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ASSESSMENT OF OIL QUANTIFICATION METHODS IN SOYBEAN AND CHIA SEEDS AND CHARACTERIZATION OF OIL AND PROTEIN IN MUTANT CHIA (*SALVIA HISPANICA* L.) SEEDS

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(*SALVIA HISPANICA* L.) SEEDS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the College of
Agriculture, Food and Environment
at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2017

ABSTRACT

ASSESSMENT OF OIL QUANTIFICATION METHODS IN SOYBEAN AND CHIA
SEEDS AND CHARACTERIZATION OF OIL AND PROTEIN IN MUTANT CHIA
(*SALVIA HISPANICA* L.) SEEDS

This thesis includes two main parts:
  I. Evaluation of techniques for oil (total lipid) quantification of chia and soybean
      seeds.
      This study evaluated 10 different methods of seed oil quantification, including
      some methods that have not been applied to oilseeds before. The main aim of this
      study was to find one or more techniques that are easy, inexpensive, safe and fast
      with a small amount of ground seeds. The Soxhlet method was used as a standard
      to compared between techniques of oil quantification. The oil extraction by the
      Soxhlet method was evaluated with two solvents petroleum ether and acetone.
      There is not a statistically significant difference between petroleum ether and
      acetone solvents. No significant differences for the amount of oil recovered via the
      Soxhlet method were found between Medium Moisture Content (MMC >10%) and
      Low Moisture Content (LMC < 4.0 %). The Folch technique provided higher
      percentages of oil extraction than Bligh and Dyer and hexane-isopropanol
      techniques. There is not a statistically significant difference (P =0.0844) between
      Soxhlet method and Folch method but less than the Soxhlet method. A supercritical
      fluid extraction (HCH) method provided lower yield of oil extraction compared
      with the Soxhlet method for three varieties of bias samples. A Direct
      Transesterification (DT) method with LMC and MMC provided a statistically
      significant difference than the Soxhlet method. The DT with LMC provided higher
      yield than DT with MMC between samples but lower than the Soxhlet method. A
      Double Direct Transesterification (DDT) following Griffiths protocol provided
      more accurate results with the stir bar technique than sonication technique. 11
      oilseeds bias samples (10 bias samples of soybean and one of chia) covering an oil
      content range of 15.4 to 32.6% showed, there is a significant difference between
      the Soxhlet and DDTG method and high oil quantification found with DDTG. A
      Double Direct Transesterification following Qiao et al. (2015) provided similar oil
      extraction to the Griffiths et al. (2010) method and also there is a significant
      difference between the Soxhlet and DDTQ method and higher oil recovered with

DDTQ. The Bead Beating Extraction (BBE) protocol showed, there is a statistically significant difference ($P < 0.001$) than the Soxhlet method. The BBE provided high oil quantification comparing with the Soxhlet method. The BBE provided the best results since it is the easiest, cheapest and fastest oil quantification method. A Nile red fluorescence technique yield no clear results.

II.   Characterization of oil and protein in mutant chia (*Salvia hispanica* L.) seeds.
The objectives of this study were to (1) measure heritability in chia plants of levels of oil and protein content and seed yield and (2) Compare differences between two locations over two years for oil and protein percentages in chia and seeds yield. A population of 180 M3 mutant individual chia plants was harvested and for which forty M4 chia seeds were planted based on seed composition characteristics, with six plants representing each characteristic (high and lower oil, protein, and density and high yield). The forty M4 chia seeds were planted, with two replications for two locations have been chosen in Kentucky one on Spindletop farm and the other in Quicksand farm. The M5 progeny seeds from plants grown in Spindletop and Quicksand contained significantly ($P < 0.05$) more protein than did seeds from the M4 parents. There were significant differences ($P < 0.05$) between locations where Quicksand obtain higher oil content than Spindletop. There was no significant difference between M5 parents seeds and M4 progeny seeds and higher yield kg/ha at Quicksand than Spindletop.

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July 18, 2017
Date

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</div>

This work is dedicated to my mother and my sister

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CHAPTER 1: Literature Review

ASSESSMENT OF OIL QUANTIFICATION METHODS IN SOYBEAN AND CHIA SEEDS

Oilseeds are a essential important food ingredients of the human diet due to containing naturals antioxidants and comprise a large portion of human food such as cereals, nuts, and their derived products. (Wanasundara et al., 1997). Oilseeds are constituted of various fatty acids composition that are grouped into saturated and unsaturated fatty acids. The fatty acids produce energy for different biochemical reactions in the human body. Oilseeds can be used in numerous medicines and cosmetics (Pandharinath, 2015; Warra et al., 2012). Oils usually are used for cooking and can be extracted from various sources such as vegetable, corn, canola, peanut, olive, coconut, soybean, sesame, sunflower, flaxseed, grape seed, pumpkin seed, safflower, argon, rice bran, palm, palm kernel , almond, avocado, cottonseed, hemp, mustard, macadamia, groundnut, tea seed, walnut, cashew, castor, colza, hazelnut, linseed, manila, mongongo nut, mustard, pecan, perilla, pine nut, pistachio, poppy seed, rapeseed, watermelon seed, jojoba, and diacylglycerol (DAG)(Chang, 2013).

Oilseeds Production

In 2013, the United Nations expected the world population will increase from 7.4 billion people to 9.6 billion people by 2050. With the growing world population, the demand for food will increase. To cover the demand of oil seeds required, production is expected to increase from 133 million tons to 282 million tons. (Ouilly et al., 2017). The main production of oilseeds is in the temperate areas. More than 60 percent of oilseeds production in the world comes from the USA and Europe, and less than 5 percent from the tropical areas including Africa, Malaysia, and Indonesia. Oil production in the tropical areas comprises of coconut, oil plam, groundnut, and cotton (Sharma et al., 2012). Biotechnological improvement of the fatty acid composition can increase demand for oils in the human diet, animals feed, and industrial materials (Lu et al., 2011). Improved quality and quantity vegetable oils for human consumption can enhance cardiovascular health and fitness. Lu et al.(2011) reported the main challenge for oilseeds biotechnology production have been increasing the yield of crops oil and increasing areas of oilseeds production to cover global demand (Lu et al., 2011).

Due to increased demand and motivation for production of oil from various crops seeds (soybean, chia, flax, canola, almond, sesame, sunflower, borage, safflower, crambe, comfrey, guayule, cuphea, broomcorn, jojoba, lesquerella, kenaf, meadowfoam, lupine, milkweed, vernonia perilla and others oil crops), we evaluated and modified current protocols to quantify oil in different varieties of oilseed of plants. For many decades, numerous techniques were used to measure lipid quantification from oilseeds, animal's lipids, algae, and yeasts. There are several reports quantifying lipids from samples of small and large seeds. Two important crops of oilseed are used as standards in the current study: soybean and chia seeds.

Soybean (Glycine max L.)

One of the most important oilseed crops in the world is soybean (*Glycine max* L.). Soybean is an annual crop and belongs to the *Leguminosae Fabaceae* family [sub-family *Faboideae* or *Papilionoideae*]. Soybean has numerous varieties with different colors such as yellow, black, green and brown seeds. Soybean is planted in several tropical and subtropical regions of the world (Alves et al., 2011). Soybean is a legume crop native to East Asia and is now grown worldwide (Aykroyd & Doughty, 1982). Cultivated soybean originated in China (T. Hymowitz & Newell, 1981). Soybean is a self-pollinated plant and can reproduce by seed (Roebbelen et al., 1989). The production of soybean is believed to have started during the Shang dynasty (1500-110 B.C.) or maybe earlier. China began trading with other continents between the first century and the 15th century and soybean was one of the most prevalent exports. China advanced the introduction of soybean to Japan, Korea, Indonesia, the Philippines, Vietnam, Thailand, Malaysia, Myanmar, Nepal, and northern India (Theodore Hymowitz, 1990; T Hymowitz et al., 1980). Samuel Bowen brought soybean for the first time from China to North America in 1765. He suggested to Henry Yonge, the Surveyor General of the Colony of Georgia, to plant soybean on Bowen's farm near Savannah, GA (Theodore Hymowitz, 2004).

Another person who introduced the soybean to North America was Benjamin Franklin. During 1770, he sent the soybean seeds to a botanist named John Bartram, who planted them in his garden near Philadelphia, PA (Theodore Hymowitz & Harlan, 1983). In 1851, soybean finally spread through Illinois and the "Corn Belt" (Robbelen et al., 1987). The largest soybean producer is the United States (U.S.A.), with 34% share in the

world's production, followed by Brazil (29%), Argentina (19%), China (6%), India (4%), Paraguay (3%), Canada (2%), and others 4% (Mattson et al., 2004). The United States and Brazil are the main producers and exporters of soybean (Jenkins et al., 2008). With the increased use of soybean in the areas of livestock feed, meal, and vegetable oil (Hatje, 1989), soybean was grown primarily as a forage crop until 1941 in U.S.A.

Currently, soybean is produced mainly for its protein and oil content. Soybean is an important economic crop, with an unique seed composition. It has 21% oil, 40% protein, 34% carbohydrates and 5% ash (Alves et al., 2011), and the levels of its seeds protein and oil composition are inversely proportional to each other (Chung et al., 2003; Diers et al., 1992; S. Lee et al., 1996). Although soybean protein is mainly used as a farm animals feed, soybean is also significant for many food products and industrial applications. The soybean oil is used for margarine, shortenings, and other fat and oil products in the food industry, as well as nonfood applications (Glaudemans et al., 1998). Like any other legumes crop, soybean is suitable for crop rotation due to its ability to fix nitrogen from the air (Anwar et al., 2016).

Chia (Salvia hispanica L.)

Chia is a member of the Lamiaceae or mint family (Ayerza, 2010; Jamboonsri, 2010). Chia is native to Central Mexico and northern Guatemala. Chia was one of the most important crops in México for 5,500 years. Chia was a staple crop of the Aztecs. after corn and beans, chia was a third most important food for more than 11 million people in the Aztec empire (Coates, 2011). In 1753, chia was classified by a Swedish botanist Carl Von Linneo who named it Salvia hispanica, which means in Latin "Spanish plant to cure or save" (Urbina, 1887). In Nahua language, the word Chian (today named chia) means oily. In 2010, Australia was the largest producer of chia seeds in the world. Chia appeared in advertisements and on shelves of mainstream supermarkets around the country (Loyer, 2016). In 2014 the production increased by 367,000 hectares in countries including Argentina, Bolivia,and Paraguay (Sosa, 2016). The biggest issue facing the USA and temperate countries is that chia was cultivated in a tropical area with short day lengths and only in agricultural zones between 20° 55' N to 25°05' S. The USA sites are in higher

latitude areas, such as 32° 14 N (Tucson, Arizona, USA) and 38°2'26.1"N (Lexington, Kentucky, USA) .

The chia plant cannot produce seeds because the plant faces frost and is killed before seeds matures. Chia is an annual summer crop in Kentucky. The first new chia varieties, specifically in Lexington KY, were developed by mutation, ethyl methane sulfonate (EMS) and gamma radiation (Jamboonsri, 2010). In general, the main elements in chia seeds comprise of 25–40% oil (Ixtaina et al., 2011; Timilsena et al., 2016), 17–24% protein and 18–30% dietary fibers (Timilsena et al., 2017). Chia seeds can reduce low-density lipoprotein (LDL) and serum triglycerides (TG), and can increase high-density lipoprotein (HDL) (I. Fernandez et al., 2008). The protein content of the chia seed is greater than that of grain seeds such as 10% in wheat, 11% in maize, and 8% in rice (Coates, 1996; Herman et al., 2016). Chia can be used whole, as a meal, raw, and roasted and the whole chia seed is beneficial for animal feed, as it increases the nutritious value of the resulting poultry, meat, and eggs. Chia may be the best source of beneficial soluble fiber (Ayerza and Coates, 2004; J. P. Cahill, 2003). Chia seed oil is abundant in polyunsaturated fatty acids (PUFA), (60 to 67% α-linolenic (ALA) and 20% linoleic (LA) acids) which makes chia seed oil more susceptible to oxidation (Dąbrowski et al., 2016). Chia seeds oil is important due to the amount of ALA and LA that are fundamental fatty acids. These two fatty acids in chia oil contain up to 80 percent of the overall fatty acid composition. A high percent of ALA and LA makes chia seeds oil one of the healthiest oils (Timilsena et al., 2017). The chia seeds are abundant in several phytochemicals such as sterols, carotenoids, phenolics, tocopherols, squalene, andwaxes (Dąbrowski et al., 2016). Chia seeds are recognized for their nutritional value and use as supplemental products such as sports nutrition, pasta, yogurts, bread, and hamburgers (Franklin & Hongu, 2016).

