was

added into the SCC, its polarity increased. Hence, some higher polarity molecules were also extracted. In addition, the extraction yield was also higher.

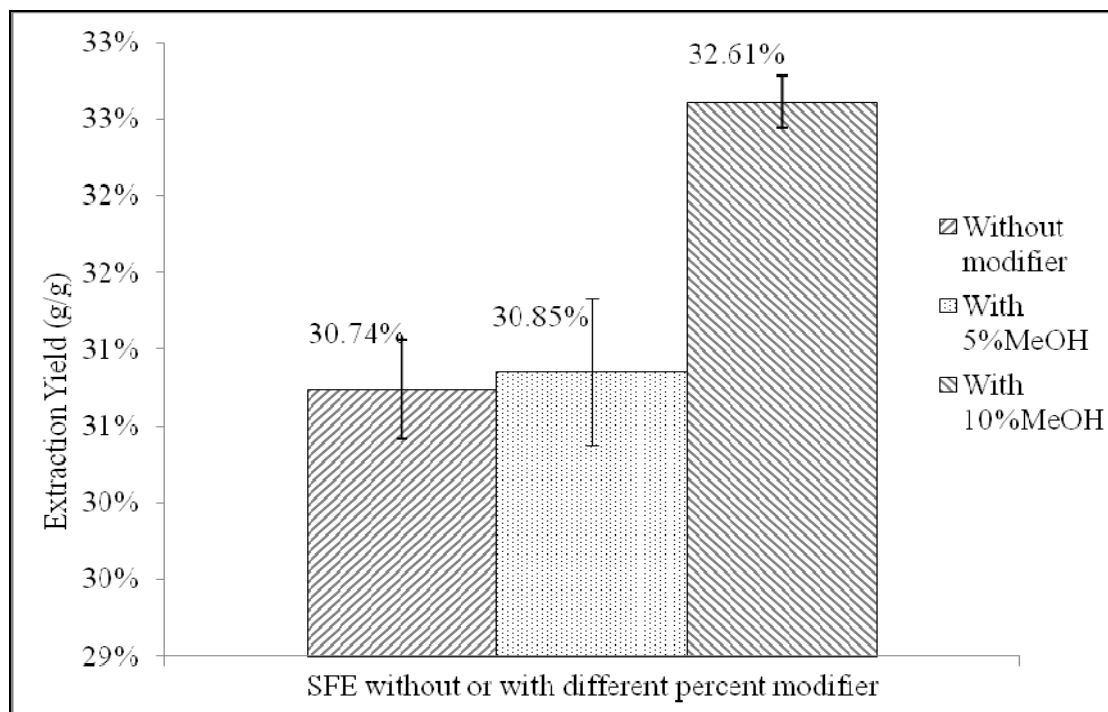


Figure 4-2 Modifier's effect on supercritical fluid extraction yields

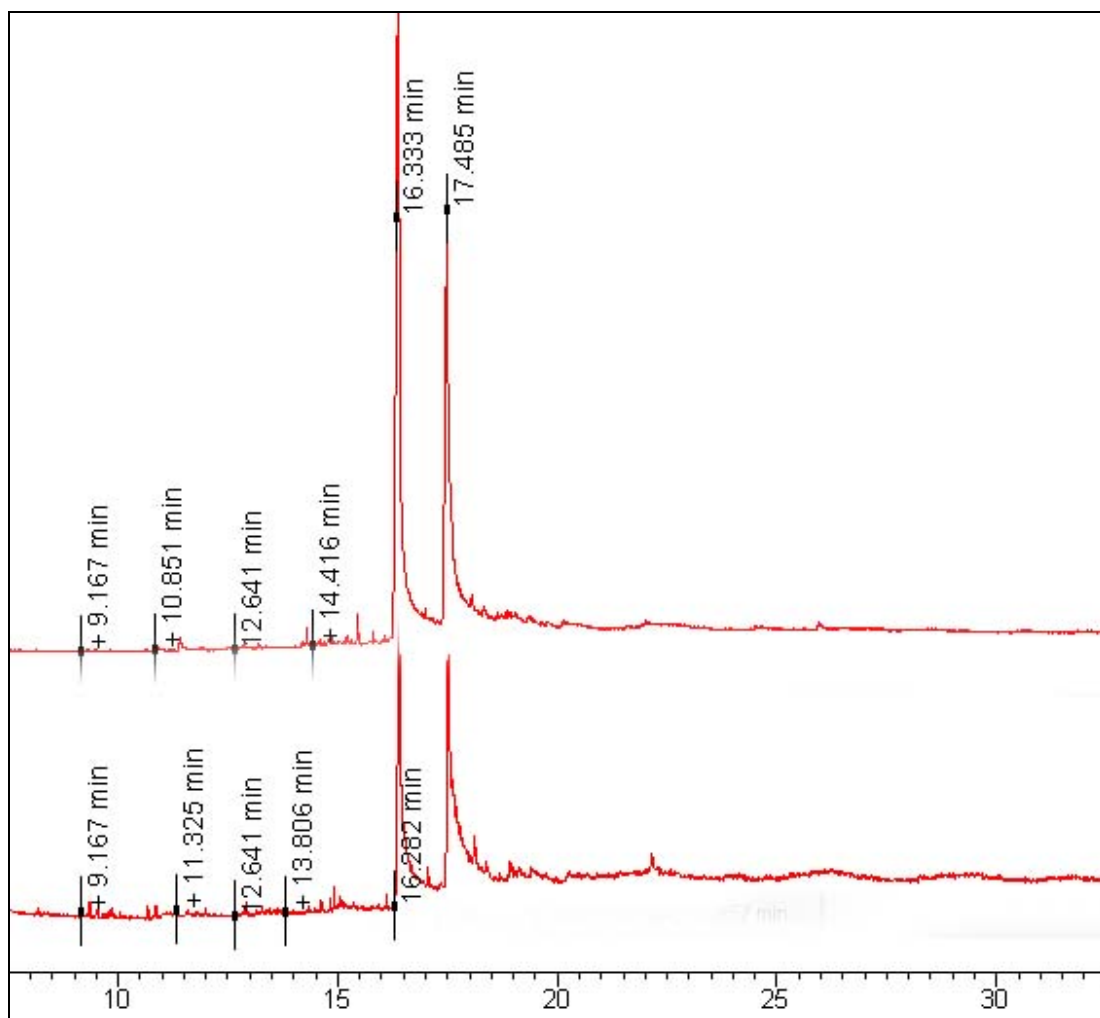
4.3.3 Gas Chromatography-Mass Spectrometry (GC-MS)

