Value-addition of cold pressed hemp seed oil and oil by-products through ultrasonic bleaching and heat treatment: Evaluation of chlorophyll, oxidative stability and antioxidant activity

By

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ABSTRACT

Cold pressed Hemp (*Cannabis sativa L*) seed oil, which contains a favorable ratio of omega-6/omega-3 and a considerable amount of vitamin E, is a high nutrition value product with various health benefits. However, the presence of a large amount of chlorophyll makes the oil highly susceptible to photo-oxidation and limits its applications. Chlorophyll and other pigments in edible oils are commonly reduced through oil bleaching process. The objective of this study was to reduce the chlorophyll content in cold pressed hemp seed oil using ultrasonic bleaching and evaluate the impacts on carotene content, peroxide value, conjugated diene, total phenolic content and oxidative stability. The bleaching efficiency increased significantly as the ultrasound power (0 to 60% pressure amplitude) and concentration of bleaching clay (20 to 40 g/kg) increased. At 20% ultrasound power, the chlorophyll reduction (%) of different clays was found to be 99.4% (industrial clay) > 97.8% (activated bentonite) > 82.7% (sepiolite) > 47.1% (non-activated bentonite). Ultrasonic bleaching significantly reduced (p<0.05) the total chlorophyll content, total carotene content and primary oxidation products of hemp seed oil. During accelerated oxidation tests (40 and 60 °C), the developments of peroxide value and conjugated diene were significantly (p<0.05) slower for hemp seed oil treated with ultrasonic bleaching compared to the control oil. Enhanced oxidative stability was observed in hemp seed oil after ultrasonic bleaching. In addition, another objective of this study was to evaluate the impacts of solvent and heat treatment on phenolic profile and antioxidant activity of cold pressed canola and hemp meals. For canola meal, a solvent-mixture of aqueous methanol (70%) and aqueous acetone (70%) in a ratio of 1:1 (v/v) extracted (p<0.05) slightly higher total phenolic content (11.3 mg SAE/g), while aqueous methanol (70%) extracts exhibited higher DPPH scavenging effect (40.4%). For hemp meal, aqueous acetone (80%) extracts exhibited higher total phenolic content (6.0 mg GAE/g) and DPPH scavenging effect (12.5%). Total phenolic content and antioxidant activity

in canola meal extracts were significantly higher (p<0.05) than those in hemp meal extracts. After heat treatments at temperature from 140 to 180 ℃, canola and hemp meal extracts showed equal or lower total phenolic content and DPPH scavenging effect.

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LIST OF ABBREVIATIONS

ABT20%: Hemp seed oil treated with Activated Bentonite and 20% Ultrasound Power

ANOVA: Analysis of Variance

ALA: α-linolenic acids

ASE: Accelerated solvent extraction

CD: Conjugated Diene

GAE: Gallic acid equivalent

HPLC-DAD: High performance liquid chromatography-Diode array detector

IC20%: Hemp seed oil treated with Industrial clay and 20% Ultrasound Power

LA: linoleic acids

PUFA: polyunsaturated fatty acid

PV: Peroxide Value

RT: Room Temperature.

SAE: Sinapic acid equivalent

SP20%: Hemp seed oil treated with Sepiolite and 20% Ultrasound Power

TAG: triacylglycerols

THC: delta-9-tetrahydrocannabinol

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CHAPTER 1

1. GENERAL INTRODUCTION

1.1 Introduction

Industrial hemp, a plant species of *Cannabis sativa L*, has been a versatile agricultural crop grown for fiber, building materials, biofuel, cosmetics, food and animal feed in Canada since the 18th century (Callaway, 2004a; Small, 2015). Although hemp belongs to the same species as marijuana, the psychoactive chemical delta-9-tetrahydrocannabinol (THC) is less than 0.3% according to Health Canada and products made from industrial hemp have no psychoactive effects when consumed. The seeds of *Finola* cultivar, a variety of industrial hemp, have over 36% of oil content (Callaway & Pate, 2009). Hemp seed oil typically has over 80% of polyunsaturated fatty acid (PUFA) with a desirable ratio (3:1) of linoleic acids (LA) and α-linolenic acids (ALA). Recently, the potential health benefits of hemp seed oil have become particular interest. For example, the effects on lowering cholesterol level and controlling high blood pressure were investigated in several studies (De Petrocellis, Melck, Bisogno, & Di Marzo, 2000; Kunos et al., 2000; Sapino, Carlotti, Peira, & Gallarate, 2005). Besides, hemp seed oil is also used to manufacture body care products for skin problems such as dry skin, topical eczema, acne and atopic dermatitis (Callaway et al., 2005; Sapino et al., 2005).

Hemp seed oil is commonly extracted mechanically by screw press or cold press, which usually operates at a low temperature below 50 ℃ without the use of chemicals. This oil pressing technique provides many advantages, including superior flavor and aroma (Matthäus & Spener, 2008; Morar et al., 2010). In addition, the cold press technique extracts and preserves a large amount of bioactive minor components, most of which are potent antioxidants that inhibit oil oxidation as well as reduce oxidative stress in the human body (Velioglu, Mazza, Gao, & Oomah, 1998). For cold pressed hemp seed oil, most of the health

benefits are attributed to its unique bioactive minor components, such as phytosterols, polyphenols, tocopherols, carotenoids and other vitamins and dietary minerals (Matthäus & Brühl, 2008).

However, there are some undesirable minor components retained in the oil during cold pressing. These minor components lower the oil quality and stability through affecting the color, flavor, aroma, clarity and nutritional value of the oil. Chlorophyll, one of the undesirable minor components in hemp seed oil, varies the oil color from dark to light green. The presence of large amounts of chlorophyll also causes the poor stability of hemp seed oil. Furthermore, chlorophyll and its derivatives are photosensitizer as well as strong prooxidants, which can induce the quick oxidation of PUFAs and consequently reduce the shelf-life of hemp seed oil (Callaway, 2004b; Ghazani & Marangoni, 2013; Matthäus & Brühl, 2008). Conventional refining and bleaching processes are typically utilized to reduce the pigments from the crude vegetable oils; however, the prolong treatment time and excessive use of bleaching clay result in other problems, including more oil losses, higher production costs and intensifying oxidation during processing (Gunawan et al., 2010; Hussin, Aroua, & Daud, 2011). Therefore, it is necessary to develop an effective and sustainable technique for reducing the pigments from cold pressed hemp seed oil.

Phenolic compounds are one of the desirable minor components existing in most of vegetable oils impacting antioxidant and antimicrobial activities (Nowak, Kujawa, Zadernowski, Roczniak, & KozŁowska, 1992). Phenolic acids, namely *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, ferulic acid and sinapic acid, were found in hemp seed oil extracts (Siger, Nogala-Kalucka, & Lampart-Szczapa, 2008).

Canola (*Brassic napus*) oil, the most consumed vegetable oil in Canada, is a rich source of phenolic compounds such as sinapic acid, sinapine, 1-O-β-D-glucopyranosyl sinapate and 4-vinylsyringol (Koski, Pekkarinen, Hopia, Wähälä, & Heinonen, 2003; Thiyam, Kuhlmann,

Stöckmann, & Schwarz, 2004).

However, the content of phenolic compounds decreases significantly during the oil refining processes. On the other hand, a large portion of phenolic compounds remains in the oil pressing by-products, namely hemp and canola meals. In addition, previous studies reported that the high temperature generated by seed roasting and oil pressing caused the decarboxylation of endogenous phenolic acids and formed the active antioxidant component (Koski et al., 2003; Siger, Kaczmarek, & Rudzińska, 2015; Zago et al., 2015). Therefore, there is a strong interest in extracting these phenolic compounds from canola and hemp meals and using as natural antioxidants for functional food and non-food applications.

1.2 Major Objectives

The long term objective of this research was to reduce the chlorophyll and other pigments in cold pressed hemp seed oil using ultrasonic bleaching and evaluate the impacts on oil quality and oxidative stability. Another objective was to determine and compare the content and antioxidant activity of phenolic compounds from cold pressed canola and hemp meals. The specific objectives are indicated below:

1. To reduce the chlorophyll from cold pressed hemp seed oil by combined use of ultrasound and bleaching clays.
2. To evaluate the impact of ultrasonic bleaching on chlorophyll and carotene content, total phenolic content, peroxide value, conjugated diene and oxidative stability of cold pressed hemp seed oil.
3. To evaluate the impact of solvent on extraction of phenolic compounds from cold pressed canola and hemp meals.
4. To evaluate the impact of heat treatment on phenolic profiles and antioxidant activities of cold pressed canola and hemp meals.

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CHAPTER 2

2. LITERATURE REVIEW

2.1 MANUSCRIPT 1: Hemp Seed Oil: Minor Components and Oil Quality

2.1.1 Summary

Owing to its high content of omega-6/omega-3 fatty acids and bioactive minor components with antioxidant activities, hemp seed oil is now recognized for its health benefits by a large number of consumers. This paper primarily discusses the profile of minor components in hemp seed oil and their beneficial and adverse effects on oil quality. While tocopherols, polyphenols and phytosterols prevent oxidative deterioration of hemp seed oil, the high amount of chlorophyll can be detrimental to oil quality.

Key words: Hemp seed oil, free fatty acids, chlorophyll, carotenoids, tocopherol, polyphenols

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2.1.2 Introduction

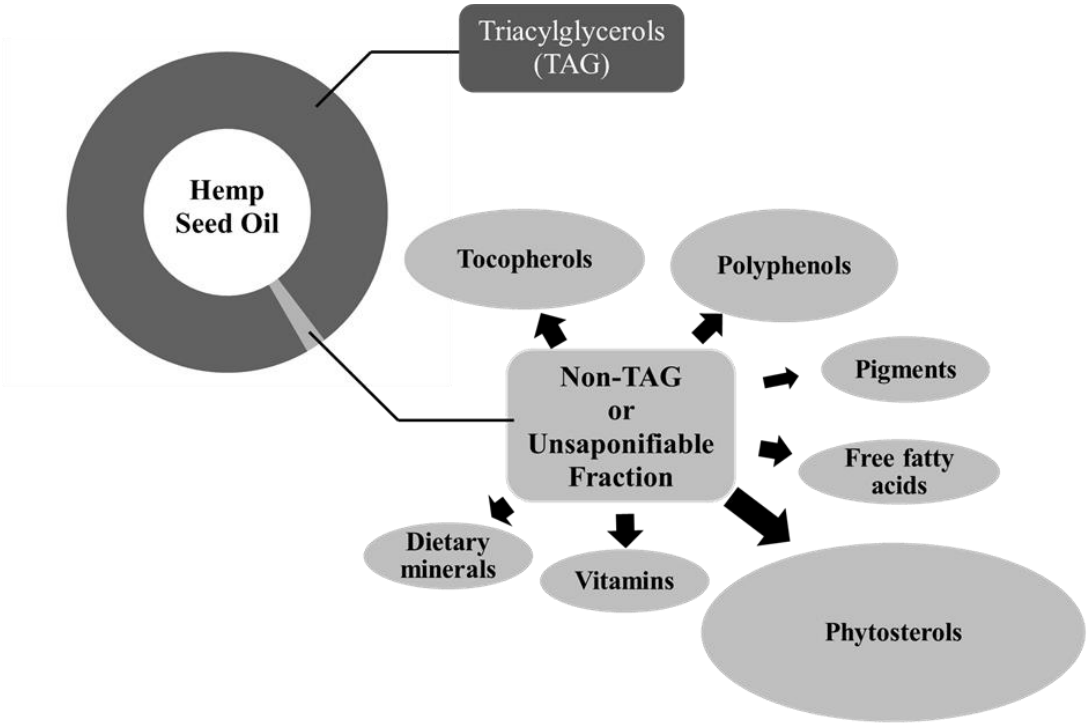
Hemp (*Cannabis sativa L.*), one of the oldest versatile plants, has been cultivated as a source of food, dietary oil, fibre and medicine for thousands of years in Europe and China. Since the last century, hemp has become a valuable industrial crop for production of fibre for clothes and for animal feed in North America. Despite its versatility, the cultivation of hemp was prohibited in many Western countries since 1930s because of the presence of the phytochemical drug component δ-9-tetrahydrocannabinol (THC). After the establishment of a standard for THC in hemp in the late 1990s, hemp varieties with less than 0.3% THC were rediscovered and legally permitted for use as a food in Canada and United States.

Hemp oil is one of the major components that accounts for about 35% of the seed. It is an important product because of its nutritional and health benefits which include lowering of cholesterol and blood pressure and prevention of cardiovascular diseases and cancers (Matthäus & Brühl, 2008; Sapino, Carlotti, Peira, & Gallarate, 2005). In addition, the oil is also used in the pharmaceutical and cosmetic industries as a moisturizer to reduce the dryness of skin (Sapino et al., 2005).

Most of the nutritional and health benefits of hemp seed oil are attributed not only to its high content of polyunsaturated fatty acids (PUFAs), but also to the considerable amount of other bioactive minor components. In general, hemp seed oil consists mainly of triacylglycerols (TAG). The remainder accounts for approximately 1.5–2% non-TAG substances or unsaponifiable fraction and is an important source of minor components (Figure 2.1). Since hemp seed oil is produced by cold pressing, in which neither high temperature nor chemical treatment are used, a large amount of minor components are extracted into the oil. Some of these minor components such as tocopherols and polyphenols have strong antioxidant properties that protect the oil from oxidation as well as provide health benefits to humans. On the other hand, some minor components are undesirable as they lower

the oil quality by affecting color, flavor, clarity, nutritional value, and oxidative stability of the oil. Therefore, this review focuses on the composition and function of minor components, and their contribution to health and quality of hemp seed oil.

Figure 2.1 Endogenous minor components of hemp seed oil



2.1.3 Bioactive minor components of hemp seed oil

Tocopherols are widely known bioactive compounds present in hemp seed oil. Other minor components include polyphenols, carotenoids, phytosterols, other vitamins and dietary minerals that contribute to the nutritional value and quality of the oil.

2.1.3.1 Tocopherols and Tocotrienols

Four different isomers of tocopherols and tocotrienols are found in the oil, namely α-, β-, γ- and δ, of which γ-tocopherol exhibits the highest antioxidant activity (Ghazani & Marangoni, 2013). In the case of hemp seed oil, it contains much higher levels of tocopherols compared to many of edible oils, while tocotrienols are present in very low amounts. Consequently, hemp seed oil manufacturers usually market their products as a good source of vitamin E with high antioxidant activity.

The total amount of tocopherols in hemp seed oil ranges from 80 to 150 mg/100 g oil, with γ-tocopherol as the predominant isomer accounting for 85–91% of the total amount of tocopherols. The amount varies depending on the varieties, agronomic conditions, processing methods and storage conditions. The other three isomers are present at much lower levels, with a ratio in hemp seed oil of 5:2:90:3 for α-, β-, γ-, and δ-tocopherol, respectively (Matthäus & Brühl, 2008). The total tocopherol content of hemp seed oil is higher than canola oil (60-70 mg/100 g), sunflower oil (~90 mg/100 g) and soybean oil (~115 mg/100 g) (Ghazani & Marangoni, 2013).

The high antioxidant activity in the oil is attributed to the tocopherols which slow down the oxidative deterioration of oil during storage. In addition, because of their free radicals scavenging ability, tocopherols are also helpful in preventing degenerative diseases, such as cardiovascular disease, Alzheimer's disease, and certain types of cancer (Matthäus & Brühl, 2008). However, tocopherols are vulnerable when exposed to heat, light and alkali as they easily form oxidation products during processing and storage. The oxidation of tocopherol

would cause the loss of the antioxidant properties in the oil (Ghazani & Marangoni, 2013).

2.1.3.2 Polyphenols

Phenolic compounds are well known as the natural hydrophilic antioxidants and antimicrobial compounds presented in most vegetable oils. In addition to the health benefits exerted *in vivo*, phenolic compounds also have significant impact on the stability, sensory and nutritional characteristics of oils.

The general method for determining the total phenolic content of oil is the Folin-Ciocalteu assay, standardized with different pure phenolic acids, such as gallic, sinapic and caffeic acids. The total phenolic content in hemp seed oil, evaluated as gallic acid equivalents (GAE), ranged from 44–188 mg/100 g oil. When measured as caffeic acid equivalents (CAE), this oil showed 2.45 mg/100 g total phenolic content. In comparison, hemp seed oil contained higher phenolic content than flaxseed oil (137 mg/100 g) and canola oil (59 mg/100 g) expressed as GAE (Teh & Birch, 2013), and soybean oil (1.48 mg/ 100 g), canola oil (1.31 mg/100 g), sunflower oil (1.20 mg/100 g) and flax seed oil (1.14 mg/100 g) expressed as CAE (Siger, Nogala-Kalucka, & Lampart-Szczapa, 2008). The oil's nutritional characteristics are not only affected by the total amount of phenolic content but also related to the composition of phenolic compounds. Using reversed phase HPLC, the dominant phenolic acids in hemp seed oil were *p*-hydroxybenzoic acid (6.0 μg/100 g), followed by sinapic acid (3.0 μg/100 g), vanillic acids (2.0 μg/ 100 g) and *p*-coumaric acid (2.0 μg/100 g) (Siger et al., 2008).

The phenolic compounds in hemp seed oil contribute to both oil's oxidative stability and health benefits. Even though the hemp seed oil has a high content of PUFAs that are susceptible to oxidation, the antioxidant properties of the phenolic compounds prevent the oil deterioration by quenching any free radical produced by lipid oxidation (Siger et al., 2008). In addition, the ability of the phenolic compounds to scavenge free radicals also plays a role

in preventing oxidative damage to DNA, lipids, and proteins in the human body, the cause of many chronic diseases (Eskin & Tamir, 2006).

An important group of polyphenols, the flavonoids, are composed of two phenyl rings and a heterocyclic ring, namely flavanone, flavones and anthocyanidin. It was reported that the total flavonoid content, detected as luteolin equivalents, was 19.50 mg/ 100 g in the hemp seed oil which was comparable to the flaxseed oil (18.75 mg/100 g) and canola oil (16.41 mg/100 g) (Teh & Birch, 2013). These bioactive compounds are widely distributed in different vegetables oils and exhibit antioxidant properties, some of which proved potent in protecting LDL from oxidation (Eskin & Tamir, 2006).

2.1.3.3 Phytosterols

Matthaus et al. (2008) found the total amount of phytosterols in hemp seed oil ranged from 3.9 to 6.7 g/kg oil in ten different varieties, among which β-sitosterol accounted for 70% of the total phytosterols, followed by campesterol (15%) and Δ5-avenasterol (7%) (Matthäus & Brühl, 2008). Chromatographic analyses showed that the amount of total sterols in the unsaponifiable fraction of hemp seed oil was 2.8 g/ kg. These results confirmed the predominant phytosterols were β-sitosterol and campesterol, accounting for 1.9 g/kg and 0.5 g/kg respectively. In comparison to other common vegetable oils, the total phytosterols content in hemp seed oil was similar to that found in canola oil (6.9 g/kg), but higher than in soybean (4.6 g/kg) and sunflower oil (4.1 g/kg) oils (Ghazani & Marangoni, 2013).

The health benefits of phytosterols are associated with lowering the risk of cardiovascular diseases, as they inhibit absorption of cholesterol from dietary fat, which ultimately reduces the LDL cholesterol. Results of clinical studies showed that a daily intake of 2 g of phytosterols could reduce absorption of dietary cholesterols by 40–50%, resulting in a decrease of 6–10% total serum cholesterol and 8–14% LDL cholesterol (Ghazani & Marangoni, 2013). Some phytosterols have anti-polymerization activity, which can improve

the oxidative stability of oil at high frying temperatures.

2.1.3.4 Carotenoids

Carotenoids are natural pigments produced from chloroplasts in most of plant cells and in some other photosynthetic organisms. Two classes of carotenoids are generally found in vegetable oils, xanthophylls and carotenes. While xanthophylls are only viewed as yellow pigments in oils, carotenes, especially β-carotene, can act as an antioxidant and reduce the risk of degenerative diseases.

Carotenoids can be extracted along with chlorophylls by methanol, diethyl ether or other organic solvents, and determined by spectrophotometer at wavelength between 400 and 500 nm. The total carotene in hemp seed oil reported by Aladic et al (2015) were 3.14 mg/100 g and 12.5 mg/100 g produced by cold press process and supercritical fluid extraction, respectively. The amounts of β-carotene in commercial hemp seed oils were reported to be 3.36-5.34 mg/100 g.

The presence of carotenoids in hemp seed oil can also protect chlorophylls from degradation and prevent any color change during storage. In addition, β-carotene is converted to vitamin A by the body and is beneficial to vision.

2.1.3.5 Other beneficial minor components

Even though vitamins and dietary minerals account only for a small portion of unsaponifiable fraction of hemp seed oil, they are also essential nutrients for human metabolism. The presence of dietary minerals such as calcium (Ca), potassium (K), magnesium (Mg), iron (Fe), sodium (Na), manganese (Mn), zinc (Zn) and copper (Cu) were also found in hemp seed oil and these play a critical role in variety of biological pathways in human metabolism.

2.1.4 Adverse effect of minor components on quality of hemp seed oil

The undesirable minor components found in hemp seed oil, such as free fatty acids and

chlorophylls, act as pro-oxidants, which trigger or accelerate oil oxidation and hence lower the oil quality.