Numerous studies utilized solvents extraction to recover lipids from the cells by quantifying lipids either gravimetrically or by Gas chromatography (GC) (Griffiths et al., 2010). GC is used to quantify total or indiviual fatty acids in extracted lipids in samples (Liu, 1994). Transesterification can determine changing saponified lipids content in biological samples (such as triacylglycerols (TAG) and phospholipids) to fatty acid methyl esters (FAMEs) by adding a surplus of methanol and a catalyst to the reaction (Carrapiso & García, 2000; Griffiths et al., 2010; Liu, 1994). Several extraction protocols are reported

in the literature for lipid extraction from soybean and chia seeds such as Soxhlet. (1879), Folch et al. (1957), Bligh and Dyer (1959), Hexane: isopropanol (HIP) Hara & Radin. (1978), Hot compressed hexane (HCH), Direct Transesterification (DT)- Li et al. (2006); Zhang et al. (2009), Double Direct Transesterification- Griffiths et al. (2010), Double Direct Transesterification – Qiao et al. (2015), Bead Beating Extraction (BBE) - Sitepu et al. (2012), and Nile red/fluorescence. Some of these methods involve reading volumetrically the lipid content while others use GC-FID. Total fats and total lipids are described as the amount of all fatty acids shown as triglyceride equivalents and include the total of all fatty acids of monoglycerides, diglycerides, and triglycerides, free fatty acids, phospholipids, and sterols (Ullah et al., 2011). Eller and King. (1998) stated that the traditional description of lipids indicates that each of the varied substances that are soluble in organic solvents include monoglycerides, diglycerides, triglycerides, free fatty acids, lipoproteins, sterols, phospholipids, hydrocarbons, and waxes (Eller & King, 1998).

Organic Solvents Used

In general, solvent extraction comprises of equilibrating the solvents with the samples matrix (Ullah et al., 2011). Numerous studies have used different solvents of oil extraction for different materials such as seeds, algae, leaves, and roots. The fact is that various lipids have different polarities and one individual solvent can not be used to extract different lipids. The total lipid extracted can be determined by the nature of the single solvent used to accomplish the lipid extraction (Phukan et al., 2011). Numerous solvents can use for Soxhlet extractions depending on their relevant properties, polarity and boiling point. For example, Petroleum ether , Hexane, Cyclohexane, Isooctane, Toluene, Benzene, Diethyl ether, Dichloromethane, Isopropanol, Chloroform, Acetone, Methanol, and Ethanol have polarity 0.1, 0.1, 0.2, 0.4 , 2.4, 2.7, 2.8, 3.1, 3.9, 4.1, 5.1, 5.1, and 5.2 and boiling point °C 35.0–60, 69.0, 80.7, 99.2, 110.0-111.0, 80.0, 34.6, 39.8, 40.0, 82.0, 60.5–61.5, 56.0, 64.7 and 78.0 respectively (Ramluckan et al., 2014).

Petroleum Ether as an Extraction Solvent

Petroleum Ether has non-polar properties and has two sides charged. It can penetrate into the matrix of feed. Due to its lack of linkage, it has O-H ends that then might interfere with the extraction process (Nwabueze & Okocha, 2008). Petroleum ether can be

used as an extraction solvent because it is non-polar, cheap and flammable. Petroleum ether has a high solvent extraction capacity, and it does not impact the properties of oil, is non-toxic, stable, and volatile (Masime et al., 2017).

Acetone as an Extraction Solvent

Acetone is a polar solvent recommended for extraction of vegetable oils such as other polar solvents ethanol and methanol. According to Dąbrowski et al. (2016) acetone is the most effective solvent for the extraction of total lipids and bioactive components, especially phenolic compounds and carotenoids. They also stated that acetone is the most efficient solvent to extract the oil with the Soxhlet method beacause its higher polarity enables it to recover more amphiphilic compounds. The acetone solvent is able to extract 95% of lipids, which is 3% more than hexane. The oil obtained by extraction with acetone was additionally characterized by the highest oxidative stability (Dąbrowski et al., 2016). Nwabueze and Okocha. (2008) reported that the oil extraction with acetone is recommended for food use on safety grounds (Nwabueze & Okocha, 2008).

Other Solvents

The hexane is considered a good solvent to extract free non-polar lipids like triglycerides, but hexane is a poor solvent for polar lipids like free fatty acids and phospholipids. In the case of the existence of bound lipids, polar solvents such as chloroform/methanol or diethyl ether might be required to extract lipid compounds. In another case, the polar solvents can be used to extract more non-fat compounds like carbohydrates (sugars or starches), amino acids, peptides, and also water (Eller & King, 1998). Lipids contain a diverse biological substances group that primarily comprises of non-polar compounds, such as triglycerides, diglycerides, monoglycerides, and sterols, and polar compounds, such as free fatty acids, phospholipids, and sphingolipids. Lipids bind covalently to carbohydrates to form glycolipids and proteins to form lipoproteins. A solvent that is high solubility and adequately polar must be utilized to remove lipids compounds from binding sites with lipoproteins, glycolipids, and cell membranes (Manirakiza et al., 2001). The quantitative extraction of groups of the lipids components requires the breakage of bonds and interactions among non-fat compounds. Chloroform and n-hexane are the organic nonpolar solvents and are used for interrupting hydrophobic and ion-dipole interactions including hydrophobic lipid chain and non-polar amino acids. Methanol is an

organic polar solvent with high insulation constant that is used to break hydrogen bonds suchas lipid hydroxyl, carboxyl, or amino groups, and nonlipid compounds.Various methods used to determine total fat (TF) and fatty acids (FA), including trans fatty acids (TFA), in diverse foods were evaluated, including gravimetric methods and gas chromatography with flame ionization detector (GC/FID), in conformity with a modified AOAC 996.06 method. (Aued-Pimentel et al., 2010).

Factors

There are numerous factors that can influenc the solvent efficiency through the extraction of a vegetable oil, including the solvent properties, the temperature used during the extraction process, solvent to material ratio, and the moisture content or dry weight, distribution of particle size, pressure stress, and the number of cycles during the extraction procedure.

Moisture content

Moisture content is one of the environment factors that could influenc oil extraction between solvents and biomass. Numerous researchers claim that the moisture content can affect oil yield have among extraction techniques used with the different organic solvents such as recently a study has reported that the flax seeds for biodiesel extraction obtained the highest oil yield of approximately 93% when moisture content was 4% (da Silva Marineli et al., 2014). The effect of dry weight and moisture content of soybean flours via Soxhlet extraction method was described by Canessa and Snyder. (1991). The percentages of moisture content depend on the relative humidity of the atmosphere around. The percentages of oil extraction can be increased by increasing moisture contents of the samples. The increased percentage of oils recovered might be due to increased phospholipid extraction (Canessa & Snyder, 1991). According to Mercer and Armenta. (2011) micro-algae has a high level of moisture content, which tend to acts as a barrier against dissemination of oil out of the cell because the moisture prevents dispersal of CO_2 inside the samples (Mercer & Armenta, 2011).

Heating temperature:

Temperature is one the most important factors that affect the recovery of oil. Usually, high temperatures provide improved extraction efficiencies. On the other hand, the higher temperatures might cause the degradation of oil extracts (Wang & Weller, 2006).

In the Soxhlet extraction process, the solvents are recovered during evaporation. Therefore the temperatures of evaporation and extraction have an important consequence on the final products' quality (Mamidipally & Liu, 2004).

Pressure

The pressure is one of the other factors that can affect the extraction rate of oil. It is reported that increasing pressure can increase the extraction rate by increaseing solubility of oil (Salgın et al., 2006). According to Zhao and Zhang. (2013), increasing pressure and extraction time significantly increased the oil production. They reported there was a relationship between impact of the pressure and temperature on the oil extracted. Increased temperature with high pressures could result in increased oil extraction (Zhao & Zhang, 2013).

Particle size

Recent studies have detected that the efficiency of a solvent during extraction is increased by reducing the particle size of the material during grinding (Masime et al., 2017). Other studies investigated that the oil production increased with the declining particle size (Zhao & Zhang, 2013). The smaller size particles obtained higher yields. The impact of intra-particle diffusion appears to grow significantly for large particles producing a significant decline in the oil yield (Salgın et al., 2006). Smaller particle size can produce the highest yield in shorter extraction time (Goula, 2013).

MAIN EXTRACTION METHODS OF TOTAL LIPID QUANTIFICATION

Soxhlet Extraction Method (Soxhlet, 1879)

The oldest and the most universally accepted protocol for determining oil and lipid content of seeds, foods, and feeds is the Soxhlet technique. The method was developed by von Soxhlet in 1879 as a new extraction system (Soxhlet extractor) (Soxhlet, 1879). Among different techniques found for extraction of lipids, the Soxhlet is the method commonly used for more than a century. Numerous studies have supported the fact that Soxhlet is a standard method. Currently, the Soxhlet is the essential reference that the performance of other techniques are compared. The original Soxhlet procedure was used to determine the content of fat in milk. Soxhlet extractor was adapted and developed for the continous extraction of a liquid with either a lighter or heavier solvent (De Castro & Garcıa-Ayuso,

1998). Soxhlet extraction was used as a starting point for a variation of modifications. Taylor et all. (1993) accomplished the analytical measurement of oil content in oilseeds by using the Soxhlet method. Soxhlet method depend on extraction medium of organic solvents (Taylor et al., 1993). Ullah et al. (2011) reported the Soxhlet extraction is the oldest method for performing solvent extraction and it is the official method used by both AOAC and American Oil Chemists' Society (AOCS) (Ullah et al., 2011).

Folch et al. (1957) and Bligh and Dyer (1959)

The original Folch method was used to determine total lipids in animal tissues (Folch et al., 1957). The Bligh and Dyer method was used to determine total lipids in frozen fish. The Bligh and Dyer method is considered the standard technique used to determine total lipids in biological tissues like microorganisms (Breil et al., 2017). The Bligh and Dyer method is the most accepted method for manually extracting all plant lipids. Recently Breil et al. (2017) have called the Bligh and Dyer or Folch methods gold standards for the analysis of extracted lipids. They stated microorganisms and biological tissue were measured with methanol, chloroform, and water that were added to the samples with two steps of extraction. The phases were separated and the lipids were quantified in the chloroform phase (Breil et al., 2017).

The Bligh and Dyer method is a slight modification and improvement of Folch method. In 1959, Bligh and Dyer cite Folch method and they claimed their technique was much faster (10 min) and used much less solvent than Folch method, but otherwise gave similar lipid yields. Bligh and Dyer also mention slightly more lipid yield with acid hydrolysis. Iverson et al. (2001) showed a comparison of the Folch and Bligh and Dyer protocols for lipid extraction from fish muscle. They claim the Bligh and Dyer method produced lower lipids than expected compared to the Folch method. When the samples contain less than 2% lipids, the Blight and Dyer method underestimated the lipid contents, and this underestimation increased significantly with increasing lipid content in the samples. For the sample with the highest percentage of lipids, the Bligh and Dyer method underestimated the lipid content by 50 percent (Iverson et al., 2001).