Figure 4-3 shows the GC-MS spectrums of tallow seeds of Sep 25th and Aug 8th, which were extracted at 40 °C, 300 bar without a modifier. The main peaks' retention times of these two spectrums were almost the same but the peak heights were different, which indicates that the main components of the different Chinese tallow seed were similar but the content was different. This is supported by the extraction yield analysis 4.3.1. In addition, the weak small peaks had many differences, which implies the minor components are different. Of different collecting times, the chemical composition of tallow seed is different.

Figure 4-4 shows the GC-MS spectrums of tallow seed of date (1) extracted with and without a modifier. These three extractions were operated at 40 °C, 300 bar. From the spectrums, there is one more peak for the 10% MeOH extracted tallow oil and 5% MeOH extracted tallow oil around 19.8 min. After comparison with standard GC-MS library, this peak was thought to be 1,2-Benzenedicarboxylic acid, diisooctyl ester. It indicates that SCC with MeOH as a modifier can extract more components from tallow seed. In addition, the 10% MeOH extracted tallow oil (Figure 4-4 top) has one more peak at 16.09 min., which was thought to be Hexadecanoic acid, methyl ester, than 5% MeOH extracted tallow oil. This implies that with increasing content of MeOH in SCC, the polarity of SCC increases and more polar chemicals can be extracted.