2.1.4.1 Free fatty acids

The fatty acids lower the oil quality by affecting the odor, flavor and other characteristics and are viewed as important quality indicator of oil. The free fatty acid in a commercial hemp seed oil product ranged 0–2.0% as oleic acid.

Due to the high concentration of PUFAs, the free fatty acids of hemp seed oil are mainly α-linolenic acid (ALA) and γ-linolenic acid (GLA). These can account for up to 2% of the oil composition, and in the free form these PUFAs are highly susceptible to oxidation, especially under conditions of high temperature or when exposed to light and oxygen. Compared to canola oil and olive oil, hemp seed oil is not stable mainly because of its high PUFAs, suggesting it is unsuitable for frying.

2.1.4.2 Chlorophyll pigments

Chlorophylls are fat-soluble pigments present in many crude vegetable oils. Chlorophylls along with its derivatives, namely pheophytin and pyropheophytin, are powerful pro-oxidants that may lower the oil quality by oxidation. The two main isomers found in hemp seed oil are chlorophyll-*a* and chlorophyll-*b*. They both have maximum absorbance at wavelengths between 640 nm to 670 nm, so that the amount of these isomers in methanol and diethyl ether extracts can be rapidly determined by spectrophotometry.

In the case of hemp seed oil, the intensive dark green color feature is associated with the high content of total chlorophylls. The total chlorophyll content of cold pressed hemp seed oil was found to be 98.6 μg/g, with 59.22 μg/g chlorophyll-*a* and 39.45 μg/g chlorophyll-*b*. In comparison, the supercritical fluid extracted hemp seed oil had twice the amount of total chlorophyll content, which was 228.79 μg/g with 193.50 μg/g and 35.39 μg/g chlorophyll-*a* and *b*, respectively. Similarly, the total chlorophyll content expressed as

pheophytin was 75.21 μg/g, which was significantly higher than canola oil (0.86 μg/g) and flax seed oil (6.78 μg/g) (Teh & Birch, 2013).

The high chlorophyll content can have many adverse effects on hemp seed oil. Firstly, chlorophylls are susceptible to photo-oxidation under the exposure of light, which causes the color change of the oil from dark green to yellow. In addition, the high content of chlorophyll could give rise to the instability of free fatty acids in the oil, accelerating the rancidity and result in a rancid aroma (Matthäus & Brühl, 2008). Moreover, even though these color pigments can be removed by conventional refining and bleaching processes, the longer processing time and larger amount of bleaching clay required by hemp seed oil with high chlorophyll content would cause the considerable loss of desirable components and higher costs of the production. Therefore, an efficient and environmental-friendly bleaching method is needed to reduce chlorophyll in hemp seed oil.

2.1.4.3 Other minor components adversely affecting oil quality

Small amount of moisture content, as well as primary and secondary oxidation products were found in the hemp seed oil. Lower moisture can keep the bacteria, such as *E. coli* and *Salmonella*, and mold away from growing in the oil, which prolong the shelf-life. Primary oxidation products, such as conjugated hydroperoxides, and secondary oxidation products, such as non-volatile aldehydes and ketone are formed during the oxidation of free fatty acids, which can be measured as the oil quality parameters, such as peroxide value and *p*-anisidine value, respectively. The excess amount of trace metals in crude oil such as iron, copper, calcium, sodium could reduce the refining efficiency, cause the oil oxidation and even harmful to human health. These metal ions can be removed by chelating agents during processing.

2.1.5 Conclusion

The health benefits and nutritional values of hemp seed oil are attributed to its unique

fatty acids composition and bioactive minor components. These minor components, such as tocopherols, phenolic compounds and phytosterols act as antioxidants, which can prevent the oil from deterioration and contribute to the health benefits to humans. However, the adverse minor components, especially high amount of chlorophylls in hemp seed oil, could lower oil quality and reduce oxidative stability of oil. Removal or reduction of chlorophyll and other adverse minor components would prolong the shelf life and improve the hemp seed oil quality, making it a much sought after oil by creating an important niche market.

2.2 Lipid Oxidation and Oil Bleaching

2.2.1 Lipid oxidation

2.2.1.1 Mechanism of lipid oxidation

Lipids are one of the most common constituents in food systems as well as an important nutrient for humans. Since the energy barrier to initiate lipid oxidation is lower than those to initiate oxidation of proteins, carbohydrates, or nucleic acids (Kołakowska & Bartosz, 2013), lipid oxidation becomes the major factor that most limits shelf-life and degrades quality of foods (Schaich, Shahidi, Zhong, & Eskin, 2013). Lipid oxidation primarily occurs between oxygen and unsaturated fatty acids in forms of triacylglycerols, phospholipids, free fatty acids and esterified fatty acids. It is a complex phenomenon recognized as a free radical chain reaction, which follows three major stages: initiation, propagation and termination (Laguerre, Lecomte, & Villeneuve, 2007).

Lipid oxidation is induced through initiators to remove a hydrogen atom from lipid molecule (LH) and create lipid allyl radicals (L·). The most common initiators or catalysts include heat, ultraviolet, metal ions, enzymes, chlorophyll and other pigments. Once allyl radicals are formed, they react quickly with oxygen and other chemical species to generate more free radicals, and hence initiate the oxidation processes.

In the propagation stage, the unstable free radicals, which are chemical species with an unpaired electron, react with oxygen to form more reactive radicals: peroxyl radicals (LOO). Then the concentration of peroxyl radicals increase quickly so that they are able to abstract hydrogen from adjacent lipid molecules, forming lipid hydroperoxides (LOOH) as well as generating new allyl radicals. These processes establish the free radical chain reaction and provide the driving force to continue the reactions (Schaich et al., 2013). Many isomers of hydroperoxides are formed and accumulated during these processes. Hydroperoxides formed from unsaturated fatty acids become stabilized by forming conjugated dienes and trienes

through the rearrangement of double bonds. Primary oxidation products namely hydroperoxides, conjugated dienes and trienes are produced during the propagation stage (Laguerre et al., 2007).

The propagation stage keeps continuing as long as hydrogen source and oxygen are available. Then the chain reactions terminated by free radicals transforming into non-radicals. The termination stage involves mechanisms including radical recombinations and scission reactions. Secondary oxidation products, such as aldehydes, alcohols and volatile ketones, are generated by the decomposition of hydroperoxides during this process. Oxidation of oil becomes slower since secondary oxidation products are stable, but off-flavor and unpleasant odors become noticeable in oils (Schaich et al., 2013).

2.2.1.2 Factors affecting oil oxidation

As for edible oils, the most important factors that affect the oxidation can be mainly divided into three categories: fatty acid composition, minor components and environmental conditions.

Edible oils oxidize much faster as the degree of unsaturation increases. Fatty acids with more than one double bone are highly susceptible to rapid oxidation. For example, fatty acids with two double bones oxidized 28 times faster than those with one double bone (Schaich et al., 2013). Hemp seed oil contains higher PUFA than other vegetable oils (Table 2.1). The major fatty acids in hemp seed oil are linoleic acid and α-linolenic acid, which have two and three double bones respectively. Therefore, virgin hemp seed oil shows poorer oxidative stability and shorter shelf-life compared to other vegetable oils. A Rancimat test performed at 120 ℃ showed that virgin hemp seed oil deteriorated in less than 1 hour, while virgin canola and olive oil that contain lower PUFA oxidized after 4 and 6 hours respectively (Matthäus & Brühl, 2008).

Table 2.1 Typical unsaturated fatty acids (%) of hemp seed oil and other vegetable oils [a]

Vegetable oils	Oleic acid (18:1)	Linoleic acid (18:2)	α-Linolenic acid (18:3)	PUFA (%)
Hemp seed oil	9	56	22	84
Flaxseed oil	15	15	61	76
Sunflower oil	22	63	<1	63
Canola oil	60	23	13	36
Soybean oil	23	55	8	63
Corn oil	25	60	1	60
Olive oil	76	8	<1	8

[a] Modified from Callaway (2004).

The reaction between fatty acid double bone and triplet oxygen does not occur spontaneously because it requires very high activation energy (Laguerre et al., 2007). Many minor components in edible oils can produce radicals that initiate and catalyze oil oxidation by generating the lipid allyl radicals and setting off the chain reactions. Even trace amounts of these radicals can cause large changes of oxidation rate. Metal ions such as iron, copper, cobalt are commonly presented in crude oils and they are active initiators that easily form initial radicals by abstracting electrons from fatty acid double bones (Schaich et al., 2013). Pigments, originated from oil seeds and plants, are powerful photosensitizers in vegetable oils. Chlorophyll can initiate oil oxidation rapidly by absorbing low-energy visible light (wavelength>4 00 nm) and transforming into reactive species. Chlorophyll is considered one of the notorious oxidation catalysts because a trace amount remaining in oil can greatly lower oxidative stability. It is interesting that some pigments in vegetable oils act as either

prooxidants or antioxidants. For instance, lower concentrations of carotenoids, especially β-carotene, the typical yellow pigments, are singlet oxygen quenchers that prevent free radicals production. On the other hand, high concentrations of carotenoids existed in the oils can produce active carotenoid alkoxyl and peroxy radicals that act as prooxidants (Schaich et al., 2013).

In addition, environmental factors namely light, temperature and oxygen play important roles in edible oil oxidation. Ultraviolet (UV) light catalyzes oxidation through inducing hemolytic scission of hydroperoxides and generating chain reaction initiator such as alkoxy radicals, peroxyl radicals and hydroxyl radicals (Schaich et al., 2013). Visible light at wavelength above 400 nm lacks the energy to directly initiate oxidation, but it can induce the photo-oxidation through active photosensitizers namely chlorophylls and initiate oil oxidation indirectly. It is well known that a higher temperature accelerates oil oxidation, especially during processing and storage (Shahidi & Spurvey, 1996). At low to moderate temperatures, heat accelerates the decomposition of hydroperoxide (LOOH), so that increases the rate of oxidation. When oils are heated at a higher temperature above 150 ℃, for instance deep frying, thermal effect causes scissions of acyl chains to generate radicals that initiate oxidation with the presence of oxygen (Schaich et al., 2013).

Furthermore, oil processing affects oxidation through altering the fatty acid composition, amounts of minor components as well as introducing heat, light and oxygen to oil systems. Therefore, when evaluating the oxidative stability of an oil product, the processing procedures cannot be ignored. The effects of processing, especially bleaching, on oil oxidation are discussed in the next section.

2.2.2 Oil bleaching

Vegetables oils obtained by mechanical pressing or solvent extraction without altering the nature of oils are considered virgin oils (Matthäus & Spener, 2008). These crude oils may

also be subjected to a series of refining processes namely degumming, neutralization, bleaching, deodorization to produce refined oils, which have better stability characteristics but less unique flavor and color attributes. The purpose of refining processes is to produce a bland oil by removing the non-TAG components such as phospholipids, free fatty acid, pigments, waxes as well as hydroperoxides and secondary oxidation products (Shahidi, 2013). These refining processes usually involve high temperatures above 80 ℃ and chemical agents (Ghazani & Marangoni, 2013). As for hemp seed oil, it mainly consists of PUFAs that are sensitive to heat. Therefore, hemp seed oil is usually produced only by cold pressing and does not undergo conventional refining processes, which preserves the prime quality of the fatty acids as well as retains large amounts of minor components, especially chlorophylls (Dunford, 2015).

2.2.2.1 Conventional bleaching and effects on oil quality

Bleaching is typically performed to remove pigments, such as chlorophylls and carotenoids. The effects of bleaching primarily involve three types of mechanisms: adsorption, heating and chemical oxidation (Gunstone, 2013).

Chemical oxidation bleaching is never used for edible oils, while it is usually used to produce oils for industrial materials, for instance soap making. Heat bleaching converts the pigments into colorless compounds by heating the oils above 175 ℃, but the decomposition products of pigments are left in the oil. These decomposition products along with the high temperature treatment cause adverse effects on oil quality (Shahidi, 2013).

The most common method for bleaching edible oils is absorption through using different types of absorbents or bleaching clay namely active carbon, naturally active clay, activated clay, synthetic silicates and aluminum (Zschau, 2001). Many large molecule compounds, not only pigments but also metal complexes and oxidation products, can be removed by the adsorptive effect of bleaching clays. During the bleaching process, clay

particles with large surface areas and porosity come in contact with the molecules of undesirable components by different interactions. For example physical interactions through Van der Waals force and molecular sieves traps molecules inside the pores of clays, or chemical interactions via ionic bonds (Kuuluvainen et al., 2015). In order to improve the bleaching efficiency, bleaching clays are usually activated by sulfuric acid or hydrochloric acid with an elevated temperature to increase the surface area by replacing the metal ions with hydrogen (Shahidi, 2005). In addition to surface area, other attributes of bleaching clay such as particle size, pore distribution and capillary structure also play significant roles in bleaching efficiency (Galan, 1996).

The bleaching process primarily enhances the oil's color and oxidative stability by removing the undesirable components, while it might also have some adverse effects on oils. Some bioactive minor components with antioxidant activity can be also adsorbed and removed by bleaching clay. Besides, using bleaching clay at a high temperature for prolonged treatment time may lead to hydrolysis; acid activated clays may increase the free fatty acid level after the interactions with oils, while excessive uses of bleaching clay may cause oil retention, filtration and disposal problems (Hussin, Aroua, & Daud, 2011; Smouse, 1995). It was reported that reducing the chlorophyll to a relatively low concentration can prevent the rapid oxidation of oils in the presence of light (Mag, 1989). Nevertheless previous studies showed that activated bleaching clay significantly reduced the chlorophyll content in oils that had a moderated to low initial concentration of chlorophyll (Didi, Makhoukhi, Azzouz, & Villemin, 2009; Makhoukhi, Didi, Villemin, & Azzouz, 2009). Conventional bleaching, however, is not appropriate for hemp seed oil which contains a large amount of chlorophyll. Removing such high concentration of chlorophyll requires more clay and longer treatment time, which could increase the chance of oxidation.

2.2.2.2 Novel bleaching methods

There are strong interests in developing novel bleaching methods for vegetable oils. Recently, ultrasound was utilized alone or in combination with conventional bleaching methods to improve the bleaching efficiency (Abedi, Sahari, Barzegar, & Azizi, 2015; Jahouach-Rabai et al., 2008; Su et al., 2013).

Ultrasound generally refers to sound waves with frequency (>20 kHz) beyond human hearing. When ultrasound interacts with liquid medium, an acoustic phenomenon known as cavitation occurs through the intense interactions between ultrasonic waves, liquid and dissolved gas (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012). From microcosmic view, the fluctuating pressure created by ultrasonic waves causes molecule oscillations of dissolved gas, while macroscopically cavitation is the rapid formation and collapse of air bubbles (Suslick et al., 1999). Consequently, cavitation can trigger a series of physical and chemical reactions, such as shock waves, strong shear forces and free radicals, which can be applied to food industry for many purposes (Su et al., 2013).

Currently, ultrasound technology has been widely used for food processing. Ultrasound-assisted extraction of bioactive compounds, especially phenolic compounds, showed the advantages of requiring simple equipment, shorter extraction time and less solvent consumption to obtain higher extraction yields (Santos-Buelga, Gonzalez-Manzano, Dueñas, & Gonzalez-Paramas, 2012). Applications of ultrasound in filtration, emulsification and viscosity modification are well established in food processing (Chandrapala et al., 2012). Furthermore, ultrasound has also been applied in oil processing. For example during oil extraction, ultrasound treatment improves the oil yield without altering the fatty acids composition (Li, Pordesimo, & Weiss, 2004). In addition, the degumming process with ultrasound significantly reduced the use of acid and water, lowering the free fatty acid and moisture content in the oils (Moulton & Mounts, 1990).

In term of applications in oil bleaching, a previous study was conducted to evaluate the effect of bleaching using high power ultrasound (20 kHz) on the fatty acids and minor compounds of olive oil (Jahouach-Rabai et al., 2008). The main advantage was the decrease in bleaching time and temperature, minimizing the harmful effects of acid-activated clays. However, this study also detected a rancid odour in oils after the ultrasound treatment, probably due to the formation of volatile compounds through sonodegradation of olive oil. Another study performed by Su et al. (2013) investigated different conditions of ultrasonic bleaching, including power of ultrasound, sonication time, treatment temperature, type and amount of absorbents. The overall bleaching effects for rapeseed oil were enhanced as the ultrasound power, temperature and treatment time increased, but the concentration of primary oxidation products increased as well. Recently, the bleaching conditions (ultrasound power, temperature, amount of clay, treatment time) were optimized by monitoring the reduction of pigments in soybean oil (Abedi et al., 2015). Compared to conventional bleaching method, ultrasonic bleaching reduced the clay amount, temperature, and time up to 35%, 35% and 10%, respectively. The above studies indicated the potential of ultrasound in application of the oil bleaching process. However, data from previous studies were limited to olive, rapeseed and soybean oils. There is no report available for hemp seed oil which is high in chlorophyll content and has unique flavor. Consequently, further study is needed for developing an improved bleaching method for hemp seed oil.

2.3 Endogenous Phenolic Compounds and Bioactivities of Canola and Hemp

Many researches showed the presence of large amounts of phenolic compounds in crude vegetables oils and their by-products. Recently, the potential values of these phenolic compounds have been investigated and recognized. This section introduces the endogenous phenolic compounds in hemp and canola, respectively. The extraction methods and potential applications of these phenolic compounds are also discussed.

2.3.1 Endogenous phenolic compounds from hemp and canola

Few studies are available on the phenolic profile of hemp products. Table 2.2 summarizes the phenolic compounds reported in hemp meal and oil from previous studies. Most of these phenolic compounds were identified and quantified by HPLC using the internal and external standards of the individual phenolic acids. According to Teh et al. (2014), phenolic compounds detected in hemp meal were caffeic acid, quercetin and luteolin, while Pojić et al. (2014) found gallic acid, *p*-hydroxybenzoic acid, catechin , vanillic acid, ferulic acid and sinapic acid in hemp meal fractions with different particle sizes. Furthermore, two unique phenolic compounds, namely *N*-*trans*-caffeoyltyramine and cannabisin B, were identified in hemp meal fraction and expressed in *trans*-cinnamic acid equivalents. These two compounds were isolated and identified as antioxidants with potent free radical-scavenging activity (T. Chen et al., 2012, 2013). As for the hemp seed oil, the phenolic compounds detected by Siger et al. (2008) were mainly in accord with that of Pojić et al. (2014), although the concentrations were much lower in the oil compared to the meal. However, the phenolic compounds detected in hemp are not consistent with previous studies.

Table 2.2 Phenolic compounds from hemp meal and oil

Sample	Phenolic compounds	Concentration (mg/100g)	Reference
Hemp Meal	Caffeic acid	8.27	(Teh et al., 2014)
	Quercetin	64.89	
	Luteolin	40.04	
	Gallic acid	0.082	(Pojić et al., 2014)
	p-hydroxybenzoic acid	7.86	
	Catechin	49.8	
	Vanillic acid	0.035	
	Ferulic acid	4.74	
	Sinapic acid	2.22	
	N-trans-caffeoyltyramine	15.2*	
	Cannabisin B	6.49*	
Hemp Oil	*p*-hydroxybenzoic acid	0.006	(Siger et al., 2008)
	Sinapic acid	0.003	
	Vanillic acid	0.002	
	p-coumaric acid	0.002	
	Ferulic acid	0.001	

*Expressed in *trans*-cinnamic acid equivalents.

Canola oil is the most consumed vegetable oils in Canada, and the phenolic profiles of canola seed, meal and oil have been intensively investigated by previous studies (Table 2.3) in the last decade. The predominant phenolic compounds in canola products are sinapic acids and its derivatives, namely sinapine and sinapoyl glucose (Khattab, Eskin, Aliani, & Thiyam, 2010; Siger, Kaczmarek, & Rudzińska, 2015). Other phenolic compounds, such as gallic acid, *p*-coumaric acid, caffeic acid, ferulic acid and quercetin were also detected in canola products (Siger et al., 2008; Teh et al., 2014). Seeds and meals of canola generally contain higher phenolic content than crude oil. Canolol, a decarboxylation product of sinapic acid, was identified in crude oil, but the concentration decreased significantly after oil refining (Y. Chen, Thiyam-Holländer, Barthet, & Aachary, 2014). Canolol is usually expressed as sinapic

acid equivalents. Previous studies suggested that canolol was primarily responsible for the antioxidant activity of crude canola oils (Y. Chen et al., 2014; Wakamatsu et al., 2005).

Table 2.3 Phenolic compounds from canola seed, meal and oil

Sample	Phenolic acid	Concentration (mg/100g)	Reference
Canola Seed	Sinapine	859-1189	(Khattab et al., 2010)
	Sinapoyl glucose	50-665	
	Sinapic acid	9-59	
Canola Meal	Sinapine	611-1011	
	Sinapoyl glucose	135-199	
	Sinapic acid	32-41	
	Gallic acid	88.98	(Teh et al., 2014)
	p-coumaric acid	32.11	
	Caffeic acid	452.67	
	Ferulic acid	11.22	
	Quercetin	62.29	
Canola Oil	Sinapine	0.078	(Siger et al., 2015)
	Sinapic acid	0.236	(Siger et al., 2008)
		3.05	(Y. Chen et al., 2014)
	Trans-sinapic acid	0.497	(Siger et al., 2015)
	Cis-sinapic acid	0.005	
	Canolol	0.069	
		69.7*	(Y. Chen et al., 2014)
	p-coumaric acid	0.013	(Siger et al., 2008)
	Ferulic acid	0.041	(Siger et al., 2015)
		0.005	(Siger et al., 2008)
	p-hydroxybenzoic acid	0.001	
	Caffeic acid	0.0003	

*Expressed in sinapic acid equivalents (SAE).