The Bligh and Dyer and Folch methods employ a solvent mixture of chloroform/methanol, which is widely used for total lipid quantitation in the biological samples such as meat products and fish. These methods are recommended as starting point

for the determination of total lipids in foods when it is unknown which techniques of analysis can be used for the food. These methods are chosen as the source of lipid extraction, fatty acid definition, and other characterizations, due to the capacity of the solvents mixture to extract various lipid classes without altering their molecule structure (Ashraf-Khorassani et al., 2002).

Reis et al. (2013) also compared five extraction protocols including Folch et al. and Bligh and Dyer and they concluded that in general, the Folch technique was the most productive method for extracting wide range lipids in LDL. The main component of oils was measured with triacylglycerol, which is a polar lipid (Reis et al., 2013). A recent relevant publication on improved oil extraction created a technique called Accelerated Solvent Extraction (ASE) (Yao & Schaich, 2015). They evaluate that ASE recovers twice the lipid levels from extruded products as Soxhlet (Strange et al., 1997). Yao and Schaich (2015) did not directly compare ASE to Soxhlet but did to an acid hydrolysis techniques. Regulatory services uses "acid hydrolysis" in addition to Soxhlet and has details on the acid hydrolysis technique and the Bligh and Dyer protocol. They report the ASE protocol yield 80% of the acid hydrolysis technique and the Bligh and Dyer protocol. They imply ASE has greater lipid yield than Soxhlet but did not include Soxhlet in their study. Yao and Schaich (2015) did not provide much detail on their ASE protocol but did mention that the extraction was achieved in Dionex 350 Accelerated Solvent Extractor. In this system 24 samples could be extracted in an automated sequence. They used different extraction solvents including chloroform, chloroform/methanol (2:1, v/v), hexane, methanol, hexane/methanol (2:1, v/v) and petroleum ether (Yao & Schaich, 2015).

Hexane: isopropanol (HIP) lipid extraction method Reis et al. (2013)

The original HIP extraction method was used to determine lipids contained in tissues by using hexane: isopropanol and then washing the extraction and removing non-lipid residuals by aqueous sodium sulfate (Hara & Radin, 1978). Hussain et al. (2015) evaluated three lipids extraction methods including Soxhlet, Hexane: isopropanol, and Bligh and Dyer methods. They freeze-dryed and oven-dryed Chlorella Vulgaris- algal biomass to appraise how these techniques impacted the yield of lipid, fatty acid composition, and quality of algal biodiesel. They stated HIP method uses a less toxic ratio

from hexane/isopropanol solvent with 3:2 (v/v) for lipids extraction, and it was a better method to extract lipid from algae that is oven-dried (Hussain et al., 2015). Reis et al. (2013) recommended the hexane-isopropanol method was the most efficient method for extracting polar lipids (Reis et al., 2013). Han et al. (2011) claim that the most accurate methods to measure lipid content are gravimetric methods due to the ability to measure the lipids weight directly (Han et al., 2011).

According to Ullah et al. (2011), the gravimetry methods are common techniques for measuring total fats. The advantages of these processes are that they are more quantitative and less dependent on user analysis than volumetric methods. Therefore, gravimetry methods might be implemented with the least equipment and need less skilled workers, making these methods simpler to achieve when compared to gas chromatography with mass spectrometric detection (GC-MS) based techniques (Ullah et al., 2011).

The disadvantage of the gravimetric methods was reported by Hounslow et al. (2017). They proved that Han et al. (2011) did not study the issues lipids weight of accuracy with gravimetric methods. Hounslow et al. stated that the analysis of gravimetric methods did not permit compositional analysis without mass spectrometry and involves considerable amounts of biomass. These techniques are limited by the weighing accuracy of a balance, and the lowest detection level of lipids is usually up to 10 mg dry lipid extraction. Therefore, the gravimetric methods are not the best methods for all experiments (Hounslow et al., 2017) to measure total lipid contents. Elnajjar et al., (2017) evaluated the Folch procedure and found that it required much shorter time than the Soxhlet method for oil extraction. Usually, the Folch method needs around 1 hour to extract oil while Soxhlet needs 12 hours (Elnajjar et al., 2017).

Supercritical extraction method or Hot compressed hexane HCH:

Supercritical hexane extraction has not been applied to lipid quantification of oilseeds but likely might be superior, as was found with algae and similar protocols for extruded products (Shin et al., 2014; Strange et al., 1997). Supercritical fluids are theoretically superior to usual solvents for lipid extraction. Mark Crocker's group at the Center for Applied Energy Research (CAER) in North Lexington reported almost three times the yield of lipid extraction from algae (*Scenedesmus sp*.) using supercritically hot

hexane (≥ 235°C, 31 bars) than Soxhlet and a slightly higher yield than the Bligh and Dyer protocol (Shin et al., 2014). Nguyen. (2016) has used Scenedesmus sp. at the optimum conditions of 300 rpm for two hours (HCH-2-300) by using supercritical hexane extraction, and the yield of lipids extracted was 8.96% lower than in the previous finding in Shin et al. (2014).

Nguyen explianed that the higher lipids yield obtained by Shin et al. (2014) is due to the higher lipid content of *Scededesmus* algae (up to 40 percent) (Nguyen, 2016). Zhao and Zhang. (2013) informed that the oil yield extracted from Moringa oleifera seeds by the supercritical fluid extraction technique was slightly lower than that of the Soxhlet extraction (Zhao & Zhang, 2013).

Direct Transesterification(DT) or Direct Methylation

In 1963, Abel and Peterson reported the first successfully achieved Direct transesterification (DT) technique using a chemical composition of carboxylic acid methyl esters for the classification of microorganisms (Abel & Peterson, 1963). Direct transesterification technique is defined as the one-step reaction that is executed in the same test tube while avoiding the step of purifying and extracting. According to Lepage and Roy. (1984) the direct transesterification method was observed in human milk and adipose tissue and compared to the Folch extraction technique. DT increased the fatty acid concentration of human milk by 11.4% and adipose tissue by15.8% because it extracted over 96% of triglycerides and fatty acid compositions (C6:0 to C24:1). They stated that DT process was particularly beneficial for the recovery of the highly volatile triglycerides medium chain, and it does not require the addition of an antioxidant to protect unsaturated fatty acids. (Lepage & Roy, 1984). Li et al. (2006) improved DT method used to determine total oil extracted from *Arabidopsis thaliana*. *Arabidopsis* is used as a model for plant biology research, and it offers an attractive system for studying oil seed. In *Arabidopsis* seeds, the almost entire of the fatty acids content is esterified as form of triacylglycerols (TAG) while the rest is created from membrane lipids and diacylglycerols. The increased amount of oil is extracted from seeds, which typically involves grinding seeds so that organic solvents (Li et al., 2006) can penetrate the cell wall. Li et al. (2006) provided accurate results for quantifying oil content of *Arabidopsis* seeds and the factors that can influence these results.

The DT method was described as fast and the seed amount required is minimum (Li et al., 2006). According to Frigo-Vaz and Wang. (2014) the direct acid methylation is fast technique, high-purity and might complete during 2 hours period comparing with the Soxhlet method (Frigo-Vaz & Wang, 2014). Zhang et al. (2009) measured the oil content of seeds by followed the protocol of Li et al. (2006), and they added slight modifications. They have randomly selected ten seeds of *Arabidopsis* after putting 30 seeds in desiccators for 48 h and after water content is stable, then measured the weight of ten seeds. They added one microgram of standard (tri-heptadecanoic) and 2 mL of 2.5% (v/v) concentration of sulfuric acid in methanol, and they kept the tube at 90°C for 90 min. Gas chromatography was used to analyze fatty acid methyl ester extracts. (Zhang et al., 2009).

Lemões et al., 2016 reported the DT for microalgae provided higher fatty acid methyl esters (FAMEs) produced in dry biomass. (Lemões et al., 2016). According to Hoarau et al. (2016) DT for wet microalgal biomass produced more FAMEs than Folch (77–93 %) and Bligh and Dyer (19–63 %) methods. Increased FAMEs production by direct methanolysis is attributed to the increased ability to penetrate the cell and the fewer number of steps in this process. The FAMEs producted by direct methanolysis of wet biomass was lower than dry biomass. Notwithstanding the biomass moisture content, dry biomass provided more oil yield than wet biomass (Hoarau et al., 2016).

Double Direct Transesterification (DDT)

I. *Griffiths et al. (2010) method:*

Griffiths et al. (2010) method called Direct Transesterification (DT) method using two catalysts by Two Sides Direct Transesterification (TSDT). In the present study we change name (TSDT) to (DDT) for the same purpose. Double Direct Transesterification Griffiths (DDTG) method converts saponifiable oils directly to fatty acid methyl esters (FAMEs) that can be determined by Gas chromatography with flame ionization detector (GC-FID). This extraction has a single step with smaller ground seeds samples than the Folch and Bligh and Dyer methods. This technique should be faster due to the elimination of an extraction step.

Griffiths et al. (2010) compared the effeciency of DT, which uses transesterification and gas chromatography to measure the content of the total fatty acid extracted in three microalgae species to the Folch method, the Bligh and Dyer method and the Smedes and

Askland method. DT presents a reliable and more accurate method than the other extraction methods to quantify the total fatty acid content in microalgae. The DDTG was more effective when combining acidic and basic catalysts than when using each catalyst individually for samples containing water (Griffiths et al., 2010). They studied two catalysts in sequences as well as the reaction impact of water content on the effectiveness of DT. Total lipid content of microalgae is typically measured by the Folch or the Bligh and Dyer techniques of solvent extraction followed by quantification of fatty acids such as gravimetrically or chromatography. The Folch method was a successful method of oil extraction, but Direct transesterification provided a higher percentage of oil extraction from the cell due to the gravimetrical methods being incomplete. (Griffiths et al., 2010).

Higher levels of fatty acid content in the cells were found with DT in comparison with the extraction-transesterification methods. DT has been used to quantify fatty acids in cement and concrete (van Hille & Griffiths, 2016). The DDTG is described as an accurate, fast technique and uses small seed samples with two standards (glyceryl triheptadecanoate (C17-TAG) and methyl nonadecanoate (C19-ME)). Tri-17:0 was added prior to the reaction as a standard of quantitative lipids and then C19-ME was added in the final step of solvent extraction to confirm that extraction was achieved. The current study modifies the protocol by combining standards tri-17:0 and methyl-19:0 and by changing the standard solution to 10 µL inernal standards in toluene per 1 mg biomass.

II.   *Qiao et al.(2015) method*

Double Direct Transesterification Qiao (DDTQ) is similar to the Griffiths et al. (2010) method. Qiao at et.(2015) used a progressive combination of alkaline catalysts methanol (MeOH) and acid catalysts Acetyl chloride (AcCl) that were found to improve the extraction yield of fatty acids in *Phaeodactylum tricornutum*. The Qiao et al (2015) claimed their combinations is superior for extracting the total lipid yield from an algae using a 2-step acid and base transesterification technique is equivalent to AOAC 991.39 (Qiao et al., 2015). DDTQ transformed intracellular lipids to saponifiable lipids that were then transesterified in situ to FAMEs. The Qiao technique, as well as the Griffiths technique, have reported that small sample sizes were enough to test lipids content because the reactions occurred in one tube, therefore the extraction steps were eliminated. Qiao technique provided an extract yield above 96 %. This yield was greater than that of

traditional solvent extraction methods (the Folch, the Bligh and Dyer, or the Soxhlet methods) and had higher levels of fatty acid content.

The Qiao et al. (2015) protocol replaced BF_3 with AcCl/MeOH, and it was easier to prepare, has high stability, is cheaper, uses safer chemicals and is more readily executed than Griffiths protocol. The DDTQ allowed up to 10 % water content without affecting the results in the total reaction volume (Qiao et al., 2015). The present study compares the Qiao et al. (2015) protocol to the Griffiths et al. (2010) protocol since Qiao protocol may be more efficient. Publications citing these mentioned articles were also examined, but no additional highly relevant references were found.