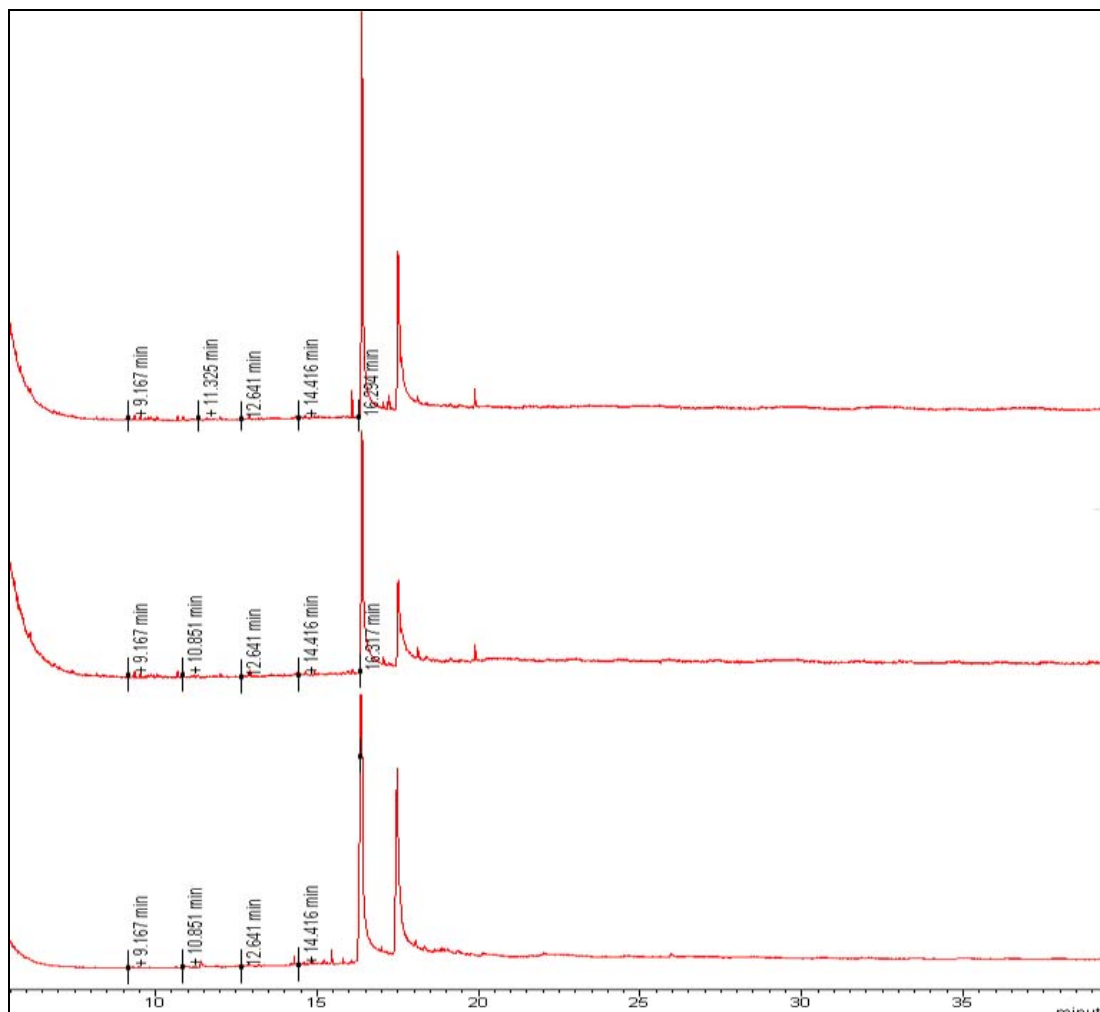
4.4 Summary

Chinese tallow tree seed oil is an import renewable source of oil. The collection time can significantly affect the seed oil content. To optimize collocation time, further study is needed. A modifier, methanol, can increase the polarity of supercritical carbon dioxide and thus increase the extraction yield, and some molecules with more polarity will be extracted.



Top spectrum is from Chinese tallow seed of date (1); bottom spectrum is from Chinese tallow seed of date (2).

Figure 4-3 GC spectra of Chinese tallow seeds of Sep 25th and Aug 8th



The top spectrum is with 10% MeOH; the middle spectrum is with 5% MeOH; the bottom is without MeOH.

Figure 4-4 Modifier's effect on GC spectrums of supercritical fluid extraction Chinese tallow seed oil

4.5 References

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Chapter 5. Conclusions

There are several conclusions that can be derived from this study:

From Chapter 2, it is concluded that the sample particle size of oxeye daisy (*Chrysanthemum leucanthemum* L.) seed affects the extraction yields. The extraction temperature and extraction pressure significantly affects the extraction rate. The microwave assisted extraction and supercritical fluid extraction oxeye daisy showed stronger antioxidant ability than Soxhlet extracted oxeye daisy oil. The oxeye daisy oil could block ultraviolet wave. The antioxidant property and ultraviolet wave block property give the oxeye daisy seed oil a potential to be sun blocker additive oil. The fatty acid profile of oxeye daisy oil is composed of unsaturated 18 carbon chain and saturated 10 carbon chain.

From Chapter 3, it is concluded that supercritical fluid extraction of three cedar oils was more efficient than Soxhlet extraction for yield and extraction time. Soxhlet extraction can cause decomposition of cedar oil components. Statistical analysis showed that supercritical fluid extracted cedar oils showed stronger antifungal activities than Soxhlet extracted cedar oils against both brown-rot and white-rot fungi. Supercritical carbon dioxide extracted Port-Orford cedar (*Chamaecyparis lawsoniana*) oil and supercritical carbon dioxide extracted Alaska yellow cedar (AYC) (*Chamaecyparis nootkatensis*) oil showed strong antifungal activities *in vitro*, which is encouraging for further study. The wood-derived oils that were evaluated in this study have potential for developing environment-friendly wood preservatives.

From Chapter 4, it is concluded that the collecting time can significantly affect the Chinese tallow seed oil content. To get optimized collecting time, further study is needed. The modifier, methanol, can increase the polarity of supercritical carbon dioxide. If some polar components are to be extracted, methanol can be used as a modifier.

Supercritical fluid extraction is an effective method to extract oil from the heartwood of trees and seeds. It has several advantages over Soxhlet extraction. It can be applied to many natural products extraction.

Vita

The author was born in Dandong, China, in January, 1984. He earned the degree of Bachelor of Chemical Engineering of Forest Products from the Department of Material Science and Engineering at Northeast Forestry University in 2006. The author then joined the wood science program at Louisiana State University as a master student of science under the supervision of Dr. Todd F. Shupe in August, 2007. The author's study focused on supercritical fluid extraction of plant oils.