The brief extraction and determination procedures of phenolic compounds were summarized from previous literatures (Table 2.4) (Abuzaytoun & Shahidi, 2006; Chavan & Amarowicz, 2013; Y. Chen et al., 2014; Kajdzanoska, Petreska, & Stefova, 2011; Khattab et al., 2010; Khattab, Rempel, Suh, & Thiyam, 2012; Pojić et al., 2014; Siger et al., 2008; Teh et

al., 2014; Teh & Birch, 2013; Yu, Zhou, & Parry, 2005). There are various methods used for extracting phenolic compounds from different plant materials, among which solvent extraction is the most widely used method. Various assisted techniques are used to enhance the extraction efficiency, including vortexing, heating, ultrasounds, microwaves, pressurized or supercritical liquids (Santos-Buelga et al., 2012). The type of solvent is the most important factor for extraction. Since phenolic compounds mostly show high polarity, polar solvents should be used in order to achieve higher yield (Dai & Mumper, 2010). Generally, methanol, ethanol, isopropanol, acetone, ethyl acetate and the combination of these solvents as well as their aqueous solution are used for phenolic extraction (Table 2.4). Centrifugation and filtration are usually carried out to separate the solid residues and extracts after solvent extraction. Furthermore, vacuum evaporation is sometimes applied to purify and isolate the target phenolic compounds.

Phenolic compounds from various plant sources have been identified as natural antioxidants (F Shahidi, 2000). Antioxidants are defined as substances that when present at low concentrations compared with those of the oxidizable substrate, significantly delay or inhibit the oxidation of lipids, proteins, DNA and carbohydrates (Halliwell & Gutteridge, 1989; F Shahidi, 2000)

By the mechanism of action, antioxidants can be classified in two categories: primary or chain-breaking antioxidants and secondary or preventative antioxidants. Primary antioxidants can break the chain reaction of oxidation by donating hydrogen ions and generating stable radicals. Secondary antioxidants can retard the rate of oxidation by several mechanisms, including chelation of metals, regeneration of primary antioxidants, decomposition of hydroperoxides, and scavenging of oxygen (F. Shahidi & Zhong, 2005).

The structures of natural phenolic antioxidants allow them to form low-energy radicals that terminate the free radical chain reactions of oxidation (Karovičová & Šimko, 2000). The

molecular structure of phenolic antioxidants consists of one aromatic carbon ring and several functional groups namely carboxyl, hydroxyl and methoxyl groups. The most important structural features affecting the antioxidant activities of phenolic compounds is the configuration and total number of these functional groups (Klaudia Jomova, Michael Lawson, 2013). Generally, phenolic compounds with these electron-donating functional groups can delay or inhibit oxidation by reacting with peroxyl radicals or alkoxyl radicals to produce unreactive phenoxyl radicals. These phenoxyl radicals are relatively stable and they can further interfere with the free-radical chain reaction by forming non-radical compounds (Antolovich, Prenzler, Patsalides, Mcdonald, & Robards, 2002).

The protective effects of phenolic antioxidants in food systems can prolong shelf-life by protecting it from oxidation-induced deterioration, such as fat rancidity and color changes (Venskutonis, 2013). In addition, in health-related aspects, it was reported that antioxidants have the potential to protect the body against cell damage caused by reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) (F. Shahidi & Zhong, 2005).

Table 2.4 Extraction and determination methods of phenolic compounds from plant materials

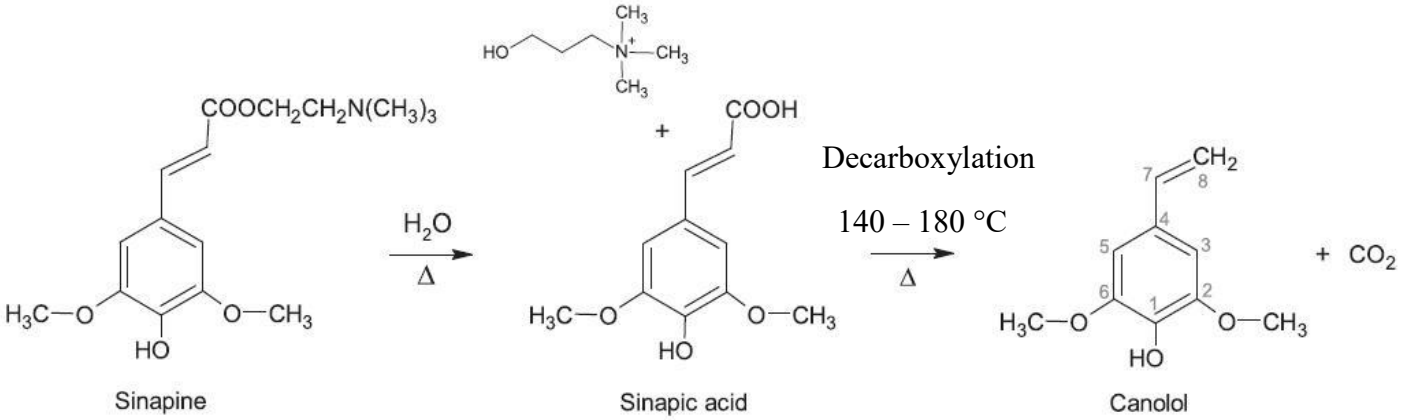
Sample	Weight (g)	Dissolved in Solvent	Extraction Solvent	Extraction Process	Separation	Determination Methods	References
Hemp oil	20 g	200 ml hexane	3×40 ml methanol	Shake	Vacuum Evaporation	Folin- Ciocalteu	(Abuzaytoun & Shahidi, 2006)
Hemp oil	2.5 g	Hexane	Methanol	Solid phase extraction column*	-	Folin-Ciocalteu& HPLC	(Siger et al., 2008)
Hemp oil	1 g	-	3×2 ml methanol	-	-	Folin-Ciocalteu	(Yu et al., 2005)
Hemp meal	1 g	-	80 ml 80% methanol	10 min ultrasonic bath, macerate for 2 h	Filtration	HPLC	(Pojić et al., 2014)
Hemp oil	10 g	50 ml hexane	3×20 ml 60% methanol	-	Vacuum Evaporation	Folin-Ciocalteu	(Teh & Birch, 2013)
Hemp seed cake	6 g	-	100 ml 100% or 80% methanol/ethanol/acetone	1h magnetic stirrer	Filtration	Folin-Ciocalteu & HPLC	(Teh et al., 2014)
Canola oil	2.5 g	5 ml *n*-hexane	2×5 ml 17.28 mol/L aqueous methanol	2 min vortex	10 min 2236 rpm centrifugation	HPLC	(Khattab et al., 2012)
Canola oil	2 g	-	2×5 ml 70% methanol	3 min vortex	5 min 5000 rpm centrifugation	Folin-Ciocalteu & HPLC	(Y. Chen et al., 2014)
Canola seed/meal	1 g	-	3×9 ml 70% methanol/isopropanol/ethanol	1 min ultra-sonication	10 min 50009 g centrifugation	HPLC	(Khattab et al., 2010)
Beach Pea meal	25 g	-	200 ml 80% methanol/ethane/acetone	15-90 min 80 °C water bath	Filtration	HPLC	(Chavan & Amarowicz, 2013)
Strawberry	5 g	-	Solvent mixture (acetone, methanol and acetic acid)	15 min sonication	37 °C Rotary-Evaporation	HPLC	(Kajdzanoska et al., 2011)

2.3.2 Influence of thermal treatment on phenolic compounds

Canolol, also known 4-vinylsyringol, was first isolated from crude canola oil, which exhibits potent antioxidant activity and free radical scavenging capacity in biological systems (Koski, Pekkarinen, Hopia, Wähälä, & Heinonen, 2003).

It was reported that canolol content became higher in crude canola oils obtained from roasted seeds, but the content decreased after oil refining (Y. Chen et al., 2014; Wakamatsu et al., 2005). The formation of canolol is due to the hydrolysis of sinapine and the further thermal decarboxylation of sinapic acid (Figure 2.2) (Zago et al., 2015). Therefore, canola seed subjected to high temperature generated by seed roasting or oil pressing can produce a large amount of canolol, since sinapic acid derivatives are the predominant phenolic compounds in canola seeds (Aachary & Thiyam-Holländer, 2013). Terpinc et al. (2011) reported that the 4-vinyl derivatives namely 4-vinylphenol, 4-vinylguaiacol, 4-vinylsyringol, 4-vinylcatechol were formed by the thermal decarboxylation of the corresponding phenolic acids: *p*-coumaric, ferulic, sinapic and caffeic acid respectively. This finding suggests that thermal treatment for canola and hemp products could produce novel phenolic compounds that exhibit higher antioxidant activity. In a previous study, the optimum temperature for canolol formation was found at 160 ℃ (Spielmeyer, Wagner, & Jahreis, 2009), while another study conducted by Siger et al. (2015) reported that canola seed roasted at 180 ℃ produced the highest canolol content in cold pressed oil. The thermal treatments performed in the previous studies included roasting, oven heating and microwave. Further investigation is needed to determine the optimum thermal condition for producing canolol.

Figure 2.2 Reactions of hydrolysis of sinapine and decarboxylation of sinapic acid into canolol, modified from Zago et al. (2015)



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CHAPTER 3

MANUSCRIPT 2: A NEW ULTRASOUND-ASSISTED BLEACHING TECHNIQUE FOR IMPACTING CHLOROPHYLL CONTENT OF COLD PRESSED HEMP SEED OIL

3.1 Abstract

Ultrasonic treatment was applied to reduce chlorophyll of cold pressed hemp seed oil in conjunction with various clays. Bleaching clays such as bentonite (activated/non-activated), sepiolite and clay from a canola oil refining industry (industrial clay) were used. Ultrasonic bleaching of hemp seed oil for 20 minutes at 20, 40 and 60% power (expressed as ultrasound amplitude) with bleaching clays (40 g/kg) reduced chlorophyll in decreasing order of effectiveness from industrial clay > activated bentonite > sepiolite > non-activated bentonite together with an increase in ultrasound power to 40%. At 20% ultrasound power, chlorophyll reduction (%) was found to be 99.4% (industrial clay) > 97.8% (activated bentonite) > 82.7% (sepiolite) > 47.1% (non-activated bentonite). The reduction in total phenolic content was 27.3%, 33.4%, 27.9% and 34.7% when treated at 20% ultrasound power with 40 g/kg clay from canola industry, activated bentonite, non-activated bentonite and sepiolite, respectively. The corresponding reduction in the peroxide value for the oil samples, due to the adsorptive nature of the clay, followed the trend of industrial clay > sepiolite > non-activated bentonite. This process proved promising for impacting hemp seed oil color through reduction of chlorophyll.

Key words Hemp; Pigments; Extraction; Clays; Phenolic compounds

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3.2 Introduction

The optimal linoleic acid to alpha-linolenic acid ratio (3:1) and the presence of gamma-linolenic acid, make hemp (*Cannabis sativa L.*) seed oil an important functional and nutritional vegetable oil (Callaway, Tennila, & Pate, 1997; Deferne & Pate, 1996). Cold pressing of hemp seed, the common method to obtain oils, could only recover~65% of the oil with a substantially high content of chlorophyll pigments. Chlorophyll initiates lipid oxidation and quality deterioration of hemp seed oil, which necessitates storage in dark or opaque containers. However, to enhance its appeal, hemp seed oil is generally marketed in transparent bottles. This can lead to a color change from green to yellow which is accompanied by a corresponding increase in oxidative deterioration and rancidity (Matthäus & Brühl, 2008).

Bleaching is commonly used during edible oil refining to reduce the pigments such as chlorophylls and carotenoids as well as to remove undesired minor components such as metals, phospholipids and oxidation products (Makhoukhi, Didi, Villemin, & Azzouz, 2009). The mechanism of bleaching involves either adsorption, heating or chemical oxidation (Gunstone & Norris, 1983). Bleaching with clays (earths) is primarily an adsorption process and is commonly practiced in the oil refining industries. This adsorption process involves complicated interactions between clay particles and undesired molecules. These interactions include physical interactions such as molecular sieves effect wherein undesired compounds are trapped inside the holes of clays, attraction effect of Van der Waals forces, and sometimes chemical reactions occurring via the ionic bonds (Kuuluvainen et al., 2015). Various attributes of bleaching clays such as particle-size distribution, micro-porosity, capillary structure and grain size affect the bleaching efficiency (Galan, 1996). Specific surface area of the bleaching clay also plays critical roles in the bleaching processes because the larger surface area of the clay indicates more opportunities of contact between the adsorbents and

the undesired compounds. In general, adsorbents from four categories can be utilized as bleaching clays, which are active carbon, naturally active clays, activated clays and other adsorbents such as alumina powder, silica gel, and aluminum and magnesium-silicate (Zschau, 2001). Specific bleaching clays such as bentonite, sepiolite and palygorskite are commonly used in bleaching of vegetable oils. Acid activation using sulfuric acid or hydrochloric acid with elevated temperature has proven to significantly improve the adsorbility of these clays (Didi, Makhoukhi, Azzouz, & Villemin, 2009; Su et al., 2013) possibly due to the increase in the surface area of the clays by replacing the large metal ions with hydrogen ions (Shahidi, 2005). Pigment removal by bleaching can prevent lipid oxidation catalyzed by chlorophyll and its derivatives. However, increasing the treatment time and amount of clay is associated with such as oil retention, filtration and environmental issues (Hussin, Aroua, & Daud, 2011); Lipid oxidation of vegetable oils would be intensified and the loss of oils and disposal costs of the clays increased. The current drawbacks of the bleaching clays necessitate exploring other sustainable, eco-friendly and rapid alternate approaches of pigment removal.

Ultrasound treatment can be an alternative to bleaching clay even though its use in bleaching of vegetable oil is limited to only a few recently reported studies (Abedi, Sahari, Barzegar, & Azizi, 2015; Su et al., 2013). In these studies, ultrasound treatment was applied to oil samples alone or in combination with bleaching clays during the bleaching process and referred to as ultrasonic bleaching. The degradation of carotenes during the ultrasonic bleaching of rapeseed oil was attributed to free radical oxidation which increased the primary oxidation products (Su et al., 2013). Previously, a non-aqueous bleaching process involving ultrasound was effectively used for olive oil (Jahouach-Rabai et al., 2008). Recently, the application of ultrasonic bleaching on soybean oil and corresponding changes in its physicochemical properties were studied (Abedi et al., 2015). There are no reports available

on the bleaching of hemp seed oil using either clay or combined application of clay and ultrasound; however, there is a report on the ultrasonic extraction of hemp seed oil (Lin et al., 2012).

Ultrasound treatment, a sustainable and eco-friendly technology (Toepfl, Mathys, Heinz, & Knorr, 2006), has already been widely applied in food industries especially for extracting the bioactive compounds such as polyphenols and carotenoids (Maki-Arvela, Hachemi, & Murzin, 2014; Santos-Buelga, Gonzalez-Manzano, Dueñas, & Gonzalez-Paramas, 2012). The low frequency energy of ultrasound (20-1000 kHz) results in a cavitation force and ensues localized pressure which disrupts plant cell wall, thereby enhancing the release of the bioactive components and facilitating the interaction between bioactive components and solvents in an extraction process. Ultrasound assisted extraction of oils from hemp seed, Jatropa, Pongamia and many other oil seeds produced higher oil yields as well as greater amount of anthocyanin from grape byproducts and phenolics from coconut shell powder, cranberry, and marula kernel oil cake (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015; Rodrigues & Pinto, 2007; Wang & Zuo, 2011). Ultrasonic extraction of phenolics and flavonoids from defatted hemp, flax and canola seed cakes were carried out and validated against results using conventional extraction methods (Teh & Birch, 2014). Utilizing ultrasound in oil bleaching is a novel application of ultrasound and a potential alternative to conventional bleaching, because it could improve the bleaching efficiency and reduce the amount of clay. However, ultrasound treatment of oil generates heat, the amount of which is directly proportional to the ultrasound power used. A general concern is that the ultrasound wave and the heat generated could not only reduce the pigments, but also have an adverse impact on the bioactive compounds and hence the quality of oil. Peroxide value (PV) is commonly used to estimate the level of oxidative deterioration in heated oils.

Ultrasound treatment is also used in the extraction of chlorophyll. In addition to the extraction techniques, solvents have a more significant impact on the extraction efficiency. Various solvents, namely diethyl ether, methanol, ethanol, acetone and chloroform have been used for the extraction of chlorophyll from different plant samples. Acetone and diethyl ether are effective solvents for extracting chlorophylls but their volatile and flammable properties are a major concern, on the other hand methanol and ethanol are not as efficient (Dere & Güneş, 1998; Ritchie, 2006). Besides, the optimal solvent for chlorophylls extraction varies depending on the plant matrices.

The main objective of this work was to reduce chlorophyll in the cold pressed hemp seed oil by simultaneous application of ultrasound and clay. Another objective was to evaluate the effect of ultrasonic bleaching on the phenolic compounds and oxidative deterioration of hemp seed oil. This is the first ever report showing the efficacy of ultrasound treatment minimizing the amount of clay required for effective removal of chlorophyll in hemp seed oil. The study extends new applications by producing hemp seed oil with varying color attributes for food and non-food applications.

3.3 Materials and Methods

3.3.1 Materials and chemicals

All chemicals were of analytical grade. Standards of chlorophyll-*a*, chlorophyll-*b* and gallic acid, cumene hydroperoxides, quercetin, diethyl ether, methanol, and chloroform were purchased from Sigma Aldrich, Canada. Cold pressed hemp seed oil, a product from Hemp Oil Canada Inc. (Ste. Agathe, Manitoba, Canada), was obtained and stored in opaque containers and flushed with nitrogen at 4 ℃. A sample of clay used for refining canola oil was obtained from a canola oil refining industry in Canada and was referred to as industrial clay in this paper. Sepiolite (original sepiolite, particle size$\leqslant$75 μm, surface area$\geqslant$223 m^2/g) and bentonite (particle size$\leqslant$75 μm, surface area$\geqslant$130 m^2/g) were purchased from Sigma-

Aldrich, Canada. A fine bentonite suspension in water (320 g/L) was treated with concentrated H_2SO_4 (1.83 kg/L) at 90 ℃ for 6 h, followed by washing the clay to neutral pH using distilled water to produce activated bentonite. The acid treated clay was dried at 100 ℃ for 6 h and ground and sieved to 75 μm particle size.

3.3.2 Extraction of chlorophylls from hemp seed oil

A modified method was used for the extraction of chlorophyll-*a* and chlorophyll-*b* (Dere & Güneş, 1998). About 0.5±0.01 g of the hemp seed oil in a 50 mL centrifuge tube was mixed thoroughly with 25 mL of extraction solvent (diethyl ether: absolute or 950 mL/L aqueous solution; methanol: absolute or 960 mL/L aqueous solution). The chlorophyll was extracted by immersing an ultrasound probe (40% power) in the mixture for 1 minute ultrasound treatment. The homogenate was centrifuged at 875 g (radius 8.7 cm) for 10 minutes at 4 ℃ and the pigment layer was collected for spectrophotometric analysis. All extractions were carried out in duplicate (Figure 3.1).

3.3.3 Quantification of chlorophyll of hemp seed oil

Standards of chlorophyll-*a* and chlorophyll-*b* were used in the respective solvents to determine maximum wavelengths of absorption against absolute solvent as blank. Based on the results, chlorophyll-*a* and chlorophyll-*b* of diethyl ether extracts were estimated at 640 nm and 663 nm respectively and that of methanol extracts were estimated at 650 nm and 664 nm respectively. Triplicate readings were recorded for statistical analysis. The concentrations (μg/g) of chlorophyll-*a* (C_a) and chlorophyll-*b* (C_b) were calculated using the following equations:

For diethyl ether:

$$C_a = 496.5 \cdot A_{663} - 39 \cdot A_{640}$$

$$C_b = 880 \cdot A_{640} - 140.5 \cdot A_{663}$$

$$C_{a+b} = 356 \cdot A_{663} + 840 \cdot A_{640}$$

For methanol:

$C_a = 782.5 \cdot A_{664} - 367 \cdot A_{650}$

$C_b = 1352.5 \cdot A_{650} - 560.5 \cdot A_{664}$

$C_{a+b} = 222 \cdot A_{664} + 985.5 \cdot A_{650}$

3.3.4 Ultrasonic bleaching of hemp seed oil

A previously reported procedure for ultrasonic bleaching of carotenoids of rapeseed oil was followed with suitable modifications (Su et al., 2013). A SONOPLUS Ultrasonic Homogenizer HD 2200 system (BANDELIN electronic GmbH & Co. KG, Heinrichstraße, Berlin, Germany) consisting of Ultrasound Convertor UW 2200, probe and HF Generator-GM 2200 was used to produce high power ultrasound with high amplitudes and frequency (20 kHz±500 Hz). Power of ultrasound treatment was the ultrasound amplitudes expressed as percentage (%). Hemp seed oil samples (25 ± 0.1 g, in duplicate) were placed in 50 mL centrifuge tubes and treated with either ultrasound (Power- 20%, 40% or 60%) under a pulsing ultrasound operating mode (pulsed cycle-4 with an active and passive interval of 4 s and 6 s respectively) or with a combination of ultrasound and bleaching clay (40 g/kg) treatment (Figure 3.1). For the ultrasonic bleaching of oil, the ultrasound probe was immersed halfway into the oil and treated for 20 minutes (four cycles of 5 minutes each with a 1 minute cooling time). Before the ultrasonic bleaching, hemp seed oil samples (25 ± 0.1 g) were mixed with 40 g/kg each of sepiolite, non-activated bentonite, acid activated bentonite and clay obtained from canola oil refining industry. The temperature of the oil was immediately recorded using a thermometer. In another set of experiment, oil samples were treated with bleaching clay (40 g/kg) without any ultrasonic treatment. Bleached oils with clay were centrifuged at 2432 g (radius 8.7 cm) for 10 minutes at 4 °C and the oils were collected and stored at 20 °C until further analysis. The results were compared with the chlorophyll content of untreated hemp seed oil (without ultrasound or bleaching clay treatment). All the

treatments were carried out in duplicates with triplicate analyses.