In generl, the Double Direct Transesterification method following Qiao et al (2015) protocol add two catalysts, Sodium hydroxide (NaOH) in methanol (MeOH) (alkaline catalysts) and Acetyl chloride (AcCl) in methanol (MeOH) (acid catalysts), but techniques following Griffiths protocol add two catalysts sodium methoxide (base catalysts) and BF_3 methanol (14% BF_3) solution (acidic catalysts) sequentially to quantify the fatty acid composition and confirm the transesterification competency.

Bead Beating Extraction (BBE)Sitepu et al. (2012):

The BeadBeating produces direct mechanical damage to cells using beads at high-speed and can be used in a laboratory and industrial sector (J.-Y. Lee et al., 2010). The improved yeast protocol for lipid quantification is described by Sitepu et al. (2012). They considers the bead beating extraction to be superior but the data were not shown. Sitepu et al. (2012) used a triplicate of 20-milligram samples of cells and then transferred the samples to two mL screw cap tubes. Next they added 1.5 mL of Folch's solvent and the screw cap tubes were filled with zirconia beads (0.5 mm). Samples of cells were homogenized in an MP extractor (Bio Fastprep®-24 homogenizer) for 30 seconds, 5X with 30 sec intervals on the ice (Sitepu et al., 2012). A major factor affecting lipid quantification is that the barrier cell walls are present for lipid removal but bead beating extraction damages the cell well. In current study this technique considered for regular GC analyses with two standards tri-17:0/19:0 ME were added before bead beating extraction so the same extract GC analyses.

Nile red/fluorescence extraction Sitepu et al. (2012)

The Nile red is a fluorescent lipophilic dye utilized to discover intracellular lipid quantity in algae, yeasts, and filamentous fungi. In the yeast, Nile red is influenced by different levels of diffusion across the cell membrane and depends on the time needed to give the highest fluorescence emission (Sitepu et al., 2012). Among the most pertinent methods for oil extraction is the Nile Red, which is used to observe the accumulation of lipids via fluorescence microscopy after the Nile Red dye is accumulated in cellular lipids. The Nile Red is commonly used as a method to quantify lipids in recent years (Rumin et al., 2015; Takeshita et al., 2015). The Nile red staining with microplate is used with a fluorescence plate reader for quantitative analysis of lipids, and it is considerd an easy and simple technique for several microalgal species(Balduyck et al., 2015; Takeshita et al., 2015).

Natunen et al. (2015) reported using 20% dimethyl sulfoxide (DMSO) and observed the stable growth phase and it gave the most stable values (Natunen et al., 2015). They found that in *Chlorella pyrenoidosa* -algae the dimethyl sulfoxide can increase Nile Red fluorescence compared to the exponential growth phase the fluorescence provided without dimethyl sulfoxide (0% v/v). The maximum stable fluorescence values were provided with 20 and 30% (*v/v*) dimethyl sulfoxide. In other species of *Isochrysis* algae, the exponential growth phase at the highest fluorescence production was with 5% (*v/v*) dimethyl sulfoxide. The fluorescence intensities were reduced with increasing concentrations of dimethyl sulfoxide (Natunen et al., 2015). Hounslow et al. (2017) described that the Nile Red method accumulated more lipids over time in 0.3 M Sodium chloride (NaCl) (Hounslow et al., 2017). The Nile red fluorescence depends on cell type and the procedure conditions to penetrate the cell membrane.

Nile red fluorescence is used with Algae and Yeast and the fluorimetric protocol apparently does not involve lipid extraction, but only penetration of the dye into the assay material. Fluorometry does need lipids to be extracted; only the fluorometric dye needs to penetrate the material. This technique is used with yeast and considered for the fluorimetric microplate protocol. Algae are highly variable particularly in cell wall characteristics, but the seed embryo tissue is more uniform. To modify this protocol for oilseeds, we added 25 microliter dimethyl sulfoxide and added 0.05 mg/ml Nile Red amount in acetone.

CHARACTERIZATION OF OIL AND PROTEIN IN MUTANT CHIA (*SALVIA HISPANICA* L.) SEEDS

Original or Native plants of chia

Chia (*Salvia hispanica* L.) is native to Central Mexico and northern Guatemala and is a member of the Lamiaceae, Labiateae, or mint family (Jamboonsri, 2010; Ayerza, 2010) Chia was extensively used in pre-Columbian Mesoamerica as a major trade commodity, and its seeds were ingredients for food, medicine, and oil (Jamboonsri, 2010). Chia was also used for utilitarian purposes, such as in foods, and was used to produce nutraceutical and supplements (Sosa, 2016). In 1753, chia was classified by the Swedish botanist Carl Von Linneo who named it *Salvia hispanica*, which means in Latin "Spanish plant to cure or save" (Urbina, 1887). In the Nahua language, the word Chian (today chia) means oily. The Aztecs used the name chia to indicate all species of the Salvia genus with high oil contents such as *Salvia hispanica* L, *Salvia tiliifolia* V, *Salvia polystachya* O, and *Salvia columbariae* B (RH Ayerza & Coates, 2006; Sosa, 2016). Chia was one of the three most important crops in México for 5,500 years. Chia is an ancient crop and was a local food along with beans, corn, and amaranth until the arrival of the Spanish. When Columbus arrived in America, these were the most important foods for more than 11 million people in the Aztec empire (Coates, 2011).

The capital of the ancient Aztec Empire, Tenochtitlan, received 5,000-15,000 tons of chia annually as a tribute from dominated nations (Ayerza & Coates, 2005). Chia was used as a religious offering to the Nahua gods (Coates, 2011). Chia was used for food, medicine and in the craft production at Olinala and Temalacatzingo Guerrero between 1500 and 1550 AD. It was a part of Azteca tribute and was sold to buy corn, gold, and cocoa (López, 2010; Sosa, 2016). Following the Spanish conquest, the use of chia was prevented for the first time for 260 years (between 1550-1810) because the Spanish forced the Aztec nation to stop domestic production. However, it survived in the mountains of Jalisco in Mexico, Michoacán, and Puebla, because numerous Nahua inhabitants that were living in the mountains continued its production in secret (J. P. Cahill, 2003; Sosa, 2016). Chia almost completely disappeared and was replaced by the crops preferred and brought by Europeans (Ayerza & Coates, 2005). After Mexico's independence from the Spanish in 1821, the prohibition on the cultivation of chia disappeared, but the cultivation of chia was

damage (Jamboonsri, 2010) and the area of the land dedicated to its production was reduced to a few hectares. The Jalisco farmers had played an important role in sustaining chia until the 1990s and chia proved to have a high nutritional value and agronomic adaptability. For 260 years chia was effectively an unknown species in the world and was recently reclassified as a modern food. Chia also was integrated with other crops that hardly survived and adapted (Sosa, 2016).

Recently, the chia plant has been investigated as a new crop (J. Cahill & Ehdaie, 2005) that has high oil content and the highest ω-3 fatty acid component among other oilseeds (Jamboonsri, 2010). In 1991, Argentinian researchers and the EUA started to study the chia plant under a research project called the "Western Argentina Regional Project¨ that integrated chia in modern cultivation. That project contributed to an increase in the worldwide area cultivated for chia from approximately 450 hectares per year or less (Ayerza, 1995) only in México in 1994 to 370,000 hectares in 13 countries in 2014 (Sosa, 2016).

Chia Production

The global demand for chia has increased during the past decade, and in 2014 the production increased by 367,000 hectacres in countries including Argentina, Bolivia, and Paraguay (Sosa, 2016). The chia plant is an important crop in countries such as the USA, Argentina, Chile, and Italy where the climate conditions make it difficult to plant chia seeds, and improved agronomic practices are needed to adapt chia in these agriculture zones (Coates, 1996; Coates, 2011). 80 percent of chia producers are in South America. The main countries producing chia are Bolivia, Argentina, Paraguay, Australia, Peru, Mexico and Nicaragua. Latin America countries have domestic markets such as Brazil, Argentina, and Chile. The major importer countries include the United States, United Kingdom and Europe (Fonseca, 2016). Argentina increased chia production from 40,000 to 120,000 hectacres between 2013 and 2014 (Peperkamp, 2015) but production of chia in northern Argentina was reduced in 2014 because of rain and diseases. The second largest producer of chia in South America is Bolivia, and its production of chia was 30,000 tons which expanded from 50,000 to 80,000 hectacres in 2014. Paraguay is third largest or sometimes a second largest producer of chia and increased the agricultures area for chia

cultivation from 30,000 to 100,000 hectacres between 2013 to 2014. In Mexico, the production area increased from 18,000 to 50,000 hectacres in 2014 (Fonseca, 2016; Peperkamp, 2015).

The biggest issue facing the USA and other countries is that chia is cultivated in a tropical area with short day lengths and only in agricultural zones between 20° 55' N to 25°05' S. The USA sites are in higher latitude areas, such as 32° 14 N (Tucson Arizona, USA) and 38°2'26.1"N (Lexington, Kentucky, USA). The chia plant cannot produce seeds in these areas because the plant faces frost and is killed before flower set (Jamboonsri et al., 2012; Sosa, 2016). If chia is planted outside this range of latitude, the yield and quality of nutrition could be very low (Daniela et al., 2013; Bochicchio et al., 2015; Sosa., 2016). In the USA and Argentina, the plant breeders and agronomists resolved this problem via plant breeding to develop varieties that have the ability to flower and produce seeds in the locations longer day-lengths than 12.5 h. These cultivars were registered as varieties that can grow in these countries, such as the USA and Argentina(Sosa, 2016). In the 20th century, numerous *Salvia* species including *Salvia hispanica* L. were introduced in the U.S.A as productive new crops (Gentry et al., 1990).

The first new chia varieties, specifically in Lexington KY, USA, were developed by mutation with Ethyl methane sulfonate (EMS) and gamma radiation, and the mutanint chia seeds produced early flowering plants and seed yields (Hildebrand et al., 2013; Jamboonsri et al., 2012). In Argentina, three new varieties were generated by individually and mass selected seeds. Mexico does not have any climate restrictions that can prevent chia growth. This country also has been developing numerous genetic lines that can be identified as the first generated variety of chia registered in the world. Chia currently can be grown in subtropical and tropical areas (Jamboonsri, 2010). The agriculture area in which chia is grown in the USA has been relatively unsuccessful compared to Argentina. The average seed yields produced in USA is 290 kg/ ha, but the global average is 350 kg/ ha (Sosa, 2016).

In 2010, Australia was the largest producer of chia seeds in the world. Chia appeared in advertisements and on shelves of mainstream supermarkets around the country. Australian chia was created by assembling old and new elements, although its continued stability is unknown (Loyer, 2016). The seed yields for commercial chia farming usually

produce from 500 to 600 kg/ha (Coates and Ayerza, 1996), but some farmers have obtained up to 1,260 kg/ha, and approximately 2,500 kg/ha has been obtained in experimental plots when irrigation and fertilizer are applied. Chia cultivation in South America and Australia exceeds Mesoamerica in the global production system due to producers of Latin American with integrated production system exposes that promoter is not only competing for the North American and European markets. But producer in South America are involved in developing markets by encouraging local consumers to uses chia. In the 1990s, there were numerous important studies in South America that focused on the international production of chia (Daniells, 2013).

Benefits of Chia

Chia (*Salvia hispanica* L.) is an annual summer crop and comprises an essential part of numerous Central American nations. Chia seeds are one of the most important economic crops after soybeans, corn, and beans. Chia seeds are one of the most important sources of polyunsaturated fatty acids (PUFA), omega-3, with up to 64 % of total lipids present, high levels of up to 24 % protein, up to 35 % oil, and up to 56 % fiber (Alfredo et al., 2009; da Silva Marineli et al., 2014). The protein content of the chia seed is greater than that of economic crop seeds such as wheat, which contains 10%, maize, which contains 11% and rice, which contains 8% (Coates, 1996; Herman et al., 2016). Chia seeds can be a useful food source because of their protein content, phytosterols, antioxidants, soluble and insoluble fiber, and minerals (Herman et al., 2016). Chia seeds are essential not only for food, but also for animal feed, medicine, oil paints, and ingredients in cosmetics (Jamboonsri, 2010), and have been cultivated for many centuries. In the last two decades, the chia seed has become more important for human health and nutrition due to its omega-3 fatty acid content, which enhances beneficial health effects (Ayerza, 2010). The whole chia seed is beneficial in animal feed as it increases the nutritious value of the resulting poultry, meat, and eggs (Ayerza and Coates, 2000; Ayerza and Coates, 2001; Ayerza and Coates, 2002). Chia may be the best source of beneficial soluble fiber identified (Ayerza and Coates, 2004; J. P. Cahill, 2003).

Chia has been established as an actual multipurpose ingredient for numerous products, such as chia seeds packets, oils, gels, flour, cereals, snacks, cookies, beverages,

animal feed, cosmetics, etc. Chia's nutritional properties are the primary reason for its popularity, with global demand increasing by 239% during 2013. There are estimates that chia demand will increas to 1.1 billion USD by 2020 (Fonseca, 2016) Numerous research has proved that benefits are derived from chia consumption by humans and animals including reducing cholesterol and triglycerides, improving bone nutrition, lowering high blood pressure, and avoiding heart disease and diabetes. Chia appeared from oblivion and today became a global superfood.

In 2013, the USA made up 47% of global consumption of chia seed food and drinks, while Canada was 12% in the Asia Pacific region was18% and Europe was 11% (Fonseca, 2016). The more accepted forms of the chia seeds are whole chia seeds and ground chia, which are added to smoothies, yogurt, juices, fruits, salads stir-fries, cereals, and for baking cookies or bread. Chia is recommended for celiac patients because it is a gluten-free product, and therefore could be used as an alternative to wheat. The hydrophilic characteristics of chia seeds can be increased to ten times more than its original size after its mucilage is soaked in water which represses feelings of hunger for a longer time (Fonseca, 2016).

Nutritive and Medicinal Values

Chia seeds are an antioxidant source of natural lipids, and chia extraction contains caffeic and chlorogenic acids and Flavonol glycosides (Taga et al., 1984). The antioxidant activity of the fiber-rich part of chia flour is observed to be higher than the activity of numerous grains and similar beverages, such as coffee, tea, wine, and orange juice.

The total dietary fiber of chia flour is 56.5 g per 100 g and the water capacity of fiber is 15.4 g per 1g (Jamboonsri, 2010). The chia seed coat is high in fiber which converts into mucilaginous and extends impressively when it is soaked in water. The chia fiber consists of glucose acid, glucuronic acid and xylose acid (Lin et al., 1994). Chia seeds contain up to 24 % protein, and the digestibility of chia protein was 79.8% in flour, 34.2% in toasted flour, 29.1% in raw seeds, 24.3% in soaked seed, and 10.9% in toasted seeds. Chia oil content is up to 35% and the oil composition of chia is high in the 18:3, α-linolenic acid,(omega-3 fatty acid). Chia diets significantly diminished triacylglycerol levels,

expanded high-density lipoprotein cholesterol and increased the content of alpha-linolenic acid in rat serum (Ayerza & Coates, 2005).

Dietary chia seeds additionally enhance insulin and adiposity resistance in dyslipeamic rats (Chicco et al., 2009). Diets supplemented with chia were found to diminish risks from some forms of diabetes, cardiovascular diseases, and cancers. Also, a chia diet reduced the tumor weight and metastasis number and restricted development and metastasis in a murine mammary gland adenocarcinoma (Espada et al., 2007). Chia seeds can reduce longterm risks and prevent cardiovascular disease more than traditional therapy and can help maintain control of glycemic and lipids in people (Kreiter, 2005; Vuksan et al., 2007; Vuksan et al., 2009). Omega-3 fatty acids have benefits for psychiatric disorders and have significant benefits in the prevention and therapy of unipolar and bipolar depression (Freeman et al., 2006).

Chia Uses

Chia leaf oil might be valuable in flavoring or fragrances and might be used as a pesticide since whiteflies and some insects appear to keep away from chia plants (Ahmed et al., 1994). Chia seeds that supplement broiler feed appeared to lead to extremely low saturated fatty acids content in dark and white meats (Ayerza and Coates, 2002). Chia seeds used in poultry feed are an important source of omega-3 in eggs (Ayerza and Coates, 2001; Ayerza and Coates, 2002). In addition, chia seeds increase the nutritioinal value of animals feed, and the vegetative parts could be a source of polyunsaturated fatty acids for ruminants (Peiretti & Gai, 2009). During the Christmas season, a product called ChiaPet is available, and it makes an unglazed ceramic tree or animals. It can fill with water and is covered with seeds of chia which grow to leaves that look like fur, hair or skin (Jamboonsri, 2010).

Kentucky area

Kentucky, USA has an area of 40,411 square miles and is about 380 miles long and 140 miles wide. The Latitude is 36° 30'N to 39° 9'N, and longitude is 81° 58'W to 89° 34'W. The average elevation of Kentucky is 228.6 m above sea level. The highest point is 1261.5 m above sea level at Black Mountain, and the lowest point is 78.3 m above sea level in the Mississippi River (NETSTATE, 2016). The state of Kentucky has an agriculture area

of 25,388,000 acres; 32% of this area is used for crop production, and 68% is used for livestock production. The main crops cultivated in Kentucky during 2015 were corn (36%), soybean (42%), tobacco (0.6%), wheat (0.4%), and alfalfa (1%). The total crop production in Kentucky from 2010 to 2015 totaled 668,635,000 tons of corn, 562,578,000 of soybean, 5,591,000 of tobacco, 2,576,000 of wheat, and 13,112,000 of alfalfa (Kim et al., 2017). In Kentucky, the major oilseed crop is soybean, and its production value was $881,100,000 in 2016 as reported by USDA (USDA, 2016).

There are many other oilseed crops grown in small areas in Kentucky, such as flax, canola, almond, sesame, sunflower, borage, safflower, crambe, comfrey, guayule, cuphea, broomcorn, jojoba, lesquerella, kenaf, meadowfoam, lupine, milkweed, and vernonia perilla. Chia is not included in the USDA report because its seeds are not planted in most USA zones (Jamboonsri, 2010). Chia is endospermic and is a diploid with only 12 chromosomes (n = 6), is a short-day plant, and is commercially produced in many countries such as Australia, Argentina, Colombia, Mexico, and Peru. Mutant chia grows in Kentucky with long- day plants and has low requirements for fertilizer, irrigation, and pesticides.

Chia belongs to the *Lamiaceae* family and is an annual seed crop that has a chromosome number of (2n = 12) (Estilai et al., 1990). The phenotype of chia plants has a 1-2 meter stem length and it is obtusely quadrangular; its leaves are ovate, opposite, and serrated. The chia flower is produced in the axillary cornered spikes that grow from bracts at the end of the branches. The shape of the corolla is tubular and it contains four stamens, two of which are sterile and larger than the others. chia seeds have an oval shape and grow in groups of four. The average seed is approximately 1 mm long and 2 mm wide, and 100 seeds weigh around 15 mg (J. Cahill & Ehdaie, 2005).

Chia mutant

The G8 variety of chia seeds used in the current study can be grown with long day length in Kentucky. The G8 variety of chia seeds was created from mutagenesis by Gamma radiation by Dr. Phillips and Dr. Hildbrand at the University of Kentucky Department Plant and Soil Sciences. The original seeds were *Shispanica cv*, or "Pinta". It is a wild, early flowering type in Mexico. It has been cultivated many years in Spindletop Farm but did not complete flowering because it could not grow in kentucky (temperate and tropical

area). The wild type Pinta were mutagenized with gamma rays (Gy) to produc early flowering plants. Two mutagenesis techniques were used. First, the seeds were soaked in ethyl methane sulfonate (EMS) solution for 6 hours and then other seeds were treated with gamma rays. M1 seed were planted in a greenhouse and M2 and M3 planted in the field of Spindletop farm in Lexington, KY (38_104700N, 84_2904100W, 298 m above sea level). Early flowering plants were transferred into a greenhouse before the frost could kill the plants after the second weeks of October, and early flowering M3 plants were collected (Jamboonsri, 2010). The daylength is recognized by leaves during the impact of red, far red and blue light on phytochromes (Ishikawa et al., 2009). The florigen causes a mobile signal identified to move from a phloem to an apical meristems. The florigen in the apical meristems makes changes in gene expression and alters the development of meristems by programming the plants to set flowers rather than leaves (Turck et al., 2008). In 2009, chia plant lines in Lexington, Kentucky that produced early flower buds and long day mutants with daylength of 14 h and 41 minutes were selected. The G8 varieties have been used in the current study with other varities created by crossing G8 with the Salba variety, which originated in Argentina, and along with G8 kummer varieties.

Ecosystem effect

Numerous studies claim that the ecosystem has a strong influence on the protein content of chia seeds, as has been reported for many other crops (Johann Vollmann et al., 2007). There is a positive correlation relationship between protein content and temperature in oilseed crops like soybean (Coates, 2011; Kumar et al., 2006; Thomas et al., 2003). The varying protein content in chia seeds among varieties was also reported for numerous other crops including sorghum (Saeed et al., 1987) and soybean (J Vollmann et al., 2000) as well as chia (Ayerza and Coates, 2004). These variations for other crops, such as soybeans and sorghum, have similarity demonstrated that changes in protein component are impacted by the environment (Saeed et al., 1987; J Vollmann et al., 2000)

According to Ayerza, (2009) the oil content increase and protein content decrease were correlated to the seed development, as well mean temperature, for chia and other crops (Ayerza, 2009). High temperatures can result in a reduction in yield, decreased seed set, and slower rate of photosynthesis (Ayerza and Coates, 2009). The elevation impact

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reported a negative relationship between protein content and elevation and might be explained by cause-effect relationship (Coates, 2011). An additional factor was altitude was reported as negative relationship between altitude and temperature is often mollified by some other factors; commonly air temperature decreases 1 Q C/ 160 m (Miller, 1975).

Coates (2011) reported that in wide-ranging oilseeds, protein content tended to decrease as altitude increased (Coates, 2011). Some reports estimated that oil content could be affected by temperatures. High temperatures reduce oil content, while low temperatures increase oil content (Ayerza, 2001; Ayerza and Coates, 2009; Cherry et al., 1985; Yaniv et al., 1995) There were not significant relationship between oil content and temperature. Higher temperatures could significantly affect chia seed content, as was verified for other seed oil crops such as soybean (Thomas et al., 2003). The temperature can influence the oil content variability through the seed growth process for chia and other crops. Commonly, the inverse relationship between temperature, altitude, and oil content is that as the altitude decreases, temperature increases (Ayerza, 2010; Thomas et al., 2003), and the percentage of oil content of some crops, including sorghum, soybean, chia, and others, tended to decrease (Ayerza, 2009; Boschin et al., 2007).

Other studies established that the chemical component of chia oil is affected by different factors like the quality of the soil and the climatic change and environment conditions. General location affected chia's growing time and seed yields, and to a lesser degree influenced protein and oil contents as well as fatty acid composition (Ayerza and Coates, 2009). The land elevation was negatively related to protein content and positively related to oil content The growing cycle length was reported to have a positive relationship with elevation (Ayerza, 2009). According to Coates. (1996) that variances in seed yields among varieties might be a result of a mixture of factors, including environment, genetics, seeding dates, row spacing, agronomic practices and their interactions (Coates, 1996). Environmental factors like temperature, rainfall, and soil type could have an affect on seed yields. However, seed composition was investigated for other oilseeds as a direct relationship with soil pH; this influence could have contributed, simultaneously with other factors to the results initiate in this study. It was also reported that the the growing period of both locations can increase with elevation (Ayerza, 2009) Planting chia in different locations could affect heritability and seed quality. We expect the outcome of the current

research to show the heritability in chia plants for high protein and oil content of different chia genetic materials. Increasing the oil percentage in chia could be beneficial for humans by enhancing animal products such as eggs, poultry feed, pork, rabbit meat, and cow's milk (Ayerza, 2009).

CHAPTER 2: Assessment of Oil Quantification Methods in Soybean and Chia Seeds.