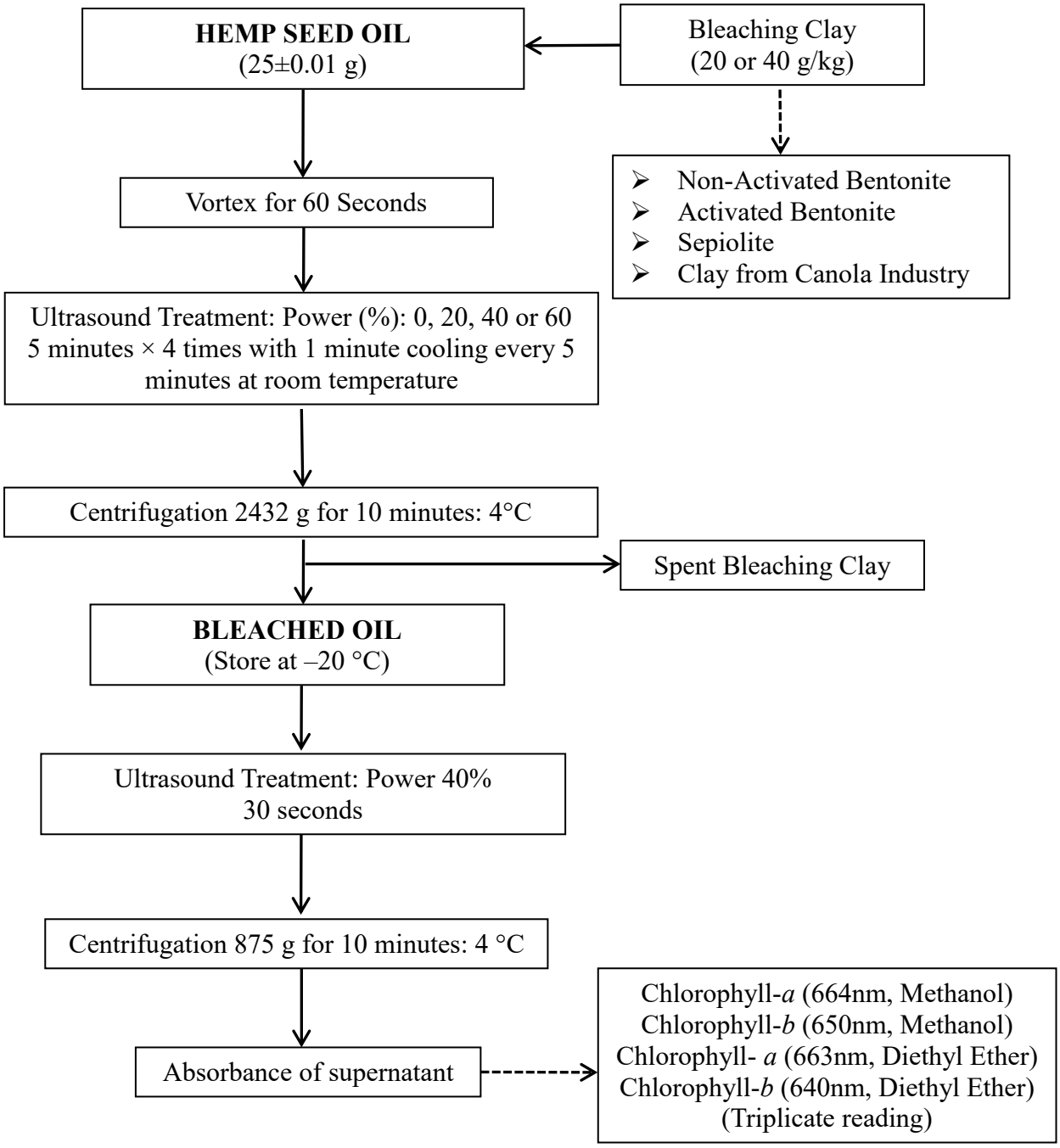
3.3.5 Total phenolic content and peroxide value

Total phenolic contents in the extracts of control and bleached hemp seed oil (40 g/kg bleaching clay and 20% power ultrasound) were estimated using the Folin-Ciocalteu reagent based on a previously reported method with slight modifications (Swain & Hillis, 1959). The results were expressed as gallic acid equivalent (GAE) μg/g of oil, at 750 nm. Peroxide value (PV) was determined by the ferric thiocyanate method with the results were calculated against a cumene hydroperoxide standard graph and expressed as mmol cumene hydroperoxide equivalent/kg oil at 500 nm (Senphan & Benjakul, 2015). The analyses were conducted in duplicate.

3.3.6 Statistical analysis

The results were reported as mean values ± standard deviations. The differences between mean values were determined by One Way Analysis of Variance (ANOVA) using SPSS Statistics version 22 (IBM, New York, USA). The post hoc multiple comparisons were conducted by Tukey's Honestly Significant Difference (HSD) Test. The statistical significance level was accepted at $p<0.05$.

Figure 3.1 Process flow chart of ultrasonic bleaching of hemp seed oil and chlorophyll extraction



3.4 Results and Discussion

3.4.1 Effects of solvents on the extractability of chlorophyll from hemp seed oil

The pigments of cold pressed hemp seed oil were extracted using methanol or diethyl ether. Absolute solvent extracted higher levels of total chlorophyll when methanol was used, while the diethyl ether showed analogous extractability of total chlorophyll for absolute and aqueous solvent. Total chlorophyll content of hemp seed oil (µg/g) was found to be 42.6±0.99 and 33.5±1.09 respectively for absolute and 960 mL/L methanol (Table 3.1). The extractability of total chlorophyll was considerably higher using diethyl ether compared to methanol. On average, diethyl ether was 1.7 fold more efficient than methanol in extracting chlorophyll. The total chlorophyll content extracted from of hemp seed oil (µg/g) was 67.3 ± 0.64 and 67.8 ± 1.11 respectively for absolute and 950 mL/L diethyl ether (Table 3.1).

Solvent properties such as polarity and safety, and solute properties such as solubility and stability should be considered when choosing the optimal solvent for extraction (Maki-Arvela et al., 2014). The optimum solvents to extract chlorophyll vary with respect to the type of plant matrices. Dere and Güneş (1998) found that methanol has higher efficiency in extracting chlorophylls and carotenoids from algae compared to diethyl ether and acetone, whereas Ritchie (2006) indicated the efficacy of acetone over methanol and ethanol for extracting chlorophylls from spinach. In the case of hemp seed oil, both methanol and diethyl ether are effective. However, each solvent has some drawbacks: diethyl ether is highly volatile and flammable, and methanol is much safer but less efficient than diethyl ether. Considering the efficiency, diethyl ether is the better solvent for extraction and quantification of chlorophyll from hemp seed oil in laboratory scale. Furthermore, a large number of experimental data about the production of pure chlorophyll pigments and accurate determination of extinction coefficients of chlorophyll-*a* and chlorophyll-*b* using diethyl ether are available (Jeffrey, Mantoura, & Wright, 1997; Porra, Thompson, & Kriedemann,

1989; Porra, 1991). Based on the above consideration, absolute diethyl ether was used for further analysis, which substantially reduced the time of extraction and need of centrifugation after the ultrasound treatment.

The relative distribution of chlorophyll-*a* and chlorophyll-*b* in the hemp seed indicated the former as the major green pigment. The approximate ratio of chlorophyll-*a* and chlorophyll-*b* is found to be 3:1 and 2:1 when absolute and 960 mL/L methanol is used for extraction. The reduction in the ratio is not attributed to the increase in the actual content of chlorophyll-*b* (Table 3.1), but the reduced extraction of chlorophyll-*a* with 960 mL/L methanol. In contrast to methanolic extraction, the chlorophyll-*a* and chlorophyll- *b* ratio was found to be higher (~3.6:1 to 3.7:1) when aqueous diethyl ether (950 mL/L) was used in comparison to non-aqueous diethyl ether (2.6:1-2.9:1). The solubility of chlorophyll-*a* and chlorophyll-*b* impacted the extraction efficiency in these solvents.

Table 3.1 Effects of solvents on chlorophyll extraction of hemp seed oil

Solvent	Chlorophyll-*a* (μg/g)	Chlorophyll-*b* (μg/g)	Total Chlorophyll (μg/g)
Methanol [1]	31.0±0.88[c]	11.6±0.12[c]	42.6±0.99[b]
Methanol (960 mL/L)	22.3±0.75[d]	11.2±0.38[c]	33.5±1.09[c]
Diethyl ether [1]	48.7±0.43[b]	18.7±0.24[a]	67.3±0.64[a]
Diethyl ether (950 mL/L)	53.0±0.46[a]	14.8±0.73[b]	67.8±1.11[a]

[a-c] Values are expressed as mean±standard deviations. Different superscript letters within the same columns are significant different (p<0.05).

[1] Absolute solvent

3.4.2 Impact of ultrasound treatment on the chlorophyll content of hemp seed oil

Hemp seed oil was treated with ultrasound (Power: 20, 40 & 60%) for 20 minutes to examine the effect of ultrasound-cavitation on the chlorophyll reduction. Table 3.2 represents the effects of ultrasound treatment on the chlorophyll content of hemp seed oil after methanolic extraction (absolute or 960 mL/L). Irrespective of the concentration of methanol, the total chlorophyll content was significantly reduced as the ultrasound power was increased from 20 to 60%. This trend was also reflected in the individual chlorophyll isomers too. The reduction in chlorophyll could be attributed to the free radicals induced oxidation during ultrasound treatment or pyrolysis leading to degradation of chlorophyll in the cavitation bubbles (Tiwari, O'Donnell, Patras, & Cullen, 2008). The destruction of chlorophyll during ultrasound treatment may result in hydroxy radicals, which act as potent pro-oxidants. Researchers matched this reaction to the hydroperoxide and free radical formation by lipoxygenase enzyme catalysis (Abedi et al., 2015; Hildebrand & Hymowitz, 1982). The present study has a distinct advantage over a similar investigation where the efficiency of pigment degradation was assessed based on Hunter Lab and Lovibond color measurement systems, while in this study the absolute amounts of chlorophyll degradation were reported.

As shown in Table 3.2, the reduction of chlorophyll-*a* was comparatively higher than that of chlorophyll-*b*, pointing to an unequal stability of these isomeric forms. Similarly, in the case of diethyl ether extraction, increasing ultrasound power drastically reduced the total chlorophyll of hemp seed oil with comparatively higher reduction of chlorophyll-a compared to chlorophyll-*b*. Several researchers reported a faster degradation of 2.5-10 times for chlorophyll-*a* compared to chlorophyll-*b*, with respect to the kinetics of chlorophyll degradation (LaJollo, Tannenbaum, & Labuza, 1971; Lorenzo & Schwartz, 1991).

The percentage reduction of total chlorophyll during ultrasound treatment (Power: 60%) was followed to understand the effect solvents (Table 3.3). Total chlorophyll reduction

was much higher when methanolic extracts were used for chlorophyll estimation, with aqueous methanol (960 mL/L) higher than absolute methanol. In the case of diethyl ether, there was no definite trend. Compared to percentage reduction, the actual reduction of total chlorophyll (μg/g) after ultrasound treatment is shown in Table 3.3 in relation to varying extraction conditions. All other types of extractions resulted in a chlorophyll reduction in a range of ~12-14 μg/g for hemp seed oil treated with ultrasound (Power: 60%).

It is evident from Table 3.3 that the percentage reduction of total chlorophyll was estimated to be higher in the case of methanolic extracts. However, in the present study, diethyl ether extraction resulted in higher values of chlorophyll compared to methanolic extraction. Normally chlorophylls are non-covalently bound to specific proteins to form chlorophyll-protein complexes (Green, Pichersky, & Kloppstech, 1991). During cold pressing of oil seeds, the chlorophyll-protein complexes are released into oil as such or as individual chlorophyll isomers due to the breaking of covalent bonds (Satoh, 1986). In hemp seed oil, chlorophyll may exist in two forms: bound and free forms. Methanol can only extract the free chlorophyll, while diethyl ether can extract part of bound chlorophyll too, which gives a higher absolute value in diethyl ether extraction (Table 3.1 & Table 3.2). This comparable amount of chlorophyll reduction (Table 3.3) possibly indicates that the degradation of chlorophyll during ultrasound treatment is mostly occurred in the free form; therefore both methanol and diethyl ether could extract it to the same extent. As a result, it is confusing to compare the percentage reduction of chlorophyll unless using the same solvent and extraction procedure.

Table 3.2 Effects of solvents on chlorophyll extraction of ultrasound treated hemp seed oil

Solvent	Ultrasound Power (%)	Chlorophyll-*a* (μg/g)	Chlorophyll-*b* (μg/g)	Total Chlorophyll (μg/g)
Methanol [1]	0	31.0±0.88^{h}	11.6±0.12^{g}	42.6±0.99^{f}
	20	29.6±0.44^{i}	10.8±0.10^{hi}	40.4±0.41^{g}
	40	26.3±0.54^{j}	10.2±0.30^{ij}	36.6±0.78^{h}
	60	20.2±0.47^{l}	8.1±0.15^{l}	28.2±0.46^{k}
Methanol (960 mL/L)	0	22.3±0.75^{k}	11.2±0.38^{gh}	33.5±1.09^{i}
	20	20.2±0.15^{l}	9.9±0.22^{j}	30.1±0.37^{j}
	40	17.5±0.07^{m}	9.1±0.18^{k}	26.6±0.21^{l}
	60	13.0±0.05^{n}	7.3±0.10^{m}	20.3±0.06^{m}
Diethyl ether [1]	0	48.7±0.43^{cd}	18.7±0.24^{a}	67.3±0.64^{a}
	20	47.8±0.49^{d}	17.5±0.20^{b}	65.3±0.61^{b}
	40	45.6±0.59^{e}	16.9±0.28^{c}	62.4±0.85^{c}
	60	39.6±0.19^{g}	13.7±0.17^{f}	53.3±0.36^{e}
Diethyl ether (950 mL/L)	0	53.0±0.46^{a}	14.8±0.73^{e}	67.8±1.11^{a}
	20	51.8±0.11^{b}	15.5±0.07^{d}	67.3±0.15^{a}
	40	49.5±0.30^{c}	15.2±0.10^{de}	64.7±0.33^{b}
	60	42.3±0.14^{f}	13.3±0.47^{f}	55.6±0.50^{d}

[a-n] Values are expressed as mean±standard deviations. Different superscript letters within the same columns are significant different (p<0.05).

[1] Absolute solvent

Table 3.3 Percentage and actual reduction of chlorophyll from ultrasound treated (P 60%) hemp seed oil

Solvent	Percentage (%)	Actual reduction (μg/g)
Methanol [1]	33.7	14.4
Methanol (960 mL/L)	39.4	13.2
Diethyl ether [1]	20.9	14.1
Diethyl ether (950 mL/L)	17.9	12.1

[1] Absolute solvent

3.4.3 Reduction of chlorophyll from hemp seed oil by ultrasonic bleaching with clays

Various bleaching clays were evaluated to establish their efficiency for removing chlorophyll in hemp seed oil simultaneously with ultrasound treatment (Figure 3.2). Bentonite (both activated and non-activated), sepiolite and a clay used for refining canola oil from a canola oil refining industry in Canada were used at 40 g/kg concentration as per previously published study (Su et al., 2013). Table 3.4 shows the changes in the chlorophyll-*a*, chlorophyll-*b* and total chlorophyll content of oil samples applied with ultrasonic bleaching with various bleaching clays. The results indicated that the ultrasonic bleaching of oil with clay significantly reduced the chlorophyll content compared to conventional bleaching using clay alone. Generally, the reduction of chlorophyll was increased as the power of ultrasound increased from 20 to 40% and indicated the following trend for chlorophyll reduction was: Clay from canola industry > activated bentonite > sepiolite > non-activated bentonite. At 60% ultrasound power, however, the total chlorophyll contents of oils bleached with clay from canola industry and activated bentonite were 4.7±0.11 μg/g and 7.5±0.77 μg/g respectively. This was higher than that of their respective samples treated at 40% ultrasound power. It is assumed that at the higher ultrasound power, the adsorptive efficiency of bleaching clay is being lost, resulting in the leaching out of some of the chlorophyll pigments back to the oil. This slight increase in the chlorophyll content may also be attributed to some of the degradation products of pigments, which showed maximum absorption at the same wavelength as the intact chlorophyll molecules.

Without any ultrasound treatment, sepiolite showed a maximum reduction of chlorophyll from 66.7±1.01 μg/g (control) to 31.5±0.12 μg/g (sepiolite). With respect to the quality of oil, it is recommended to choose the lowest ultrasound power when treating the oil to minimize the possible lipid oxidation. In the present study, chlorophyll reduction using various clays at 20% ultrasound power was compared. The total chlorophyll content

remaining in the oil samples (µg/g) treated with various clays were found to be in the following order: 0.4±0.06 (clay from canola industry) < 1.5±0.10 (activated bentonite) < 11.5±0.06 (sepiolite) < 35.3±0.21 (non-activated bentonite). This corresponds to a percentage reduction in chlorophyll degradation (%) of 99.4, 97.8, 82.7, and 47.1 for clay from canola industry, activated bentonite, sepiolite and non-activated bentonite respectively (Table 3.4). The results indicated the efficacy of ultrasonic bleaching with clay from canola oil refining industry and activated bentonite were higher than with sepiolite and non-activated bentonite for removing chlorophyll pigment from hemp seed oil. Su et al. (2013) observed that such a reduction in carotene is mainly due to pigment adsorption, thermal degradation of pigment and ultrasound cavitation process. The bleaching efficiency of oil was highest when bentonite was used. In the present study, though acid activated bentonite gave the best results compared to non-activated bentonite and sepiolite, the clay samples obtained from canola oil refining industry exhibited the best bleaching efficiency.