INTRODUCTION:

Oilseeds are a necessary food ingredient of the human diet. Oilseeds contain natural antioxidants and comprise a large portion of human food, such as cereals, nuts, and their derived products (Wanasundara et al., 1997). Oilseeds are constituted of various fatty acids compositions that are grouped into saturated and unsaturated fatty acids. The fatty acids produce energy for different biochemical reactions in the human body. Oilseeds can be used in numerous medicines and cosmetics (Pandharinath., 2015). Improved quality and quantity of vegetable oils for human consumption can reduce risk of cardiovascular disease and improve fitness (Lu et al., 2011). In 2013, the United Nations expected the world population would grow from 7.4 billion people to 9.6 billion people in 2050 and with the growing world population, the demand for food will be increased. To cover the increasing demand for oil seeds, production may be increased from133 million tons to 282 million tons (Ouilly et al., 2017). According to Sharma et al (2012) the main production of oilseeds is in the moderate areas and more than 60% of oilseeds production comes from the USA and Europe (Sharma et al., 2012). Due to increased demand and motivation for the production of oil from various crops seeds, we evaluated and modified current protocols to quantify oil in different varieties of two crops, soybean and chia seeds. For many decades, numerous techniques were used to measure lipid quantification from oilseeds, animal lipids, algae, and yeasts. There are several reports that quantify lipids from samples of small and large biomass.

This study evaluated different methods of seed oil quantification, including some methods that have not been applied to oilseeds before. The objectives of this study are (1) Evaluate oil quantification methods and (2) Find the best technique for complete extraction of oils from seeds depending on characteristics such as high accuracy, easy, cheap and fast. Different methods are evaluated and tested, such as' the Soxhlet method, the Folch, Bligh and Dyer, Hexane- isopropanol (HIP) methods, a supercritical fluid extraction or Hot compressed hexane (HCH), a Direct Transesterification (DT), a Double Direct Transesterification (DDT), a Bead Beating Extraction (BBE) and Nile red fluorescence method.

The most accepted protocol for determining oil and lipid content of seeds, foods, and feeds is the Soxhlet extractor. The Soxhlet extraction is the oldest method for performing solvent extraction and it is the official method used by both AOAC and American Oil Chemists' Society (AOCS) (Ullah et al., 2011). The method was developed by von Soxhlet in 1879 as a new extraction system. The original Soxhlet procedure was used to determine the content of fat in milk (Soxhlet, 1879). The Soxhlet extraction was used as a starting point for a variety of modifications. Among different techniques found for extraction of lipids, the Soxhlet is the method most commonly used for more than a century. Numerous studies have supported the fact that Soxhlet is a standard method. Currently, the Soxhlet is the essential reference that the performance of other techniques are compared to. Two solvents have been used in this study with the Soxhlet extraction method, petroleum ether (PE) non-polar (0.1 polarity) solvent and acetone (Ac) polar (5.1 polarity) solvent.

The original Folch method was used to determine total lipids in animal tissues (Folch et al., 1957). The Bligh and Dyer method was used to determine total lipids in frozen fish. Bligh and Dyer cite the Folch method and they claimed their technique was much faster by 10 minutes and used much less solvent than the Folch method, but otherwise gave similar lipid yields (Bligh & Dyer, 1959) . The Bligh and Dyer and Folch methods employ a solvent mixture of chloroform/methanol, which is widely used for total lipid quantitation in biological samples such as meat products and fish (Ashraf-Khorassani et al., 2002). The original Hexane- isopropanol (HIP) extraction method was used to determine lipids contained in tissues (Hara & Radin, 1978) and it is the most efficient method for extracting polar lipids (Reis et al,. 2013). HIP uses a less toxic ratio of hexane/isopropanol solvent of 3:2 (v/v) for lipids extraction, and it was a better method to extract lipids from algae after it is oven-dried (Hussain et al., 2015). A supercritical extraction method, or hot compressed hexane (HCH), has not been applied to lipid quantification of oilseeds but is theoretically superior to the usual hexane solvent for lipid extraction. Mark Crocker's group at the Center for Applied Energy Research (CAER) in North Lexington reported almost three times the yield of lipid extraction from algae (*Scenedesmus sp*.) using supercritical hot hexane ($\geq 235°C$, 31 bars) than Soxhlet and a slightly higher yield than the Bligh and Dyer protocol (Shin et al., 2014).

In 1963, the first successful direct transesterification (DT) or direct acid methylation technique was achieved using a chemical composition of carboxylic acid methyl esters for the classification of microorganisms (Abel & Peterson, 1963). DT is the one-step reaction that is executed in the same test tube while avoiding the step of purifying and extracting. DT is used to determine total oil extracted from *Arabidopsis thaliana*. DT is a fast technique, the seeds amount required is minimal and the oil extracted is high-purity (Li et al., 2006). DT for dry biomass of microalgae provided higher fatty acid methyl esters (FAMEs) (Lemões et al., 2016). Double Direct Transesterification (DDT) has not been applied to lipid quantification of oilseeds. DDT converts saponifiable oils directly to FAMEs that can be determined by Gas chromatography with a flame ionization detector (GC-FID). DDT is an accurate, fast technique and uses small seed samples with two standards, glyceryl triheptadecanoate (C17-TAG) and methylnonadecanoate (C19-ME). This extraction has a single step with smaller ground seeds samples than the Folch and Bligh and Dyer methods (Griffiths et al., 2010). This technique is faster due to the elimination of an extraction step.

Two published papers used DDT with microalgae including (1) Griffiths et al. (2010) DDTG is more effective when combining acidic and basic catalysts than when using each catalyst individually for samples containing water. DDTG presents a reliable and more accurate method than the other extraction methods to quantify the total fatty acid content in microalgae (Griffiths et al., 2010). (2) Qiao et al.(2015) DDTQ is similar to the Griffiths et al. (2010) method. DDTQ used a progressive combination of alkaline catalysts and acid catalysts that were found to improve the extraction yield of fatty acids in microalgae. It was easier to prepare, has high stability, is cheaper, uses safer chemicals and is more readily executed (Qiao et al., 2015). The Bead Beating Extraction (BBE) produces direct mechanical damage to seeds using beads at high-speed and can be used in a laboratory and industrial sector (J.-Y. Lee et al., 2010). This technique has not been applied to lipid quantification of oilseeds before. The Nile red is a fluorescent lipophilic dye utilized to discover intracellular lipid quantity in algae, yeasts, and filamentous fungi. Using fluorometric microplate protocol, Nile red fluorescence was confirmed with Algae and Yeast (Sitepu et al., 2012). The Nile Red is commonly used as a method to quantify lipids in recent years (Rumin et al., 2015; Takeshita et al., 2015). The fluorimetric protocol

does not involve lipid extraction; rather just penetration of the Nile red into the assay material and it is considerd an easy and simple technique for several microalgal species.

MATERIAL AND METHODS

Materials used in this study:

The oilseed materials used consisted of ten standard soybean samples including regular oil Jack and a high oil line/ lot VgD, plus a chia_G8 2011 lot. The seeds have been provided by the Department of Plant and Soil Sciences at the University of Kentucky. Dr. Hildbrand lab and Dr. Phillips.
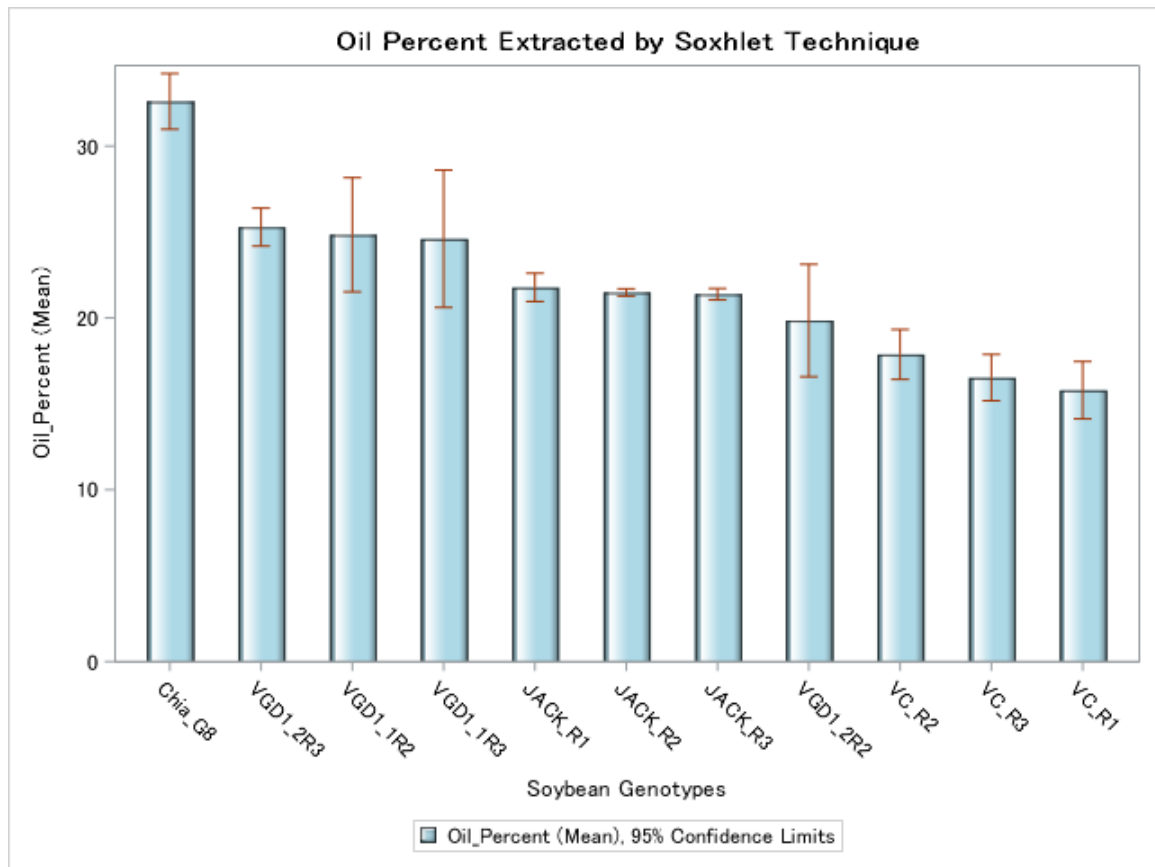
Figure 1: Eleven bias samples used for evaluated lipids quantification methods.

Ten bias samples of soybean (VC R1, VC R3, VC R2, VGD1-2 R2, JACK R3, JACK R2, JACK R1, VGD1-1R3, VGD1-1R2 and VGD1-2 R3) used for covering the range of oil from 15.4% to 25.3% and chia, G8 oil at 32.6%.

The SAS output of 10 soybean varieties in the GLM procedure provided ls-mean oil percent and confidence limits 95%, standard error and P-value:

Table 1: The GLM procedure of 10 soybean bias samples with 95 confidence interval and P-value.

Sample	Oil_Percent LSMEAN	95% Confidence Limits		Standard Error	Pr > |t|
VC_R1	15.794	14.531925	17.056075	0.6127952	<.0001
VC_R3	16.526	15.263925	17.788075	0.6127952	<.0001
VC_R2	17.678125	16.232236	19.124014	0.702045	<.0001
VGD1_2R2	19.848	18.585925	21.110075	0.6127952	<.0001
JACK_R3	21.382	20.119925	22.644075	0.6127952	<.0001
JACK_R2	21.415625	19.716504	23.114746	0.8250007	<.0001
JACK_R1	21.7089583	20.009838	23.408079	0.8250007	<.0001
VGD1_1R3	24.5389583	22.839838	26.238079	0.8250007	<.0001
VGD1_1R2	24.7722917	23.073171	26.471412	0.8250007	<.0001
VGD1_2R3	25.215625	23.516504	26.914746	0.8250007	<.0001

There is not a significant difference between replications for each sample (P= 0.62) but there are significantly different between soybean varieties (P-value <.000), less than 0.05 and $R2 = 0.9$, that means the model explains 90% the variability of the samples data around its mean. The contrast showed there is a significant difference between high vs low, mid vs low and mid vs high with P<.0001 Appendix chapter 2 Table 1-A and B

The statistical analysis shows there are significantly different between bias samples, so that can be exhibited and proved the ability to use the 10 soybean standard and chia_G8 as a benchmark (Control) for evaluating different methods involved in this study. The contrast shows there is a significant difference between high vs low, mid vs low and mid vs high with P<.0001. Generally, the oil quantification methods ran with three main bias samples JACK_R3 medium oil content 21.3% and VGD1_2R3 high oil content 25.2%, and chia_G8 with 32.6% oil content.