Figure 3.2 Color changes in hemp seed oil after ultrasonic bleaching (60% Power) with various clays (40 g/kg)

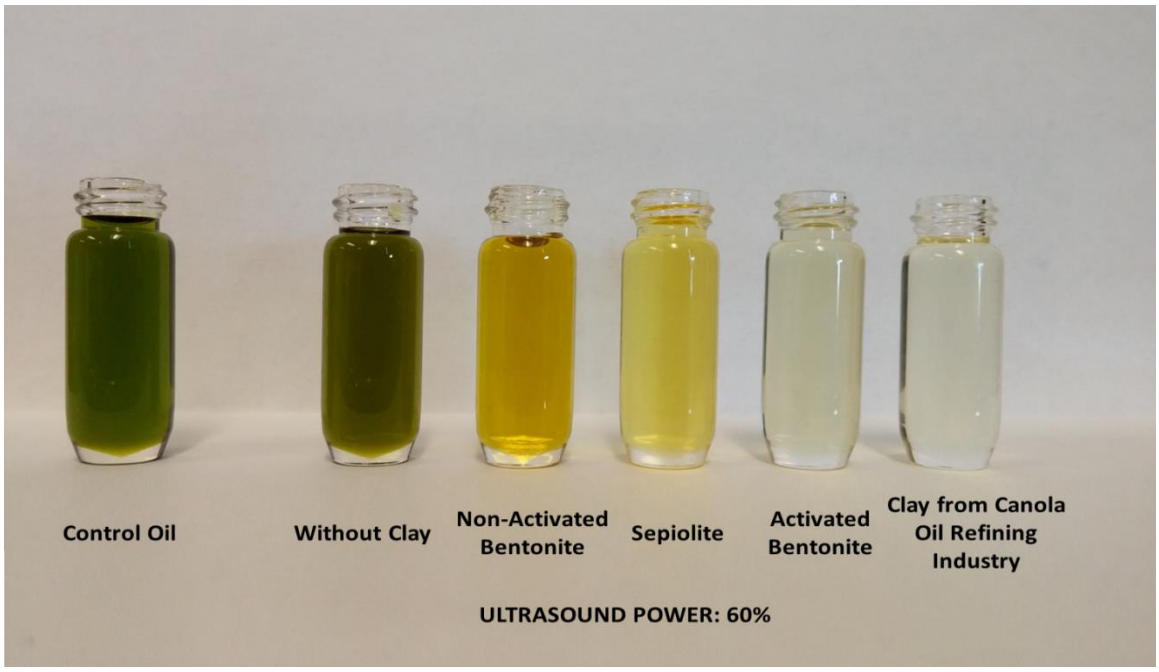


Table 3.4 Chlorophyll content of hemp seed oil applied with ultrasonic bleaching with various clays (40 g/kg)

Ultrasound Power (%)	Chlorophyll-a (µg/g)	Chlorophyll-b (µg/g)	Total Chlorophyll (µg/g)	Reduction (%)
Control Oil	49.1±0.32^{a}	17.6± 0.68^{c}	66.7±1.01^{a}	NA
		Non-Activated Bentonite		
0	43.7±0.27^{b}	22.1±0.19^{a}	65.8±0.15^{b}	1.3^{l}
20	20.3±0.15^{e}	15.0±0.06^{e}	35.3±0.21^{d}	47.1^{j}
40	16.6±0.17^{f}	13.8±0.00^{f}	30.3±0.17^{f}	54.5^{h}
60	11.1±0.22^{i}	11.5±0.19^{g}	22.6±0.41^{g}	66.1^{g}
		Activated Bentonite		
0	12.2±0.07^{g}	19.7±0.02^{b}	31.9±0.05^{e}	52.1^{i}
20	0.6±0.03^{n}	0.9±0.06^{k}	1.5±0.10^{l}	97.8^{b}
40	0.6±0.16^{n}	0.8±0.25^{k}	1.5±0.42^{l}	97.8^{b}
60	3.0±0.28^{k}	4.5±0.49^{i}	7.5±0.77^{i}	88.8^{e}
		Sepiolite		
0	24.9±0.17^{d}	6.5±0.27^{h}	31.5±0.12^{e}	52.8^{i}
20	11.5±0.06^{h}	ND	11.5±0.06^{h}	82.7^{f}
40	5.8±0.06^{j}	ND	5.8±0.06^{j}	91.3^{d}
60	1.3±0.03^{m}	ND	1.3±0.03^{l}	98.1^{b}
		Clay from Canola Oil Refining Industry		
0	40.6±0.17^{c}	15.9±0.04^{d}	56.5±0.21^{c}	15.3^{k}
20	0.2±0.03^{o}	0.2±0.03^{l}	0.4±0.06^{m}	99.4^{a}
40	0.2±0.01^{o}	0.3±0.01^{l}	0.5±0.30^{m}	99.3^{a}
60	1.8±0.03^{l}	2.9±0.08^{j}	4.7±0.11^{k}	93.0^{c}

$^{a-o}$ Values are expressed as mean±standard deviations. Different superscript letters within the same columns are significant different (p<0.05).

In the present study, we found that bleaching clay obtained from a canola oil refining industry was more efficient than the other bleaching clays in terms of chlorophyll degradation at 20% ultrasound power. The impact of reduced amount of clay (20 g/kg) in chlorophyll adsorption using clay from canola oil refining industry was shown in Table 3.5. Consequently concentration of clay at 20 g/kg proved less efficient than at 40 g/kg and the content of chlorophyll was found to be increased gradually at the higher ultrasonic power levels (defined 40 and 60%) compared to the lowest level of 20% ultrasound power. Thus it was possible to optimize the various parameters of bleaching including the concentration of clay, ultrasound power and treatment time etc.

Table 3.5 Chlorophyll content of hemp seed oil applied with ultrasonic bleaching with clay (20 g/kg) from canola oil refining industry

Ultrasound Power (%)	Concentration of pigments (μg/g)			Reduction (%)
	Chlorophyll-*a*	Chlorophyll-*b*	Total Chlorophyll	
Control Oil	49.1±0.32 [a]	17.6±0.68 [a]	66.7± 1.01 [a]	NA
0	44.1±0.33 [b]	16.9±0.08 [b]	61.0±0.42 [b]	8.5 [d]
20	0.9±0.07 [e]	1.1±0.02 [e]	2.0±0.10 [e]	97.0 [a]
40	2.2±0.03 [d]	2.8±0.01 [d]	5.0±0.04 [d]	92.5 [b]
60	5.2±0.05 [c]	6.7±0.34 [c]	11.9±0.30 [c]	82.1 [c]

[a-e] Values are expressed as mean±standard deviations. Different superscript letters within the same columns are significant different (p<0.05).

3.4.4 Effect of ultrasonic bleaching on phenolic compounds and primary oxidation products of hemp seed oil

Natural phenolic compounds, are attractive natural food additives because of their intrinsic antioxidant potential for protecting food products against lipid oxidation (Balasundram, Sundram, & Samman, 2006; Schmidt & Pokorny, 2005). In the current study, the cold pressed hemp seed oil had an average phenolic content of 162.5±2.85 μg/g GAE. Analysis of various cold pressed seed oils indicated that the total phenolic content of cold pressed hemp seed oil extract is 44.0 mg/100 g GAE (Yu, Zhou, & Parry, 2005). Similarly, hemp seed oil with a total phenolic acid content of 188.2 mg/100 g GAE was reported (Teh & Birch, 2013). In addition, phenolic content values ranging from 490 to 1194 mg/100 g GAE (defatted kernels) and 4080 to 10,920 mg/100 g GAE (defatted hull) for seeds of two hemp varieties cultivated in China was also reported (Chen et al., 2012).

We compared the total phenolic content of hemp seed oil treated with various bleaching clays, with or without ultrasound (Power: 20%). There was a considerable reduction in the total phenolic content of hemp seed oil owing to ultrasound treatment and bleaching. In the absence of bleaching clay, a 30.9% reduction in the total phenolic content was observed at 20% ultrasonic power as shown in Table 3.6, which was probably attributed to the cavitation and heat generated by ultrasound. The total phenolic content of oil treated with bleaching clays showed a lower value compared to the control oil. This reduction was further enhanced with the combined treatment of ultrasound and bleaching clays. The reduction in phenolic content was found to be 27.3%, 33.4%, 27.9% and 34.7% when clay from canola industry, activated bentonite, non-activated bentonite and sepiolite were used at 20% ultrasound power, respectively. This further reduction was attributed to the possible increased adsorption of phenolics in the presence of ultrasound irradiation.

Peroxide value (PV) is commonly used to determine the primary oxidation products,

which indicates the level of oxidative deterioration in heated oils. On an average, in the present study ~80 ℃, ~95 ℃ and ~120 ℃ were recorded at 20, 40 and 60% ultrasound power respectively, irrespective of the type of clay used. Since this high temperature generation is momentary and unlike conventional heating, its effect on oil quality may be minimal. The effect of ultrasonic bleaching on the oil quality was established based on the peroxide value of bleached and unbleached oil. Table 3.7 indicates the trend of PV in terms of mmol cumene hydroperoxide equivalent/kg of control oil and oils treated with ultrasonic bleaching (Power 20%). Overall no increase in the PV was observed with any of the samples treated using ultrasonic bleaching with clays.

The sample which was treated with ultrasound without any clay, showed an increase in the PV at 20% ultrasound power. Similarly, all the oil samples treated with bleaching clays without any ultrasound treatment showed a higher PV value than the control hemp seed oil. However, with the co-application of ultrasound and bleaching clay, the PV was significantly reduced possibly because the adsorption of oxidation products by the clay was enhanced by ultrasound power. The reduction of PV indicated the following decreasing trend: clay from canola industry > sepiolite > non-activated bentonite. An increase in primary oxidation products (conjugated hydroperoxides) in rapeseed oil at 60% ultrasound power or at 200 ℃ during the carotenoid bleaching was reported elsewhere (Su et al., 2013). The authors also observed similar content of secondary oxidation products between control and clay treated oils. In contrast to this study (Su et al., 2013), a recent study (Abedi et al., 2015) on bleaching of chlorophyll and carotenoids in soybean oil indicated a decrease in PV and TBA of oil upon increasing amount of bentonite (5-20 g/kg), which was in agreement with the findings in this study(Abedi et al., 2015).

Table 3.6 Total phenolic content of hemp seed oil applied with ultrasonic bleaching (20% Power) with various clays (40 g/kg)

Ultrasound Power (%)	Total phenolic content (μg/g) of hemp seed oil				
	Without Bleaching clay	Industrial Clay	Activated-Bentonite	Non-activated Bentonite	Sepiolite
0	162.5±2.85 [a]	142.1±4.09 [b]	128.5±3.40 [c]	128.2±4.76 [c]	126.8±4.67 [c]
20	112.3±14.18 [a]	118.1±0.77 [a]	108.2±4.97 [a]	117.1±3.92 [a]	106.0±5.37 [a]
Control oil: 162.5±2.85 μg/g					

[a-c] Values are expressed as mean±standard deviations. Different superscript letters within the same rows are significant different (p<0.05).

Table 3.7 The Peroxide value of hemp seed oil with or without ultrasonic bleaching (20% Power) using various clays (40 g/kg)

Ultrasound Power (%P)	Peroxide Value of hemp seed oil (mmol Cumene Hydroxide Equivalent/kg oil)			
	Without Bleaching clay	Industrial Clay	Non-activated Bentonite	Sepiolite
0	3.0±0.15 [b]	3.5±0.10 [ab]	3.6±0.31 [a]	3.2±0.32 [ab]
20	3.6±0.44 [a]	0.4±0.02 [d]	2.7±0.25 [b]	1.2±0.22 [c]
Control oil: 3.0±0.18 mmol/kg				

[a-c] Values are expressed as mean±standard deviations. Different superscript letters within the same rows are significant different (p<0.05).

3.5 Conclusion

The present study showed that ultrasound treatment along with the use of bleaching clay could be an effective alternative to other conventional bleaching processes. Advantages include reduction of treatment time, amount of bleaching clay, peroxide value of oil and chlorophyll content. By varying the amount of clay, duration of ultrasound treatment and its power, hemp seed oil with varying color attributes (light green through light yellow to colorless) can be produced. Since color is a major determinant of consumer acceptability, this aspect needs to be explored in further studies. Colorless hemp seed oil would be suitable for topographical applications. On the other hand, for cooking and other food applications, light green or yellow hemp seed oil are comparable to other popular oils in the market such as canola and sunflower.

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CHAPTER 4

MAMUSCRIPT 3: IMPACT OF ULTRASONIC BLEACHING ON CHLOROPHYLL CONTENT AND OXIDATIVE STABILITY OF COLD PRESSED HEMP SEED OIL

4.1 Abstract

The impact of ultrasonic bleaching on chlorophyll and oxidative stability of cold pressed hemp seed oil was evaluated in this study. 20% power of ultrasound reduced the chlorophyll content in hemp seed oil significantly (p<0.05) from 56.3 μg/g (control) to 9.9, 14.8 and 7.8 μg/g using the bleaching clays namely activated bentonite (ABT), sepiolite (SP) and industrial clay (IC). Peroxide value (PV) and conjugated diene (CD) decreased from 3.73 meq/kg and 0.102 % to 0.22, 1.94 and 0.11 meq/kg, and 0.079, 0.073 and 0.095 %, using ABT, SP and IC respectively. Accelerated oxidation test at 60 ℃ proved that the increases of PV and CD were significantly (p<0.05) slower in ultrasonic bleached oils (ABT, SP and IC) compared to the control oil. This test also supported that the lowered chlorophyll content in the treated oils remained constant. Chlorophyll in the control oil decreased from 56.3 to 36.8 μg/g after 7 days. This study indicated that oxidative stability of hemp seed oil can be enhanced after ultrasonic bleaching.

Key words Hemp seed oil; Chlorophyll; Ultrasound; Oxidative stability

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Chapter 4 was submitted as a manuscript entitled "Ultrasonic Bleaching of Cold Pressed Hemp (*Cannabis sativa L.*) Seed Oil: Effect on Reducing Chlorophyll Content and Improving Oxidative Stability" for publication in International Journal of Food Science & Technology. Authors: Jingbang Liang, Ayyappan Appukuttan Aachary, N. A. Michael Eskin, Arnold Hydamaka, Peter Eck and Usha Thiyam-Holländer.

4.2 Introduction

Industrial Hemp, a non-psychoactive variety of *Cannabis sativa L.*, is a versatile agricultural crop cultivated widely in Europe, China and North America (Johnson, 2014). Generally, the oil content of hemp seed from different cultivars ranges from 25-35% (Vonapartis, Aubin, Seguin, Mustafa, & Charron, 2015). Hemp seed oil contains a remarkably high amount (up to 80%) of polyunsaturated fatty acids (PUFAs) with an optimal ratio (3:1) of omega-6/omega-3, including 50~60% linoleic acid (LA), 20~30% α-linolenic acid (ALA). In addition, some varieties of hemp seed oil, for example *Finola*, have about 2% stearidonic acid (SDA, 18:4 ω-3) and up to 4% γ-linolenic acid (GLA, 18:3 ω-6), which are the metabolites of ALA and LA, respectively (Callaway, 2004; Deferne & Pate, 1996; Schwab et al., 2006). Cold pressed hemp seed oil is also a rich source of vitamin E compared to other vegetable oils (Abuzaytoun & Shahidi, 2006; Vonapartis et al., 2015). With its high nutritional values, hemp seed oil is considered as a functional food with important health benefits, including the effect of lowering cholesterol and blood pressure (Sapino, Carlotti, Peira, & Gallarate, 2005). A clinical study conducted by Schwab et al. (2006) found that the daily consumption of hemp seed oil can significantly affect the profile of serum lipids by increasing the proportion of LA and GLA in serum cholesteryl esters and triglycerides. A significant decrease in the ratio of total cholesterol and HDL cholesterol was observed during the hemp seed oil diet period, which implied a lower risk for cardiovascular diseases.

One of the main factors affecting the quality and shelf-life of edible oil is the oxidative deterioration that occurs during storage (Przybylski, Wu, & Eskin, 2013). Compared to microbial and enzymatic deteriorations, oxidative deterioration of edible oil occurs more quickly at room temperature because of low activation energy (Hahm & Min, 1995). Autoxidation of edible oil, a process in which oxidation products are formed, can reduce the nutritional values and result in rancidity. Consuming edible oil with a large amount of

oxidation products can be harmful to the human health (Sionek, Krygier, Ukalski, Ukalska, & Amarowicz, 2013). The rate of autoxidation is not only affected by the storage conditions, such as the presence of oxygen, temperature and light, but also depends on the types of oil and their fatty acid composition. Hemp seed oils with a large amount of PUFAs (80%) are especially prone to oxidation, because unsaturated fatty acids have reactive double bonds within their chemical structures (Miller, 2010). On the contrary, oils rich in saturated fatty acids are more stable against free radicals. Oil oxidation involves a complex series of chemical reactions, including the breakdown of triacylglycerols, release of free fatty acids and formation of primary and secondly oxidation products. As the formation of hydroperoxides is the first step of oil oxidation, peroxide value (PV) is commonly used as a quality parameter to measure the oil oxidative state (Hahm & Min, 1995). Generally, there are two types of methods to determine the peroxide value based on the reaction principle. The first type is iodometric titration, including the official methods of the American Oil Chemists' Society (AOCS Cd 8b-90) (AOCS, 2011), the Association of Analytical Communities International (AOAC 965.33) (AOAC, 2000) and the National Standard of the People's Republic of China (GB/T 5009.37) (Zhang et al., 2010). Another type of PV determination is the spectrophotometric ferric thiocyanate method or colorimetric method, including the ferrous xylenol orange (FOX) method and the International Dairy Federation (IDF) method. Other indices used to describe the oil oxidative status include acid value, conjugated diene (CD), *p*-anisidine value and thiobarbituric acid value.

Oil oxidative stability refers to oil products' resistance to the oxidation and the ability to maintain the same physical and chemical characteristics (Sionek et al., 2013). These oxidative processes usually occur from weeks to months, depending on the types of fats and storage and package conditions. Generally, regular or accelerated oxidation tests are used to determine the oxidative stability in the laboratory scale. Most of the accelerated oxidation

tests, such as Schaal oven test, Oil Stability Index (OSI) and Active Oxygen Method (AOM) are designed to facilitate the oxidation reactions by exposing the oil products to elevated temperature, controlled air flow or intensive light (Wan, 1995). The progress of oil oxidation can be monitored by measuring the formation of oxidation products or the consumption of the oxygen.

Aside from its high content of PUFA, another factor causing the instability of hemp seed oil is the presence of a substantial amount of chlorophyll. Chlorophyll and its derivatives originate from hemp seed and retained in oil during cold pressing. As photosensitive pigment, chlorophyll is susceptible to photo-oxidation and also acting as prooxidant in oils. Therefore, a large amount of chlorophyll not only results in the dark green color that limits the applications of hemp seed oil, but also increases the susceptibility to oxidation and hastens rancidity. Conventional bleaching and refining processes of edible oils, to some extent, can reduce the chlorophylls to a low level, but the prolonged treatment time and the large amounts of clay used intensify the oxidation and increase oil loss. Ultrasonic bleaching is a combination of the classic technique of bleaching edible oils with ultrasound (Jahouach-Rabai et al., 2008). Our recent study indicated that ultrasonic bleaching was an effective technique for removing up to 99% of chlorophylls from hemp seed oil (Aachary, Liang, Hydamaka, Eskin, & Thiyam-Holländer, 2016). However, the impact of ultrasonic bleaching on oxidative stability was not assessed.

Consequently, the main objective of this study was to investigate the influence of ultrasonic bleaching on chlorophyll, carotene content, peroxide value, conjugated diene and oxidative stability of hemp seed oil. The oxidative stability of oil samples was determined by the accelerated oxidation test. Oxidative stability of hemp seed oil was compared with common vegetable oils, as well as ultrasonic bleached oils.

4.3 Materials and Methods

4.3.1 Materials and chemicals

Three batches of cold pressed hemp seed oil from Hemp Oil Canada (Ste. Agathe, Manitoba, Canada) were used in this study. The fatty acid composition of hemp seed oil was shown in Table 4.1. Oil samples were shipped directly from the factory and delivered in opaque containers. Cold pressed canola oil (Mountainview Farming), refined canola oil (Canola Harvest), virgin olive oil and refined olive oil (Bertolli) were purchased from the local food market. Bleaching clay namely sepiolite and bentonite were purchased from Sigma-Aldrich (Canada). Industrial clay was obtained from a canola oil refining industry in Canada. Activation of bleaching clay was conducted by the optimized procedures reported by Didi et al. (2009) and Makhoukhi et al. (2009). Diethyl ether, chloroform, methanol, acetic acid, isooctane and cyclohexane were of analytical grade and purchased from Sigma-Aldrich and Fisher Scientific.

Table 4.1 Fatty acid composition (%) of hemp seed oil[1]

C:D[2]	Fatty Acid	Percent (%) of total Fat
14:0	Myristic acid	0.08
16:0	Palmitic acid	5.79
18:0	Stearic acid	2.28
18:1	Oleic acid (Omega 9)	10.32
18:2	Linoleic acid (LA) (Omega 6)	55.77
18:3	Alpha linolenic acid (ALA) (Omega 3)	18.33
18:3	Gamma linolenic acid (GLA) (Omega 6)	3.98
18:4	Stearidonic acid (SDA) (Omega 3)	1.18
20:0	Arachidic acid	0.79
22:0	Behenic acid	0.28
24:0	Lignoceric acid	0.13

[1] Product specification data as reported by Hemp Oil Canada Inc. (Permission pending)

[2] Ratio of carbon atom and double bond

4.3.2 Ultrasonic bleaching of hemp seed oil

Ultrasonic bleaching followed the procedures described by Aachary et al. (2016) with slight modification. Ultrasound treatment was performed by a SONOPLUS HD 2200 Ultrasonic Homogenizer (20 kHz, BANDELIN electronic GmbH & Co. KG, Heinrichstraβe, Berlin, Germany) consisting of a HF Generator (GM 2200) and an ultrasonic converter (UW 2200). Briefly, 25.00 g oil sample was weight into a 50 mL centrifuge tube and mixed thoroughly with 1.00 g of bleaching clay by vortexing. The oil and clay mixture was treated for 20 minutes with ultrasound at 20% power (expressed as percentage of pressure amplitude) and pulsation mode four (0.4s active interval per second). Oil samples were continuously flushed with nitrogen during the ultrasound treatment. The temperature of oil mixture was recorded immediately after ultrasound treatment. Bleached oil was centrifuged at 5000 RPM for 10 minutes to separate the bleaching clay until the oil was clear. The bleached oil samples were stored at -20 ℃ until further analysis. The treatments were performed in duplicates.

4.3.3 Oxidative stability

Oil oxidative stability was determined by modified Schaal oven test (Sionek et al., 2013; Wan, 1995). 25 g of oil sample was placed in a 50 mL glass test tube. The tubes were filled with nitrogen in the headspace and closed tightly, after which the oil samples were incubated in an oven at 60 ℃ and 40 ℃ to accelerate oxidation. About 1.5 mL of oil sample was collected from the glass test tube periodically and stored at -20 ℃ for quality measurements. The accelerated oxidation tests were conducted in duplicates.