Soxhlet Protocol (Soxhlet, 1879):

In the current study, we compared two different solvents acetone (Ac) and petroleum ether (PE) with Jack lab, Jack1 2015, and VGD1-2 of soybean and G8 of chia.

Soxhlet process
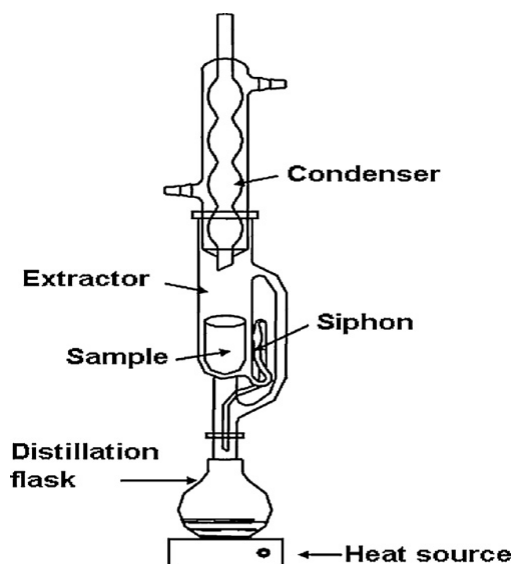
Figure 2: Conventional Soxhlet Extractor (De Castro & Priego-Capote, 2010).

A seed sample mass is placed in a thimble which is filled with the condensed solvent from a distillation flask. (Figure 2). When the solvent reaches the overflow level, the solvent is aspirated by a siphon from the thimble holder and directing the solution back into the distillation flask. This process is repeated until describe (De Castro & Priego-Capote, 2010) oil extraction. The temperature of distillation port is setting to a boiling point of the solvent utilized.(petroleum ether or acetone) In this study. Cycles are repeated many times until allowed the color in the extractor mixture changes to colorless (Chauhan et al., 2017). Usually, it ran for 35 cycles to extract 99% of the oil from seeds. Then the solvent extract is evaporated by using a condenser to dry the extract in the distillation flask, and the dried extract is weighed. Oil extraction was performed using petroleum ether and acetone as the extraction solvents and remove oil. Seeds mass is used 5 g, and 1.5 g of the ground of soybean and chia seed by a burr mill grind machine are used for extraction in a Soxhlet extraction process for at least 35 cycles.

Soxhlet Protocol

Analytical Reagent

Acetone provided from VWR, USA and petroleum ether bought from Millipore, MA, USA.

Materials

In the beginning, rinsed flasks, paper towels and string in petroleum ether for a few minutes. Dried for few minutes under the hood, and put paper towels and string into a forced draft oven for 12 hours at 103ºC. 2-3 crystals of Butylated hydroxytoluene (BHT) into the flask was added. Distillation flasks, paper towel, strings, and grounded seeds (5 grams or less for each paper) were weighted. Prepared the water bath, and replaced the water with galvanized metal beads.

Methods

These steps for each sample:

Two hundred mL, solvent (acetone or petroleum ether) in a distillation flask and ground seeds in the towel were added. Tighten the towel that has ground seeds with string, then added it to the extraction tube, which connected to the distillation flask (lower part) and the condenser (upper part) (Figure.2). Keep the whole unit upright and turn on the heat source and the cooling system for Soxhlet unit. Recorded the time for each cycle and recorded the time started and the time stopped. Dried all samples after the rotary evaporation, in vacuumed oven at 50ºC for 1 hour and measured the weight, and then increased the temperature in vacuumed oven to 103ºC for 30 minutes and weighed again.

As the Soxhlet protocol, it is added one small BHT crystal to each flask before weighing because it forbids the acceleration of oxidation of oil in the flasks. Oil oxidation makes weight determinations and further use of the oil inefficient. Normally when conducting lipid chemical analyses, including lipid quantification, it is crucial to include an effective antioxidant at an optimal level, standardly BHT at 0.001%. The samples have been checked for lipid oxidation by fatty acid analysis. The weights were recorded for the flasks with oil in the vacuum, and the number of cycles per hours and total cycles were recorded too, and the moisture content of the specific ground sample used for these Soxhlet runs should be measured.

The previous protocol of Soxhlet was used with the flasks being partly submerged in water. A poorly controlled water bath was difficult to work with and made it very hard to see how much solvent was in the bottom flasks. Various materials could be used in place, and one of them was included in the current study. We replaced the water with Armor beads. We measured the temperature of different materials in the bath instead of water, and

we needed to maintain a number of temperatures. We set up the bath for three materials to reach higher heat (temperature °C) in contact with the flasks that have solvents. The boiling and evaporation speed of solvents were recorded to find accelerated cycle time/hr. We carried out the comparison with sand, rust beads, and small beads.

Three different materials were recorded a pick of higher temperature and the best results were with small beads because they reached the highest temperature at 153°C.

Table 2: Maximum temperature among materials

Materials	Temperature
Rusted bbs	106°C
Small Armor Beads	153°C
Sand bath	122°C

Soxhlet extraction employed acetone and petroleum ether with chia, G8 and soybean, Jack varieties

In the beginning, the Soxhlet method was run with ground seeds of chia, G8 and soybean, Jack for two different times. Each run used a duplicate with 5 g ground seeds. We put 200 mL solvents into flasks, and each cycle time was measured with the temperature °C. Percentages of total lipid recovered was measured by two different solvents petroleum ether (PE) and acetone(Ac). Also, the effect of cycle time and moisture content on the percentage of oil extracted from Soxhlet was measured.

Moisture Content (MC):

One of the factors used to evaluate the Soxhlet method is used different moisture content (1) medium (higher than10%) and low (lower than 4%).

Soybean and chia seeds stated 10% MC from seeds room with humidity at 50% and 10°C. The moisture content was measured for three samples (chia, G8, and soybean, Jack1 and VgD1) then three sub-sample put in forced drying oven for 48 hours at 103°C. Three varieties from oilseeds that used with Soxhlet assessment provided more than 10% MC such as chia, G8 variety obtained 10.6% MC, soybean, Jack lab gave 10.8% MC, and soybean, VgD provided 10.9% MC (Table 3).

Table 3: Percentages of MC at 50% humidity.

No	Varieties	Seed weight (g)	Seed Weight after the dry	Moisture%	SE±
1	Chia, G8	0.2870	0.2565	10.64	0.32
2	Soybean, Jack seeds	0.4342	0.3873	10.79	0.32
3	Soybean, VgD1	0.2779	0.2476	10.91	0.08

The easier way can be reduced MC% is force dry oven but this way is not practically process due to a damage could be happened of seeds content. Recently stated that increasing temperature of the dry oven has an impact on oil content negatively. The best dry oven temperature at 30°C and it is the best drying temperature for sustain the quality and quantity of essential oil (Shahhoseini et al., 2013). In the present study, we are looking for better way to reduce the MC less than 3 or 4% without the effect of the composition of seeds:

Saturated Salt Solution Lithium Chloride (LiCl):

It is reported saturated solutions of lithium chloride create atmospheres 11.1 – 11.3% relative humidity (RH) from 5 and 50°C that could be employed for equilibrating seeds when the moisture content of the seeds must be controlled (Hay et al., 2008). The equilibrium for 11.3% relative humidity can be achieved with saturated lithium chloride at 25°C temperatures. (Fernandez, 2011). 11.4% RH equal less than 4% moisture content of soybean seeds as reporeted by Ellis et al. (1990) in soybean seeds, the logarithmic relation was continued down to the lowest moisture content indicated 3·3% that equilibrium relative humidity 11·4%. (Ellis et al., 1990).

Two different seeds materials chia and soybean (whole seeds) was tested at 25°C, 30°C, and 40°C for 9, 10 and 10 days until the samples weight constant (Table 4). The salt LiCl solution reduced of the total MC of seeds was reduced 3.5% of chia, G8 and 2.5% with soybean, Jack at 25°C. The total MC seeds was reduced 3.6% of chia, G8 and 1% with soybean, Jack at 30°C. At 40°C the total MC of seeds was reduced with chia, G8 was 4.4% and 2.7% with soybean, Jack.

Table 4: Percentages of total weight reduced by salt LiCl solution under different temp.°C.

Seeds	Temp. °C	Weight (g)	Weight (g), after SSLC used	Weight Reduced(g)	MC %
Chia, G8	25	2.16261	2.08645	0.07616	3.52
Soybean, Jack1	25	2.47242	2.40985	0.06257	2.53
Chia, G8	30	5.27342	5.08058	0.19284	3.66
Soybean, Jack1	30	5.20795	5.1586	0.04935	0.95
Chia, G8	40	2.17732	2.08263	0.09469	4.35
Soybean, Jack1	40	2.187	2.12838	0.05862	2.68