4.3.4 Analytical methods

4.3.4.1 Chlorophyll and carotene content of oil

Extraction and quantification of chlorophyll and carotene was performed by the optimized procedures described by Aachary et al (2016). Briefly, about 1.0 g of oil sample was dissolved with absolute diethyl ether in a 50 mL centrifuge tube. The solution was mixed

thoroughly by vortexing and extracted by ultrasound at a power of 40% for 1 min. The absorbance of the solution was measured at 470 nm, 640 nm and 663 nm by a DU 800 UV/Vis spectrophotometer (Beckman Coulter Inc., Mississauga, Canada) against the solvent as blank. The concentrations (μg/mL) of chlorophyll and total carotene were calculated according to the equations of Aladić et al. (2015):

$$chlorophyll\ a = 9.93 \times A_{663} - 0.78 \times A_{640}$$

$$chlorophyll\ b = 17.60 \times A_{640} - 2.81 \times A_{663}$$

$$chlorophyll\ a + b = 7.12 \times A_{663} + 16.80 \times A_{640}$$

$$Total\ carotene = \frac{(1000 \times A_{470} - 0.52 \times Chl\ a - 7.25 \times chl\ b)}{226}$$

The amount of pigment in the oil was calculated as micrograms of pigment per gram of oil (μg/g):

$$c = \frac{c_1 \times V \times R}{G}$$

Where: c - amount of pigment in oil (μg/g); c_1 - concentration of pigment (μg/mL); V - initial volume (mL); R - dilution (if any); G - measure oil mass (g)

4.3.4.2 Peroxide value

Peroxide value of oil sample was determined by both spectrophotometric method and iodometric titration method. Spectrophotometric method was performed according to the modified official International Dairy Federation (IDF) method (Shantha & Decker, 1994). The oil sample (10-50 mg, depending on the extent of oxidation) was mixed in a disposable glass tube with 9.8 ml chloroform-methanol (7:3 v/v) by vortexing for 2 to 4s. Ammonium thiocyanate solution (50 μL, 30% w/v) and 50 μL of iron (II) chloride solution were added to the solution, following by 2 to 4s vortexing to mix thoroughly. After incubation (5 minutes) at room temperature, the absorbance of the sample was measured by spectrophotometer at 500

nm against a blank that contained all the reagents except the sample. The peroxide value, expressed as milliequivalents of peroxide per kilogram of sample (meq/kg), was calculated by the following equation:

$$Peroxide\ Value = \frac{(A_s - A_b) \times m}{55.84 \times m_0 \times 2}$$

Where: A_s - absorbance of the sample; A_b - absorbance of the blank; m - slope of the calibration curve, m was determined as 41.52 for this experiment; m_0 - mass in grams of the sample; 55.84 - atomic weight of iron.

As for iodometric titration, the peroxide values of oil samples were determined by the AOCS official method Cd 8b-90 (AOCS, 2011).

4.3.4.3 Conjugated diene

Conjugated diene of oil sample was conducted by the AOCS method Ti 1a-64 (AOCS, 2009) with minor modification. 10-50 mg oil samples were weight into a test tube and dissolved in 5 mL cyclohexane. The mixture was vortexed thoroughly for 1 min. The absorbance was read at 234 nm using the solvent as blank. The readings should be between 0.2 and 0.8, otherwise change the weight of the oil sample or dilute more to get the required reading. The CD value was reported as a percentage of conjugated dienoic acid and is calculated according to the following equation:

$$Conjugated\ Diene\ (\%) = 0.84 \times (\frac{A_s}{bc} - K_0)$$

Where: A_s - absorbance of the solution at 234 nm; b - cell length (cm); c - concentration of the solution in g/L of the final dilution used for the absorption measurement.

4.3.5 Statistical analysis

The analyses were performed in triplicate and the results were reported as mean values ± standard deviations. The differences between mean values were determined by One-Way Analysis of Variance (ANOVA) and Independent-Sample T Test using SPSS Statistics version

22 (IBM, New York, USA). The post hoc multiple comparisons were conducted by Tukey's Honestly Significant Difference (HSD) Test. The statistical significance level was accepted at $p<0.05$. The relationship between analyzed parameters was evaluated by Peason's correlation coefficient using SPSS Statistics version 22 (IBM, New York, USA). Correlation is significant at the level of $p<0.01$.

4.4 Results and Discussion

4.4.1 Determination of peroxide value of oils

In this study, the peroxide value (PV) of hemp seed oil and other four common vegetable oils were measured by both the iodometric titration and spectrophotometric methods (Table 4.2). Among these oil samples, refined canola oil had the lowest PV with 1.27±0.15 and 0.75±0.01 meq/kg determined by iodometric titration and spectrophotometry, respectively. Virgin olive oil had the highest PV of 12.02±1.19 and 10.5±0.63 meq/kg, respectively. The PVs for virgin oils (canola and olive oil) are generally higher than those of refined oils. Hemp seed oil produced by cold pressed is considered virgin oil. It had a similar PV to virgin canola oil with 3.20±0.60 compared to 5.65±0 meq/kg (virgin canola oil) using iodometric titration, and 3.73±0.18 compared to 5.27±0.05 meq/kg (virgin canola oil) using spectrophotometry, respectively. The PVs of virgin canola oil and refined olive oil obtained by iodometric titration were slightly higher (p<0.05) than those obtained by spectrophotometric method. No statistical difference for peroxide values of hemp seed oil, refined canola oil and virgin olive oil were observed.

Table 4.2 Peroxide values of vegetable oils determined by different methods

Oil Samples	Peroxide Value (milliequivalents of peroxide/kg)	
	Iodometric Titration Method	Spectrophotometric method
Hemp Seed Oil	3.20±0.60[a]	3.73±0.18[a]
Refined Canola Oil	1.27±0.15[a]	0.75±0.01[a]
Virgin Canola Oil	5.65±0.00[a]	5.27±0.05[b]
Virgin Olive Oil	12.02±1.19[a]	10.5±0.63[a]
Refined Olive Oil	2.51±0.03[a]	1.77±0.02[b]

[a-b] Values are expressed as mean±standard deviations. Different superscript letters within the same rows are significantly different (p<0.05).

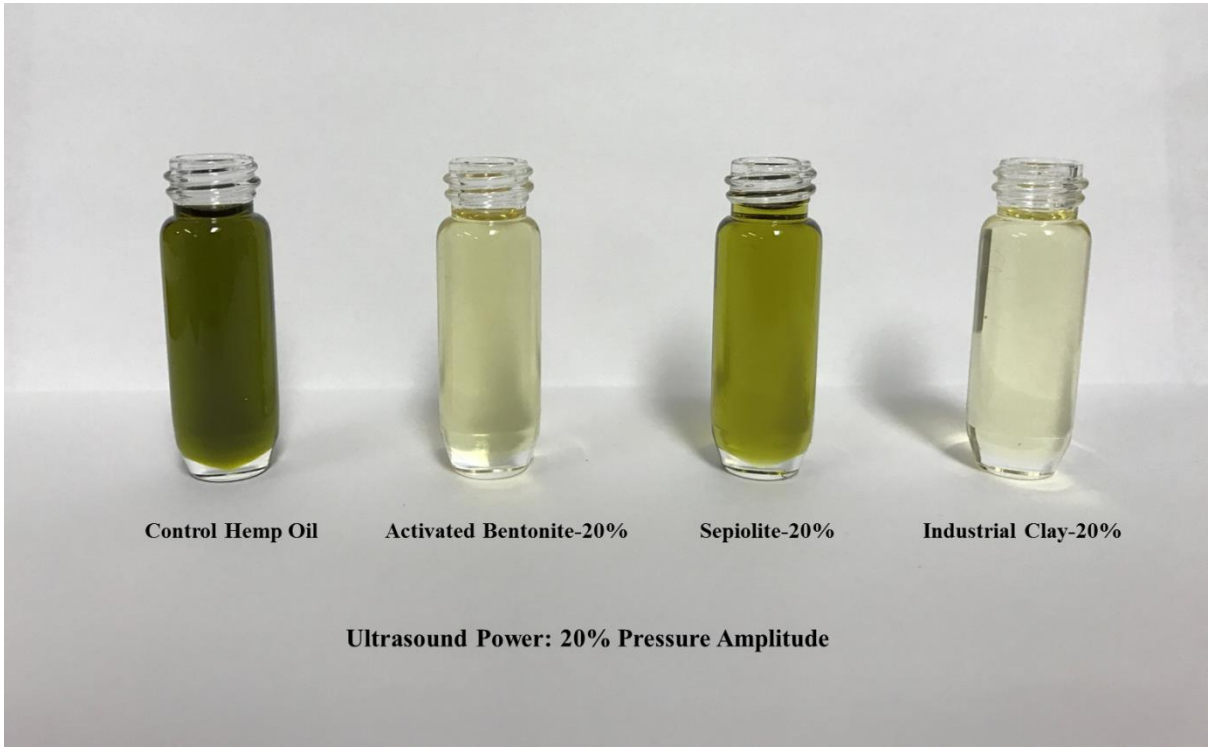
The principles of PV determination are mainly based on redox reactions. Iodometric titration is based on the oxidation of iodide ions (I^-) to iodine (I_2) with the presence of hydroperoxides, following by titrating with sodium thiosulfate with starch solution as endpoint indicator. Iodometric titration is a reliable and practical method for PV determination with no requirement for special instruments. However, this method was time consuming, labor intensive and required a large amount of oil and solvent (Dobarganes & Velasco, 2002; Ruiz, Canada, & Lendl, 2001). In addition, oil samples with large amounts of pigments, such as hemp seed oil, virgin canola oil and virgin olive oil, are difficult to observe the endpoint since the green and yellow pigments interfere with the color change during the iodometric titration. In terms of the spectrophotometric ferric thiocyanate method, the advantages are more sensitive and requiring smaller amounts of oil sample and solvent compared to iodometric titration. The rationale of this method is based on the ability of hydroperoxides to oxidize ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}). The ferric ions form chromophores with thiocyanate and the ferric thiocyanate is a red complex that can be measured by spectrophotometer at 500 nm (Eymard & Genot, 2003). Ferrous ions (Fe^{2+}) are more stable than iodide ions (I^-) against oxygen in the air or light, which increase the sensitivity of this method. In the present study, the higher PV obtained from iodometric titration is possibly due to the iodine reacted with other oxidation products that similar to hydroperoxides. Considering the fact that spectrophotometric method is a rapid method and requires smaller sample size, this method would be more appropriate for oil samples obtained from accelerate oxidation test. Therefore, the spectrophotometric ferric thiocyanate method was used for further PV analysis of oil samples in this study.

4.4.2 Impact of ultrasonic bleaching on hemp seed oil quality

The primary goal of oil bleaching process is to remove the pigments and other foreign matters by chemical and physical reactions, in order to reduce the color and obtain a lighter

oil product (Smouse, 1995). In this study, bleaching clay was combined with ultrasound as a more effective and eco-friendly way to remove the chlorophylls and other pigments in hemp seed oil. Three different types of bleaching clays (activated bentonite, sepiolite and industry clay) were applied simultaneously with ultrasound treatment at a power of 20% pressure amplitude. The effect of ultrasonic bleaching was shown in Table 4.3. The results indicated that chlorophyll and carotene content, the two major pigments in hemp seed oil, were reduced sharply after ultrasonic bleaching at 20% power (Figure 4.1). The effectiveness of ultrasonic bleaching in reducing the total chlorophyll content were 86.1% (IC20%) > 82.4% (ABT20%) > 73.7% (SP20%). Chlorophyll is also a strong prooxidant, so that reducing its level would improve the oil's oxidative stability as well as making a lighter oil product acceptable to the consumers.

Figure 4.1 Changes in color of hemp seed oil after ultrasonic bleaching (Power: 20% pressure amplitude) with various clays



Both peroxide value (PV) and conjugated diene (CD) are common parameters for assessing oil oxidation. In this study, the PV of fresh hemp seed oil was found to be 3.73±0.18 meq/kg. The PVs of hemp seed oil treated with ultrasonic bleaching were reduced to 0.22±0.01 meq/kg (ABT20%), 1.94±0.05 meq/kg (SP20%) and 0.11±0.03 meq/kg (IC20%), respectively. The CDs also decreased from 0.102±0.007% (control hemp oil) to 0.079±0.001% (ABT20%), 0.073±0.002% (SP20%) and 0.095±0.001% (IC20%), respectively. PV measures the formation of hydroperoxides, which is one of the primary oxidation products of oil oxidation. CD measures the formation of conjugated hydroperoxides by shifting in the position of double bonds in the unsaturated fatty acids (Lukešová, Dostálová, El-Moneim Mahmoud, & Svárovská, 2009). Generally, PV and CD are highly correlated for most vegetable oils at the initial stage of oil oxidation (Farhoosh & Moosavi, 2009; White, 1995). The current study also indicated the similar decreasing trend of PV and CD after ultrasonic bleaching. A previous study by Abedi et al. (2015) reported a decrease of PV and thiobarbituric acid value (TBA) in soybean oil after bleaching by ultrasonic processing with increasing amount of bleaching clay. On the contrary, Teh and Birch (2013) reported an increase of CD in rapeseed oil after ultrasound treatment at a power of 60% pressure amplitude. It is believed that the formation and collapse of bubbles generated by the ultrasound wave cause an increase of oil temperature. In this study, after the ultrasonic bleaching, oil temperature increased from room temperature to around 80 ℃, irrespective the types of clay (Table 4.3). The thermal effect generated by continuously high-power ultrasound treatment would induce oil oxidation and increase the primary oxidation products. The reductions of PV and CD, however, are possibly due to the adsorptive effect of bleaching clay. This study was in agreement with Abedi et al. (2015) that the primary and secondly oxidation products were adsorbed and eliminated using a combination of ultrasound and bleaching clay.

As mentioned above, continuous treatment of high power ultrasound would increase oil temperature, which might have adverse effects on the oil products. Carotene and total phenolic content, which are oil bioactive compounds with antioxidant activity, were observed to decrease after ultrasonic bleaching (Table 4.3). Even though increasing the ultrasound power produced a higher reduction rate of chlorophyll (Aachary et al., 2016; Abedi et al., 2015), a medium power of ultrasound would be more appropriate by minimizing the side effect of higher temperature. Therefore, in this study, ultrasound power at 20% pressure amplitude was used for oil bleaching.

Table 4.3 Impact of ultrasonic bleaching (20% Power) on oil quality

Oil Quality Parameters	Control Hemp Oil	ABT20%[1]	SP20%[2]	IC20%[3]
Total Chlorophyll (μg/g)	56.3±0.6[a]	9.9±0.7[c]	14.8±1.1[b]	7.8±1.2[d]
Total Carotene (μg/g)	23.4±0.2[a]	2.9±0.3[c]	4.0±0.3[b]	2.3±0.4[c]
Peroxide Value (meq/kg)	3.73±0.18[a]	0.22±0.01[c]	1.94±0.05[b]	0.11±0.03[c]
Conjugated Diene (%)	0.102±0.007[a]	0.079±0.001[b]	0.073±0.002[b]	0.095±0.001[a]
Total Phenolic Content* (μg/g GAE[4])	162.5±2.9[a]	108.1±5.0[c]	106.0±5.4[c]	118.1±0.8[b]
Oil Temperature (°C)	RT[5, b]	82±4[a]	83±1[a]	83±6[a]

[1-4] ABT20%: Hemp seed oil treated with Activated Bentonite and 20% Ultrasound Power; SP20%: Hemp seed oil treated with Sepiolite and 20% Ultrasound Power; IC20%: Hemp seed oil treated with Industrial clay and 20% Ultrasound Power; GAE: gallic acid equivalent; RT: Room Temperature.

* Data reported by Aachary et al. (2016).

[a-c] Values are expressed as mean±standard deviations. Different superscript letters within the same rows are significantly different (p<0.05).

4.4.3 Oxidation stability test

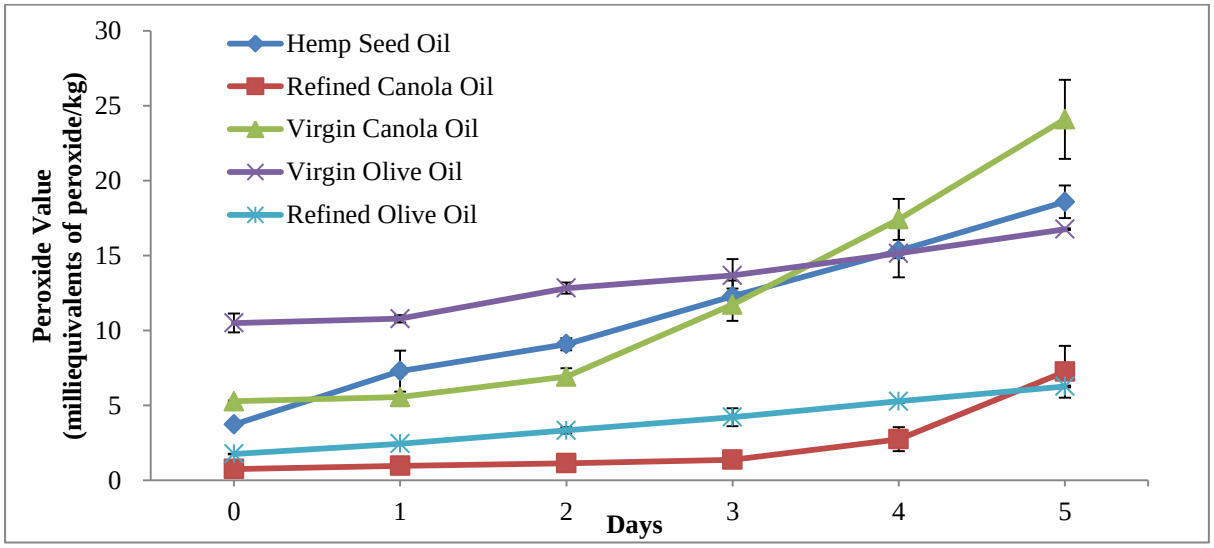
4.4.3.1 Comparison of oxidation stability of hemp, canola and olive oil

Oil quality is the present state of oil acceptability, while oil oxidative stability is the resistance to oxidation and the ability to maintain the physical and chemical properties of the oil (Sionek et al., 2013; Smouse, 1995; Velasco & Dobarganes, 2002). Many factors can affect the oil oxidative stability, including the oil endogenous components (fatty acid composition, pigments and moisture etc.), oil processing (refining, bleaching, deodorizing) as well as packaging, storage conditions and additives. In this study, oxidative stability of hemp seed oil was compared with four common vegetable oils as measured by PV.

The changes in PV of different oil during accelerated oxidation (60℃) are presented in Figure 4.2. Initially, hemp seed oil and virgin canola oil had a moderate PV (3.73±0.18 and 5.27±0.048 meq/kg), while refined canola oil and refine olive oil had low PVs (0.75±0.012 and 1.77±0.022 meq/kg). In contrast, virgin olive oil showed a relatively high initial PV as 10.5±0.63 meq/kg. During the accelerated oxidation, a continuous increase in PV was observed for all oil samples. For hemp, virgin canola and virgin olive oil, PV increased rapidly over 5 days' storage to 18.59±1.09, 24.1±2.639 and 16.76±0.053 meq/kg, respectively. On the contrary, refined canola oil and refined olive oil were more stable and their PVs slightly increased to 7.25±1.72 and 6.27±0.06 meq/kg at the end of the 5th day. Hemp oil consists of up to 80% PUFAs, while canola and olive oil have a high content of MUFAs. Unsaturated fatty acids are susceptible to oxidation. Moreover, the pigments in the oil are strong prooxidants. Therefore, the oxidative stability of virgin oil is usually lower than refined oil. During the 5 days' storage, refined canola oil and refined olive oil had significantly lower PV than the corresponding virgin canola oil and virgin olive oil (p<0.05), indicating that the oxidative stability of refined oils were better than the virgin oils at higher temperature (60℃). Consequently, reducing the chlorophyll content by ultrasonic bleaching

should also improve the oxidative stability of cold pressed hemp seed oil.

Figure 4.2 Changes in peroxide value of common vegetable oils in accelerated oxidation test (60℃)



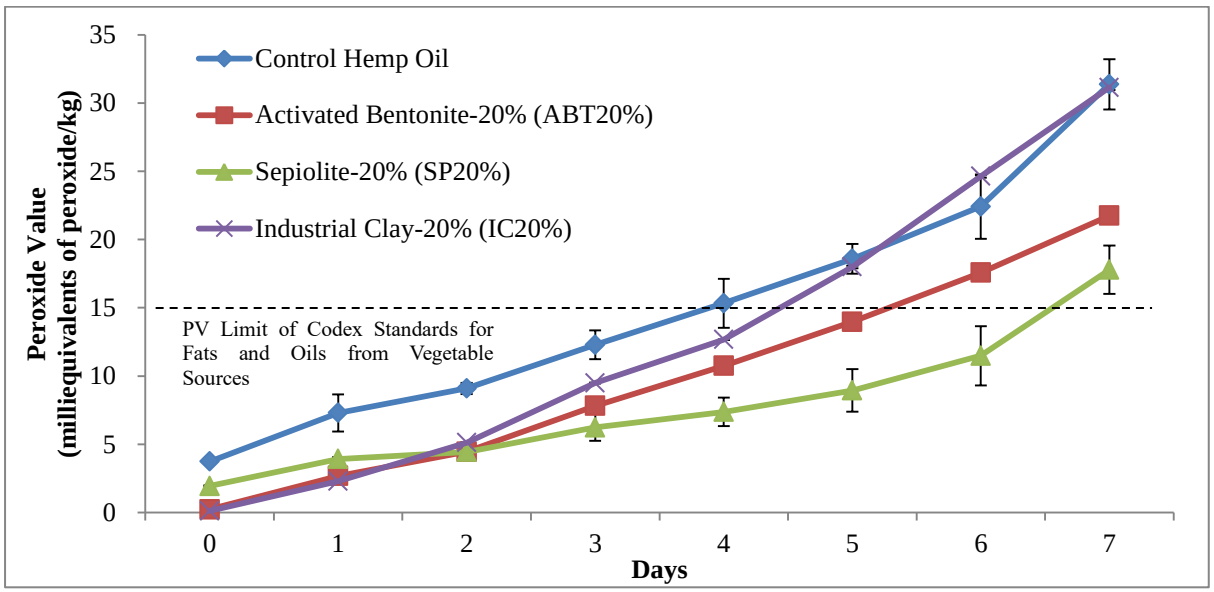
4.4.3.2 Impact of ultrasonic bleaching on oxidative stability of hemp seed oil

The oxidative stability of hemp seed oil treated by ultrasonic bleaching was evaluated by accelerated oxidation test at 60℃ and 40℃. Hemp seed oil was treated with three different types of bleaching clay, namely activated bentonite (ABT20%), sepiolite (SP20%) and industry clay (IC20%), simultaneously with ultrasound treatment at a power of 20% pressure amplitude. The changes in PV, CD and chlorophyll content during the storage at 60 ℃ were measured to determine oxidative stability of bleached oil samples.

As shown in Figure 4.3, hemp oil samples treated by ultrasonic bleaching (ABT20%, SP20% & IC20%) had lower initial PVs compared to the control hemp oil. A gradual increase in PV was observed for all oil samples stored at 60 ℃ during 7-days storage. However, the PV increased at a much slower rate for ABT20%, SP20% and IC20% compared to the control oil and the PV of these three oil samples were significantly lower (p<0.05) until the 5th day.

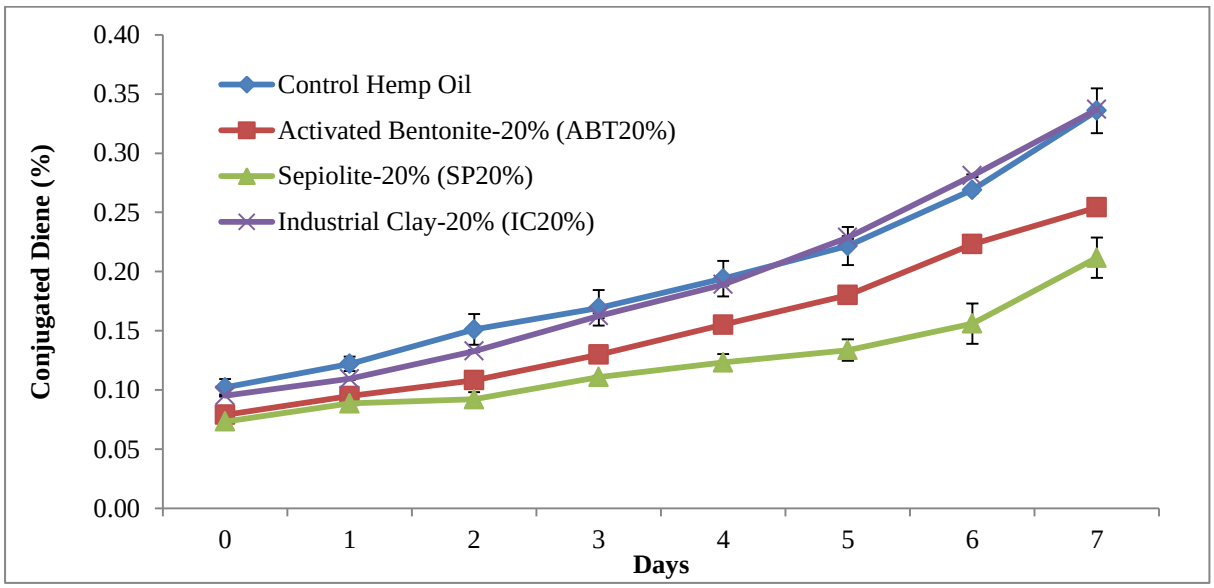
Among the different treatments, oil sample treated with SP20% had highest oxidative stability, followed by ABT20%, while IC20% was comparable to the control oil after 5 days' storage. According to the standard of the Codex Alimentarius Commission (2001), the maximum PV level for cold pressed and virgin oils is up to 15 meq/kg. The control oil sample reached this PV maximum level by 4 days at 60 ℃, while SP20% extended this period to 7 days.

Figure 4.3 Changes in peroxide value of hemp seed oil treated with ultrasonic bleaching in accelerated oxidation test (60 ℃)



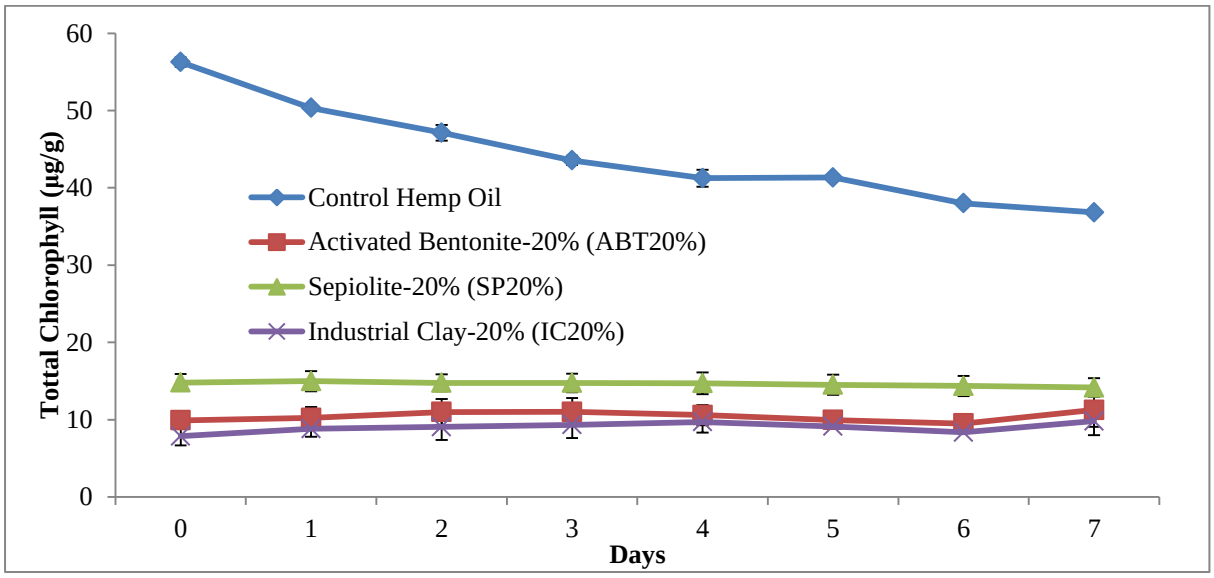
Changes in CD are presented in Figure 4.4. During the 7 days' storage, the increasing trend of CDs for the four hemp seed oil samples followed the trend for PVs. CDs for ABT20% and SP20% treated oils were significantly lower (p<0.05) than control hemp oil, while CD for IC20% treated oil was similar to control hemp oil. These results further confirmed the improved oxidative stability of hemp seed oil following the ultrasonic bleaching (ABT20% or IC20%).

Figure 4.4 Changes in conjugated diene of hemp seed oil treated with ultrasonic bleaching in accelerated oxidation test (60 ℃)



As for chlorophyll content (Figure 4.5), the control hemp oil was significantly higher (p<0.05) in chlorophyll (56.3 µg/g) in the initial stage compared to the ABT20% (9.9 µg/g), SP20% (14.8 µg/g) and IC20% (7.8 µg/g). The high chlorophyll content in the control hemp oil was not stable during the accelerated storage with the values decreasing rapidly to 36.8µg/g at the end of 7 days' storage. In contrast, the chlorophyll content in hemp seed oil samples after ultrasonic bleaching, using different clays, were significantly lower with the values remaining constant throughout 7 days as shown in Figure 4.5. The decreasing trend of chlorophyll in control hemp seed oil indicated the degradation during storage and reflected the poor stability of high chlorophyll content. On the other hand, reducing chlorophyll content to a lower concentration by ultrasonic bleaching can greatly improve the stability of hemp seed oil during storage.

Figure 4.5 Changes in total chlorophyll content of hemp seed oil treated with ultrasonic bleaching in accelerated oxidation test (60 ℃)



Correlations between PV, CD and total chlorophyll content of the control and treated hemp seed oils subjected to accelerated oxidation test (60 ℃) were assessed using the Pearson correlation coefficient (Table 4.4). PV showed a significantly positive correlation with CD (R=0.990-1.000, $p<0.01$) for all the hemp seed oil samples in this study, indicating that lipid hydroperoxides and conjugated diene formed and accumulated during the initial stage of hemp seed oil oxidation. These results were in agreement with the correlation coefficients ($R^2=0.993$) reported by Hahm & Min (1995). These authors also suggested that PV and CD can be used independently to evaluate the oxidative stability of an oil sample or to complement each other during the initial oxidation state.

On the other hand, PV had a significantly negative correlation with total chlorophyll content for the control hemp seed oil (R=-0.901, $p<0.01$). However, there is no statistically significant correlation between PV and total chlorophyll content for ultrasonic bleached oils. Control hemp seed oil contained a large amount of chlorophyll (56.3±0.6 μg/g). After ultrasonic bleaching (ABT20%, SP20% and IC20%), chlorophyll content reduced to 9.9, 14.8 and 7.8 μg/g respectively, and the content remained constant during storage.

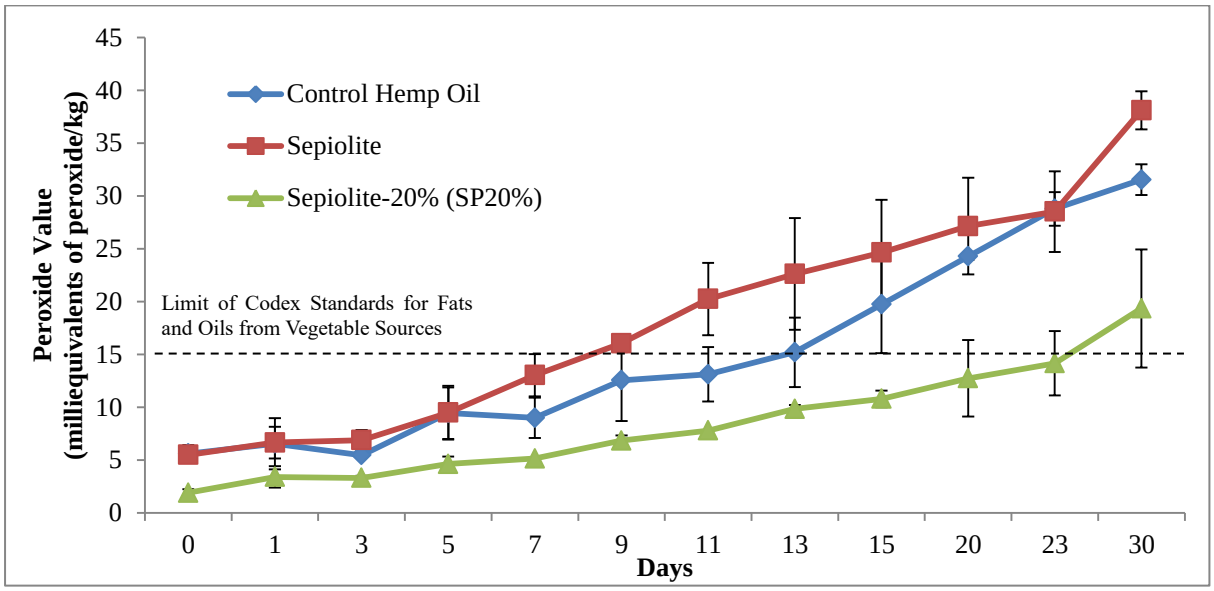
Table 4.4 Correlation between analyzed parameters during accelerated oxidation test (60 ℃) of hemp seed oil

Treatment	Peroxide Value-Conjugated Diene	Peroxide Value-Chlorophyll
	R	R
Control Hemp Oil	0.990*	-0.901*
Activated Bentonite-20% (ABT20%)	0.996*	0.028
Sepiolite-20% (SP20%)	0.996*	-0.046
Industrial Clay-20% (IC20%)	1.000*	0.215

R: Pearson correlation coefficient; *Correlation is significant at the level of $p<0.01$

The impact of ultrasonic bleaching on oxidative stability was also evaluated during accelerated storage at 40 ℃ (Figure 4.6). The effect of ultrasonic bleaching (SP20%) on PV was compared with clay bleaching without ultrasound (Sepiolite). All oil samples stored at 40 ℃ showed gradually increases in PV, although the rate of PV was slower than oil samples stored at 60 ℃. During 30 days' storage at 40 ℃, PV of hemp seed oil treated with sepiolite and 20% ultrasound increased from 1.89±0.35 meq/kg to 19.35±5.59 meq/kg, compared to from 5.48±0.58 meq/kg to 38.11±1.81meq/kg for hemp seed oil treated only with sepiolite. PVs of sepiolite-treated oil were comparable to control hemp oil and were significantly higher (p<0.05) than the ultrasonic bleaching (SP20%) treated oil. It was evident that hemp seed oil bleached by sepiolite alone did not show improvement in oxidative stability compared to the control oil; however, when combined ultrasound treatment with bleaching clay, the oxidation of hemp seed oil were slow down as measured by PV.

Figure 4.6 Changes in peroxide value of hemp seed oil treated with ultrasonic bleaching and clay bleaching without ultrasound in accelerated oxidation test (40 ℃)



Few studies are available on the oxidative stability of hemp seed oil. Matthäus and Brühl (2008) reported the development of peroxide value of virgin hemp seed oil during storage at room temperature. The PV showed a similar trend as the present study, increasing from 3 meq/kg to around 10 meq/kg after 12 weeks. Aladić et al. (2015) conducted an accelerated oxidation test (60°C) to determine the effect of additional antioxidants on oxidative stability of cold pressed hemp oil. PV of hemp oil without antioxidants increased from 2 mmol O_2/kg to 9 mmol O_2/kg within 4 days. In general, pigments in edible oil play an important role in their oxidative stability. During bleaching, pigments are adsorbed by clays and removed in the later processing. A reduction in pigments and other minor components could normally improve oil stability. However, using bleaching clays at elevated temperature for a long period of time would cause hydrolysis of fatty acids and induce oil oxidation (Smouse, 1995). Chlorophyll and its derivatives exhibit great prooxidative activity in oils. Tautorus et al. (1993) found that canola oil with additional chlorophyll showed a rapid increase in conjugated diene during accelerated storage, which proved the prooxidative effect of chlorophyll in oils. Mag (1989) reported that reducing the chlorophyll and its derivatives to a low concentration can avoid the rapid oxidation of the oil, which is in accordance with the findings of the present study.

4.5 Conclusion

As shown in the present study, ultrasonic bleaching can effectively remove a large amount of chlorophyll from cold pressed hemp seed oil producing lighter and pale colored oil. This treatment also reduced the primary oxidation products. During the accelerated oxidation tests, hemp seed oil sample treated with ultrasonic bleaching exhibited a slower development of primary oxidation products compared to the control hemp seed oil. The results of ultrasonic bleaching suggested its potential for prolonging the shelf-life. Utilizing the ultrasonic bleaching technique as an alternative to conventional bleaching would be

beneficial to the edible oil industry. This lighter hemp seed oil could be more competitive and expand its applications in the food and non-food sector through enhanced oxidative stability.

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CHAPTER 5

MANUSCRIPT 4: ANTIOXIDATIVE COMPONENTS FROM BY-PRODUCTS (MEALS) OF COLD PRESSED CANOLA AND HEMP OILS

5.1 Abstract

Cold pressed canola and hemp meals are oil processing by-products obtained after mechanical extraction at a relatively low temperature (50 ℃). These meals or press cakes retain a substantial amount of phenolic compounds. As a result, the phenolic extracts of these meals were investigated for their antioxidative properties. The content and antioxidant activity of these meal extracts are highly associated with the extraction solvents and the heat treatment conditions. In this study, the extraction of phenolic compounds from cold pressed canola and hemp meals was conducted using different solvents namely aqueous methanol (70%), aqueous acetone (80%) and a solvent-mixture of aqueous methanol (70%) and aqueous acetone (70%) in a ratio of 1:1 (v/v). For canola meal, the solvent-mixture extracted slightly higher (p<0.05) total phenolic content, while aqueous methanol (70%) extracts exhibited higher DPPH scavenging effect. For hemp meal, aqueous acetone (80%) extracts exhibited higher total phenolic content and DPPH scavenging effect. Total phenolic content and antioxidant activity in canola meal extracts were significantly higher (p<0.05) than those in hemp meal extracts. In addition, canola and hemp meals were heated at 140, 160 and 180 ℃ to determine the effect of heat treatment on phenolic profiles and antioxidant activity. The canola and hemp meal extracts showed equal or lower total phenolic content and DPPH scavenging effect when the temperature and treatment time increased.

Keywords: canola, hemp, meal, phenolic, antioxidant activity, heat, extraction

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The content and data in t chapter 5 were presented as a poster at 2nd Northern Great Plains Lipids Conference. Poster title: Impact of solvents and thermal treatment on endogenous phenolic compounds and antioxidant activity of cold-pressed canola and hemp meals. Authors: Jingbang Liang, Peter Eck, N. A. Michael Eskin and Usha Thiyam-Holländer.

5.2 Introduction

Cold pressed canola and hemp oils are extracted from oilseeds by mechanical pressing at a temperature below 50 ℃. Canola and hemp meals are by-products of cold pressed oils obtained after cold pressing. These oilseed meals are rich source of various types of phenolic compounds which are retained and preserved well in the meals after the mild cold pressing (Koski, Pekkarinen, Hopia, Wähälä, & Heinonen, 2003). Recently, antioxidant properties of naturally plant-derived phenolic compounds are of interest. These antioxidative compounds were extracted from meals and used to improve the oxidative stability of oils (Aachary, Chen, Eskin, & Thiyam-Hollander, 2014; Thiyam, Stöckmann, & Schwarz, 2006). The content and antioxidant activity of phenolic compounds from canola meal has been intensively investigated in the past decade. Consequently, many studies reported that sinapic acid derivatives, especially sinapine (the choline ester of sinapic acid), are the major phenolic compounds detected in canola meals (Khattab, Eskin, Aliani, & Thiyam, 2010; Koski et al., 2003; Thiyam, Kuhlmann, Stöckmann, & Schwarz, 2004). However, for hemp meals, only a few studies were conducted to investigate the phenolic profiles. Thus, the phenolic composition and content were not consistent as reported in different studies (Pojić et al., 2014; Teh, Bekhit, & Birch, 2014; Teh & Birch, 2013). It becomes interesting and imperative to investigate and compare the phenolic content and their antioxidant activity of these two oilseed meals obtained after cold pressing.

The yields of phenolic compounds and the antioxidant activity of canola and hemp meals are highly associated with the extraction solvents. For extraction of phenolic compounds, solvent extraction is the most commonly adopted procedure, in particular for plant-based materials. Different polar solvents namely methanol, ethanol, isopropanol, acetone, ethyl acetate and the mixture of these solvents are usually used for extraction of phenolic compounds. It was reported that aqueous solution of organic solvents were more

effective for extraction of phenolic compounds compared to the corresponding anhydrous solvents (Teh et al., 2014). This is probably due to the high polarity and hydrophilic nature of these phenolic compounds (Thiyam et al., 2006).

On the other hand, the heat generated by seed roasting and oil processing can influence the antioxidant activity of phenolic extracts through altering the composition and structure of phenolic compounds. For instance, canolol (4-vinylsyringol) is formed through the thermal decarboxylation of sinapic acid and this phenolic compound exhibited potent antioxidant activity in crude oil extracts (Aachary & Thiyam-Holländer, 2013; Koski et al., 2003). Another study also suggested that 4-vinyl derivatives namely 4-vinylphenol, 4-vinylguaiacol, 4-vinylsyringol, 4-vinylcatechol were formed by the thermal decarboxylation of the corresponding phenolic acids: *p*-coumaric, ferulic, sinapic and caffeic acid, respectively. These 4-vinyl derivatives showed higher antioxidant activity in emulsion (Terpinc et al., 2011). The above studies indicated that thermal treatment for oilseed meals may change the phenolic composition and increase the antioxidant activity by altering the structure of endogenous phenolic compounds.

Therefore, the objective of the present study was to determine and compare the content and antioxidant activity of phenolic compounds from cold pressed canola and hemp meals. In addition, the effect of solvents on phenolic extraction was evaluated in this study. Another objective was to determine the effect of heat treatment on phenolic content and antioxidant activities of canola and hemp meals.

5.3 Materials and Methods

5.3.1 Samples and chemicals

Cold pressed canola meal was obtained from Food Development Centre (Manitoba, Canada). Cold pressed hemp meal was obtained from Hemp Oil Canada (Ste. Agathe, Manitoba, Canada). These meals were ground into powders and stored at -20 ℃ before

analysis. Phenolic acid standards namely sinapic acid, gallic acid were purchased from Sigma-Aldrich (Canada). Other reagents and solvents were purchased from Sigma-Aldrich or Fisher Scientific.

5.3.2 Extraction of phenolic compounds from canola and hemp meals

The extraction of phenolic compounds was modified from previous literatures (Khattab et al., 2010; Teh et al., 2014). Briefly, 1 g of meal sample was extracted three times with 9 mL solvent using ultrasound (40% power) for 1 minute, followed by centrifugation at 5000 RPM for 10 minutes at 4 ℃. The extracts from the three extractions were combined and made up to a total volume of 30 mL. The solvents used for extraction were aqueous methanol (70%), aqueous acetone (80%) and a solvent-mixture of aqueous methanol (70%) and aqueous acetone (70%) mixture in a ratio of 1:1 (v/v). The extracts of all the samples were stored at -20 ℃ until further analysis.

5.3.3 Determination of total phenolic content

Total phenolic content of extract was determined using the Folin-Ciocalteu assay as per Khattab et al. (2010) with slight modification. Phenolic extracts (0.5 mL) were diluted to 5 mL with distilled water, followed by adding 0.5 mL Folin-Ciocalteau's phenol reagent to the solution and mixing thoroughly. After 3 minutes reactions, 1 mL 19% sodium carbonate solution was added, following by diluting the total volume to 10 mL using distilled water. After 60 minutes incubation in dark, the absorbance of the solution was measured at 750 nm using DU 800 UV/Vis spectrophotometer (Beckman Coulter Inc., Mississauga, Canada). The blank was prepared in the same way by replacing the extracts with distilled water. Gallic acid and sinapic acid standards were used for the calibration, and the results were expressed as gallic acid equivalents (GAE) and sinapic acid equivalents (SAE).

5.3.4 DPPH radical scavenging assay

The antioxidant activities of extracts were determined by the DPPH radical scavenging

assay with slight modification (Terpinc et al., 2011). Briefly, phenolic extracts (50 μL) were added into spectrophotometric cuvettes, followed by adding 2.95 mL DPPH solution (0.1 mM) and mixing by plastic stirrer. Solvent control was prepared by mixing 50 μL extraction solvent (e.g. 70% methanol) with 2.95 mL DPPH solution. The cuvettes were stored in dark for exactly 10 minutes. The absorbance was measured at 516 nm using absolute ethanol as blank. The scavenging effects of samples were calculated by the following equation:

$$Scavenging\ Effect\ (\%) = (1 - \frac{A_s}{A_c}) \times 100$$

Where: A_c - absorbance of solvent control; A_s - absorbance of sample.

Standards of sinapic acid in different concentrations were prepared to establish the calibration curve and the DPPH scavenging activity equivalent was expressed as milligram sinapic acid equivalent per gram of sample (mg SAE/g).

5.3.5 Heat treatment of canola and hemp meals

100 g cold pressed meals were placed in metal dishes. Once the oven (Thelco laboratory oven, Thermo Scientific) is preheated, each sample of cold pressed meal was heated in oven at specific temperature (140, 160, 180 ℃) for 5, 15 and 30 minutes, respectively. After the heat treatment, samples were transferred to a glass desiccator to cool down to ambient temperature. Heat treated samples were stored at 4 ℃ until further analysis. Unheated meals were used as control samples.

5.3.6 Statistical analysis

All the extractions and analyses were performed in triplicates and the results were reported as mean values ± standard deviations. The differences between mean values were determined by One Way Analysis of Variance (ANOVA) using SPSS Statistics version 22 (IBM, New York, USA). The post hoc multiple comparisons were conducted by Tukey's Honestly Significant Difference (HSD) Test. The statistical significance level was accepted at

$p<0.05$.

5.4 Results and Discussion

5.4.1 Effect of solvents on phenolic extraction from canola and hemp meals

In the present study, phenolic compounds from canola and hemp meals were extracted using aqueous solvents namely 70% methanol, 80% acetone and 70% MA (a solvent-mixture of aqueous methanol (70%) and aqueous acetone (70%) in a ratio of 1:1, v/v). The effects of these three solvents on phenolic extraction were measured using Folin-Ciocalteu assay (Table 5.1).

For canola meals, these three solvents appeared to show similar efficiency for extraction of phenolic compounds as measured by total phenolic content. 70% MA extracted slightly higher ($p<0.05$) total phenolic content compared to the other two solvents, showing 11.27±0.41 mg SAE/g and 14.64±0.50 mg GAE/g, respectively. The total phenolic content in 70% methanol extracts were 10.71±0.39 mg SAE/g and 13.96±0.49 mg GAE/g, while in 80% acetone extracts were 10.62±0.22 mg SAE/g and 13.84±0.27 mg GAE/g. There is no statistical difference between the total phenolic content extracted using 70% methanol and 80% acetone.

For hemp meals, 80% acetone was the most efficient solvent for extraction of phenolic compounds. The total phenolic content of cold pressed hemp meal extracted by different solvents was significantly different ($p<0.05$) and followed the order namely 80% acetone (4.29±0.28 mg SAE/g) > 70% MA (3.18±0.06 mg SAE/g) > 70% methanol (1.95±0.08 mg SAE/g), and 80% acetone (6.04±0.35 mg GAE/g) > 70% MA (4.67±0.08 mg GAE/g) > 70% methanol (3.12±0.10 mg GAE/g).

As shown in the present study (Table 5.1), canola meals contained higher total phenolic content than hemp meals, irrespective of the extraction solvents. Similar result for these two oilseed meals was reported in previous study (Teh et al., 2014), where total phenolic content

of canola meal (17.76 mg GAE/g) was higher than hemp meals (4.32 mg GAE/g) in anhydrous methanol extracts and other different solvent systems. The same study also reported that aqueous acetone (6.43 mg GAE/g) was more efficient than aqueous methanol (5.45 mg GAE/g) for extraction of phenolic compounds from hemp meals. In addition, Chen et al. (2012) reported that aqueous acetone extracted significantly higher total phenolic content from hemp hull and kernel compared to aqueous methanol and ethanol, which is in accordance with the finding of the present study. However, the same study suggested using aqueous acetone had a disadvantage that chlorophyll was also extracted from hemp seed, which impacted the phenolic analyses (Chen et al., 2012). Based on the previous studies for these two oilseeds, the total phenolic content in canola seed ranged from 16.09 to 22.54 mg SAE/g in 70% methanol extracts (Khattab et al., 2010). Vonapartis et al. (2015) reported that the total phenolic content in hemp seed ranged from 13.68 to 51.60 mg GAE/g. For the cold pressed oils of canola and hemp, the total phenolic content was reported 0.59 mg GAE/g in canola oil and 1.88 mg GAE/g in hemp oil, respectively (Teh & Birch, 2013). The total phenolic content in these two oilseed meals detected in the present study was slightly lower than those in the seeds, while much higher than those in the oils. Therefore, the above results suggested that the loss of a large amount of phenolic compounds in the oils was mostly retained in the oilseed meals.

In addition, the effects of solvents on antioxidant activities of phenolic extracts from these two oilseed meals were also compared in this study. The antioxidant activity was determined by DPPH scavenging assay and the results were shown in Table 5.1. The DPPH scavenging effects of standard sinapic acid in different concentrations were shown in Table 5.2. For canola meals, the DPPH scavenging effect of 70% methanol and 70% MA extracts were 40.44±2.57% and 39.66±2.89%, respectively, showing no statistical difference (p<0.05). The DPPH scavenging effect of 80% acetone extracts (36.58±1.74%) were slightly lower

(p<0.05) than the other two solvent extracts. For hemp meal, the DPPH scavenging effects in these three solvent extracts were significantly different (p<0.05), following the order namely 80% acetone (12.33±1.30%) >70% MA (8.87±0.85%) > 70% methanol (5.22±0.60%).

Teh et al (2014) reported that the DPPH scavenging effect of hemp meal extracts using aqueous acetone (12.48%) was slightly higher compared to the extracts using aqueous methanol (11.01%). Hemp kernel and hull exhibited higher DPPH and ABTS radicals scavenging capacity in aqueous acetone extracts compared to aqueous methanol and ethanol extracts (Chen et al., 2012). The results in these literatures were in agreement with the present study, indicating that aqueous acetone is more effective to extract antioxidant compounds from hemp meals. In addition, canola meal extracts exhibited significantly higher DPPH scavenging effect compared to hemp meal extracts, regardless of the extraction solvents (Table 5.1). This result was in accordance with the previous study, where the DPPH scavenging activities in canola extracts were higher than hemp meal extracts in seven different solvents (Teh et al., 2014). However, cold pressed hemp seed oil (76.2%) showed higher DPPH scavenging effect compared to the cold pressed canola oil (51.2%) (Siger, Nogala-Kalucka, & Lampart-Szczapa, 2008).

Table 5.1 Effect of solvents on total phenolic content and antioxidant activity of canola and hemp meal extracts[1]

Sample	Solvent	TPC-FC[2] (mg SAE/g)[3]	TPC-FC (mg GAE/g)[4]	DPPH Scavenging effect (%)	DPPH Scavenging activity equivalent (mg SAE/g)
Canola meal	70% Methanol	10.71±0.39[a]	13.96±0.49[a]	40.44±2.57[b]	9.69±0.60[b]
	80% Acetone	10.62±0.22[a]	13.84±0.27[a]	36.58±1.74[a]	8.74±0.39[a]
	70% MA[5]	11.27±0.41[b]	14.64±0.50[b]	39.66±2.89[b]	9.48±0.65[b]
Hemp meal	70% Methanol	1.95±0.08[a]	3.12±0.10[a]	5.22±0.60[a]	0.94±0.13[a]
	80% Acetone	4.29±0.28[c]	6.04±0.35[c]	12.33±1.30[c]	2.75±0.32[c]
	70% MA	3.18±0.06[b]	4.67±0.08[b]	8.87±0.85[b]	1.86±0.18[b]

[1]Values for each sample in the same column followed by the different superscript letter are significant different (p<0.05). [2]TPC-FC: total phenolic content calculated by Folin-Ciocalteu assay. [3]SAE: results expressed as sinapic acid equivalent. [4]GAE: results expressed as gallic acid equivalent. [5]70% MA: a solvent-mixture of aqueous methanol (70%) and aqueous acetone (70%) mixture in a ratio of 1:1, v/v.

Table 5.2 DPPH scavenging effect of standard of sinapic acid

Phenolic standard	Concentration (mg/mL)	DPPH Scavenging effect (%)	Calibration Curve
Sinapic acid	0.05	6.75±0.03	y = 120.01x + 1.2167 R^2 = 0.9881
	0.10	13.53±0.49	
	0.17	22.87±0.42	
	0.20	24.06±0.67	
	0.21	26.67±1.62	

5.4.2 Effect of heat treatment on endogenous phenolic compounds from canola and hemp meals

As shown in Table 5.3, canola and hemp meals were subjected to heat treatment at 140, 160 and 180 ℃ for 5, 15 and 30 minutes, respectively. For canola meals, the total phenolic content of heat treated meals ranged from 8.42±0.02 to 10.35±0.01 mg SAE/g, which were lower than the unheated canola meal (10.71±0.39 mg SAE/g). At 180 ℃, the total phenolic content decreased gradually as the treatment time increased from 5 to 30 minutes. In addition, subjecting the canola meals to heat treatments at temperature from 140 to 180 ℃ for 5 to 15 minutes, the DPPH scavenging effects of canola meal extracts were similar to or slightly lower than the control, but no statistical difference was observed (p<0.05). However, after heating at 180 ℃ for 30 minutes, the DPPH scavenging effect of canola meal extracts (33.94±0.44%) was significantly (p<0.05) lower compared to the control (40.44±2.57%).

For hemp meals, after the heat treatment from 140 to 180 ℃, the total phenolic content of heat treated meals (from 2.37±0.09 to 3.03±0.02 GAE/g) was significantly lower compared to control (3.12±0.10 mg GAE/g). After heating at 180 ℃ for 30 minutes, the total phenolic content in hemp meal extracts reduced (p<0.05) from 3.12±0.10 mg GAE/g (control) to 2.95±0.01 mg GAE/g. However, the lowest total phenolic content (2.37±0.09 GAE/g) was obtained from hemp meal heated at 160 ℃ for 5 minutes, suggesting that higher temperature (180 ℃) and longer treatment time (15 and 30 minutes) may cause formation of new phenolic compounds. On the other hand, even though the extracts obtained from hemp meal heated at 180 ℃ for 15 minutes showed slightly higher DPPH scavenging effect than the control, no statistical difference (p<0.05) was found between two samples. Similarly, no statistical difference (p<0.05) was observed for DPPH scavenging effect between other heated hemp meal extracts and the control hemp meal extracts.

According to Zago et al. (2015), the total phenolic content of heat treated canola meal

extracts decreased from 19.90 mg/g DM (control) to 14.24 mg/g DM (simple roasting) and 16.68 mg/g DM (microwave roasting), respectively. This result was in agreement with the present study. The decrease of total phenolic content may result from the decomposition of endogenous phenolic compounds during heating. However, some novel phenolic compound could be synthesized simultaneously. During roasting of canola seeds, the decarboxylation of sinapic acid derivatives occurs and a potent antioxidative phenolic compound known as canolol is formed (Koski et al., 2003). Another study reported that canolol (0.06 to 0.40 mg/g) was only found in canola meals that subjected to roasting and microwave treatments (Zago et al., 2015). Siger et al. (2015) reported that the content of canolol in crude canola oils increased from 11.54 μg/g to 609.94 μg/g when the canola seeds were roasted from 140 ℃ to 180 ℃ before cold pressing. The optimal thermal condition for canolol formation was roasting at 180 ℃ for 15 minutes as reported in such study. As reported by Spielmeyer et al. (2009), the canolol content increased as the roasting temperature for canola seeds increased to 160 ℃, while the canolol content decreased as the roasting temperature continue to grow up to 171 ℃. Even though the sum of phenolic compounds reduced after heating, the antioxidant activity of oil extracts increased significantly (Siger et al., 2015). Terpinc et al. (2011) found that the 4-vinyl derivatives obtained from decarboxylation of corresponding hydroxycinnamic acids showed higher antioxidant activities in emulsions determined by β-carotene bleaching method. However, lower antioxidant activities for these 4-vinyl derivatives were observed when determined by DPPH radical scavenging method, reducing power assay and superoxide anion scavenging activity assay. For phenolic compounds in hemp, there is no literature available currently for the thermal effect on content and antioxidant activity of hemp meal extracts.

Table 5.3 Effect of heat treatment on total phenolic content and antioxidant activity of canola and hemp meal extracts[1]

Thermal Conditions	Canola meal		Hemp meal	
	TPC-FC[2] (mg SAE/g)[3]	DPPH Scavenging effect (%)	TPC-FC (mg GAE/g)[4]	DPPH Scavenging effect (%)
Control	10.71±0.39^{e}	40.44±2.57^{b}	3.12±0.10^{f}	5.22±0.60^{abc}
140 ℃, 5 min	9.66±0.21^{b}	36.56±1.28^{ab}	2.47±0.03^{b}	4.98±0.33^{ab}
140 ℃, 15 min	10.07±0.24^{cd}	37.73±1.75^{ab}	2.76±0.05^{cd}	5.64±0.22^{bc}
160 ℃, 5 min	9.78±0.17^{bc}	38.20±1.85^{ab}	2.37±0.09^{a}	4.51±0.39^{a}
160 ℃, 15 min	10.12±0.13^{cd}	38.91±1.84^{b}	2.67±0.04^{c}	5.31±0.14^{abc}
180 ℃, 5 min	10.35±0.01^{de}	41.11±0.76^{b}	2.85±0.01^{d}	5.91±0.38^{bc}
180 ℃, 15 min	9.92±0.06^{bc}	38.47±0.92^{ab}	3.03±0.02^{ef}	6.15±0.28^{c}
180 ℃, 30 min	8.42±0.02^{a}	33.94±0.44^{a}	2.95±0.01^{e}	5.44±0.18^{abc}

[1]Values in the same column followed by the different superscript letter are significant different (p<0.05). ND: not detectable. [2]TPC-FC: total phenolic content calculated by Folin-Ciocalteu assay. [3]SAE: results expressed as sinapic acid equivalent. [4]GAE: results expressed as gallic acid equivalent.

5.5 Conclusion

The present study investigated the total phenolic content and antioxidant activity of the phenolic extracts from the cold pressed canola and hemp meals. The effect of three different solvents on phenolic extraction was also evaluated. The results indicated that 70% MA extracted slightly higher total phenolic content from canola meals while 70% methanol extracts exhibited higher DPPH scavenging effect. 80% acetone was the most efficient solvent for extraction of phenolic compounds from hemp meals. The total phenolic content and DPPH scavenging effect in 80% acetone extracts were significantly higher than those in the other two solvents. Significantly higher total phenolic content and antioxidant activity were observed in canola meal extracts compared to hemp meal extracts. After heat treatments at temperature from 140 to 180 ℃, canola and hemp meal extracts showed equal or lower total phenolic content and DPPH scavenging effect.

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CHAPTER 6

6. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 General Conclusions

Cold pressed hemp seed oil contains high nutritional values that beneficial for human health, but the large amount of chlorophyll limits its application and shortens its shelf-life. This thesis investigated a novel bleaching method – ultrasonic bleaching to reduce the chlorophyll content from hemp seed oil. An extraction and determination method for chlorophyll was also developed in this study. Absolute diethyl ether was found more effective in extracting chlorophyll from hemp seed oil compared to methanol. A procedure for ultrasonic bleaching using different bleaching clays was developed. At ultrasound power of 20% pressure amplitude, chlorophyll reduction was found 99.4% for industrial clay, 97.8% for activated bentonite, 82.7% for sepiolite and 47.1% for non-activated bentonite. The results indicated that ultrasonic bleaching is an effective bleaching method with the advantages of higher bleaching efficiency, shorter treatment time and less amount of bleaching clay used.

The impacts of ultrasonic bleaching on oil oxidative stability were conducted by accelerated oxidation tests (40 and 60 ℃) and evaluated by monitoring the changes in peroxide values and conjugated dienes. Ferric thiocyanate spectrophotometric method was found more appropriate for measuring peroxide value of hemp seed oil compared to iodometric titration method. Hemp seed oil treated with ultrasonic bleaching had a lower initial content of primary oxidation products, while the total phenolic content was lower than those of untreated (control) hemp seed oil. In addition, the treated hemp oil showed higher oxidative stability compared to the control hemp seed oil. The results suggested the potential of ultrasonic bleaching on prolonging the shelf-life and expanding the applications of hemp seed oil.

The antioxidative components from by-products (meals) of cold pressed canola and hemp oils were investigated and compared in this study. The effects of extraction solvent and heat treatment on phenolic extraction and their antioxidant activity were also evaluated. For canola meal, the solvent-mixture of aqueous methanol (70%) and aqueous acetone (70%) in a ratio of 1:1 (v/v) extracted slightly higher total phenolic content from canola meals while aqueous methanol (70%) extracts exhibited higher DPPH scavenging effect. For hemp meal, the total phenolic content and DPPH scavenging effect in aqueous acetone (80%) extracts were significantly higher compared to other solvent extracts. Total phenolic content and antioxidant activity in canola meal extracts were significantly higher than those in hemp meal extracts. After heat treatment from 140 to 180 °C, the canola and hemp meal extracts showed equal or lower total phenolic content and DPPH scavenging effect.

6.2 Further Perspectives

To our knowledge, this is the first published study that uses ultrasonic bleaching to reduce the chlorophyll pigments from cold pressed hemp seed oil and improve the oil oxidative stability. Furthermore, investigating the naturally plant-derived phenolic compounds from cold pressed oilseed meals and evaluating the antioxidant properties of these compounds is a promising research topic in recent decades. Further studies in the following aspects are still needed to be carried out:

1. The ultrasonic bleaching of hemp seed oil was conducted in laboratory scale in this study; therefore, this bleaching method is needed to be assessed in industrial scale. Optimization of bleaching procedures, such as ultrasound power, treatment time, temperature, type and amount of clay will be further investigated.
2. More parameters of oil quality, such as fatty acid composition, and other physical and chemicals indexes, will be measured for evaluating the impacts of ultrasonic bleaching.

3. Further studies will be conducted to determine the effect of pretreatment and optimal thermal condition for canolol formation. The bioactivities of canolol namely antimicrobial and anticancer properties will be evaluated for applications in food preservatives and functional foods. The extraction procedure of canolol in industrial scale will also be assessed.

4. In order to determine the individual main phenolic compounds in hemp seed and cold pressed hemp meals, effective extraction methods namely accelerated solvent extraction (ASE) and solid phase extraction (SPE) will be conducted for hemp seeds, meals and hulls (shells).

5. The HPLC elution condition will be developed and assessed to detect the main phenolic compounds in cold pressed canola and hemp meal extracts. An effective isolation method will be developed to isolate the individual main phenolic compounds. Antioxidant activity and anticancer properties of these isolated bioactive compounds will be evaluated using different *in vitro* and *in vivo* assays.