More details in figure 2.3 A-F

Chia, G8

C-
Temperature
30°C

Soybean, Jack

D-
Temperature
30°C

Chia, G8

E-
Temperature
40°C

37
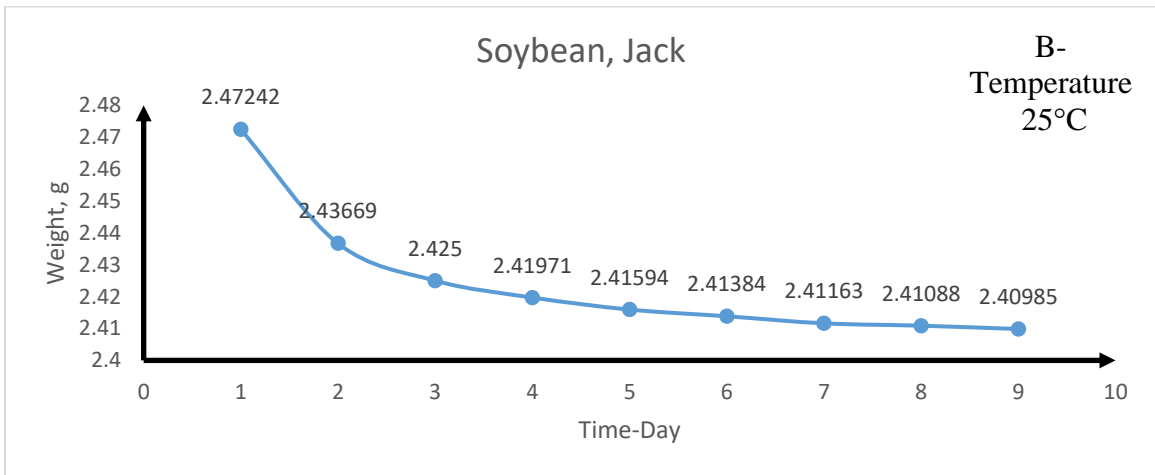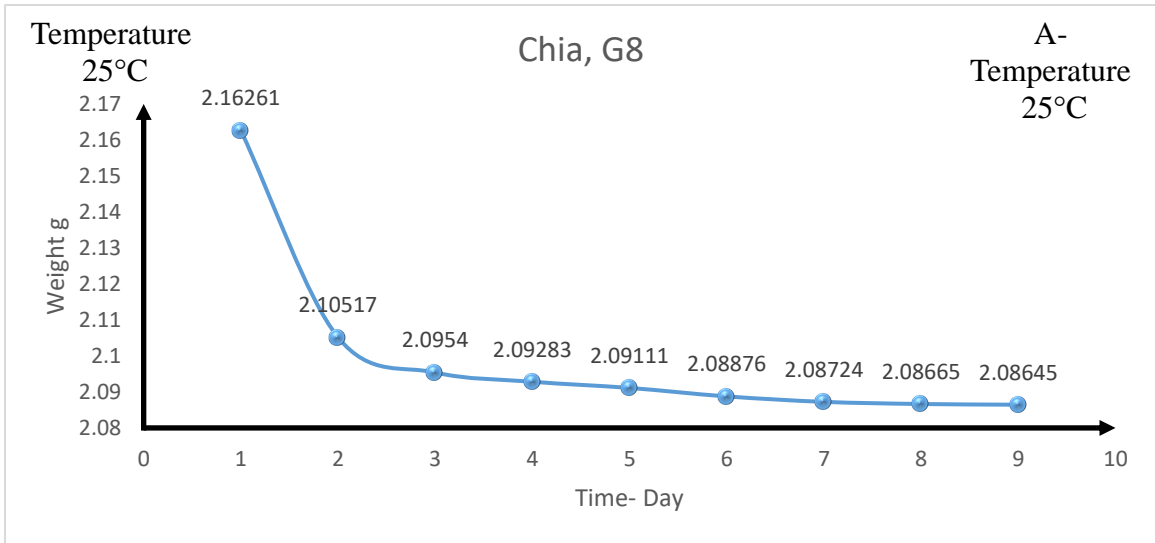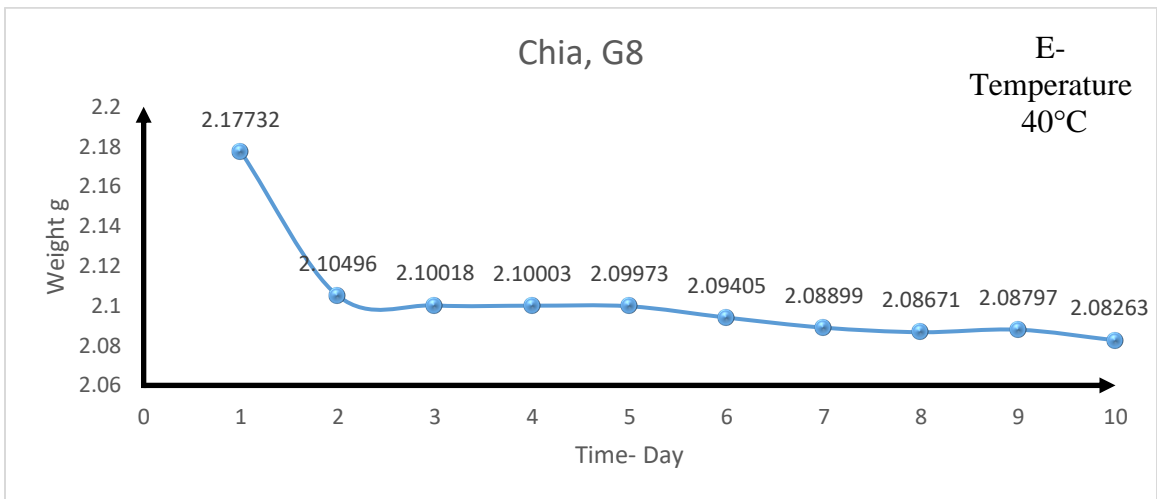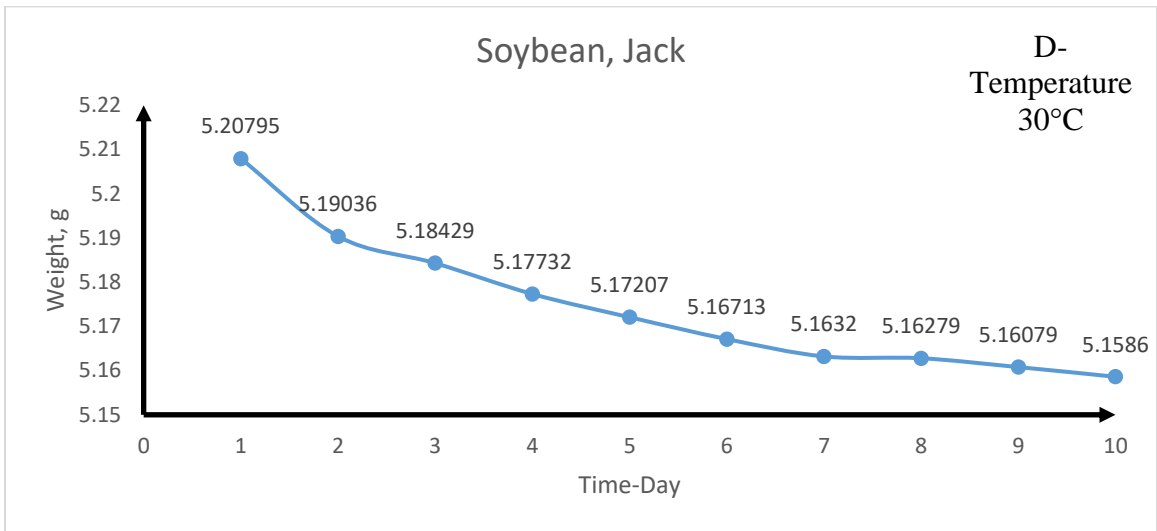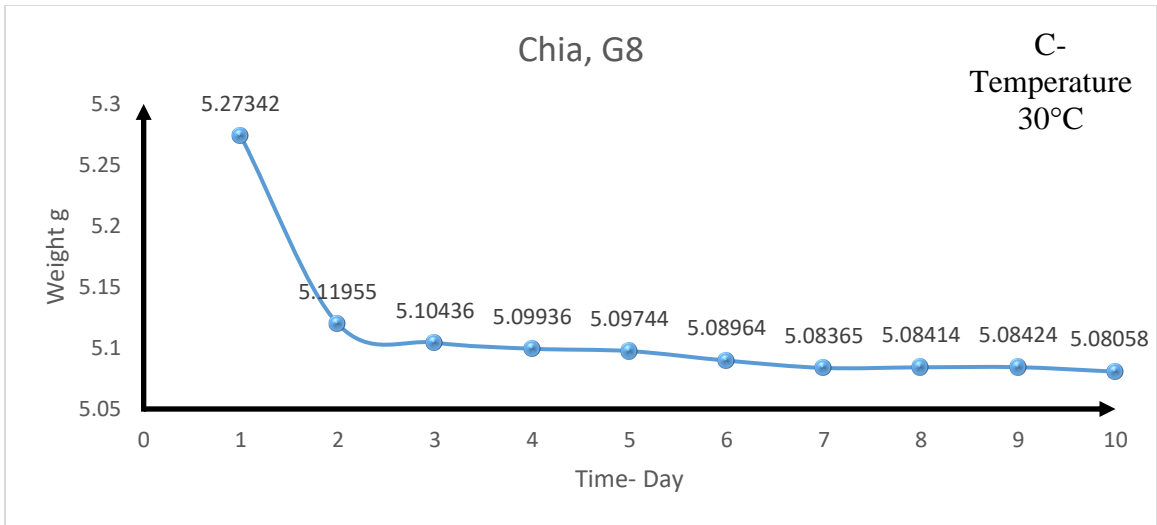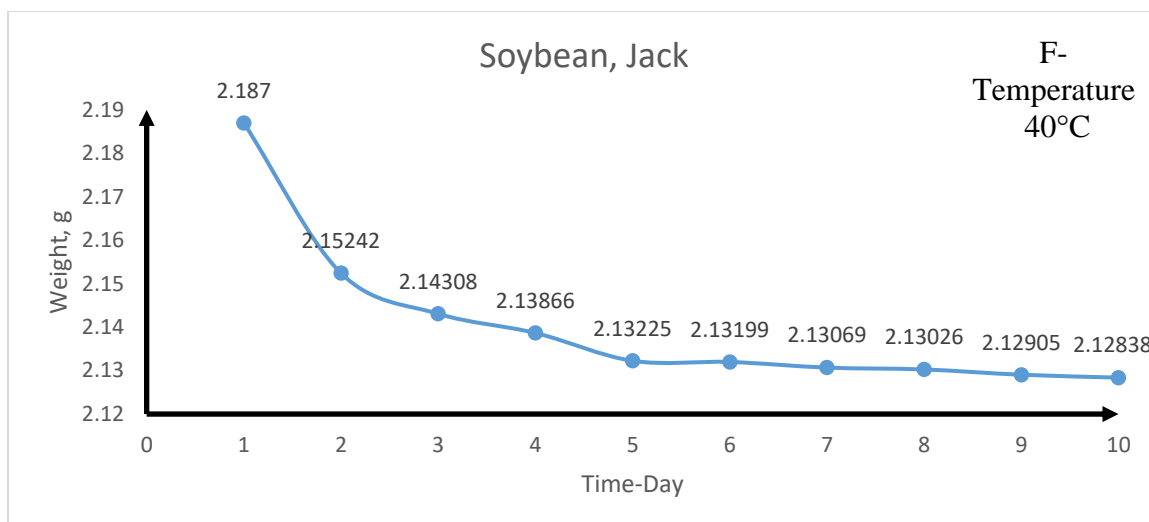
Figures 3 A-F: Weights of soybean and chia seeds in salt LiCl at different temperature °C.

Figure 3 A-F shows the moisture content (MC) of seeds in salt LiCl for 9 days at 25°C and 10 days at 30 and 40°C until the samples weight constant. The moisture content achieved different results. Then after constant seeds in salt LiCl solution, the weight under different temperatures 25, 30, and 40°C put a duplicate sub-sample of seeds materials in force dry oven for 48 hours at temperature 103°C. Reducing MC to 4% or less was not valuable by using salt solution of lithium chloride (LiCl) due to seeds weight was stable before 10 days with keep moisture content more than 5% (Table 5). on the other hand, this experiment is not efficient because it is required long time 10 days' period for reducing percentages of moisture content comparing with other ways such as freeze drying. Vijay et al (2015) evaluated the lithium chloride had low dry rate and effect on seeds quality through used (Vijay et al., 2015).

Table 5: Percentages of moisture content after 48 hrs. in force dry oven at temp.103°C.

No	Varieties	Temp. of Salt LiCl	Moiture	SE±
1	Chia G8	25°C	5.82	0.13
2	Soybean Jack	25°C	5.80	0.59
3	Chia G8	30°C	5.55	0.16
4	Soybean Jack	30°C	6.25	0.56
5	Chia G8	40°C	9.27	0.37
6	Soybean Jack	40°C	7.60	0.23

Freeze drying (FD)

One of the best methods to avoid damage that is caused by heat is freeze-drying (Ghasemi et al., 2013). We have used freeze drying (FD) with ground seeds materials of two soybean varieties (Jack and VgD) and chia (G8) for 72 hours in FD, under pressure 0149 MT and temperature -60.0°C (Table 6).

The percentages of moisture content after FD put a duplicate sub-sample of seeds materials in force dry oven for 48 hours at temperature 103°C. MC% of chia, G8 variety reduce to 1.1%, soybean, Jack variety decreased to 2.4%, and soybean, VgD variety diminished 3.2% of total MC of seeds.

Table 6: Percentages of moisture content via freeze drying.

No	Varieties	MC%	SE±
1	Chia G8	1.10	0.07
2	Soybean Jack1	2.35	0.06
3	Soybean VgD1	3.15	0.02

Folch, Bligh and Dyer, and hexane-isopropanol experiments

In the present study, the main component of the oils are triacylglycerol, is a polar lipid. We compared Folch, Bligh and Dyer, and hexane-isopropanol lipid extraction and quantification methods. Our implementation of the Folch method followed the protocol set by (Iverson et al., 2001). The Bligh and Dyer lipid extraction and quantification procedure followed the protocol of (Iverson et al., 2001) and hexane-isopropanol method that of (Reis et al., 2013).

Folch Protocol (Folch et al., 1957)

Analytical Reagent

Chloroform ($CHCl_3$) bought from Millipore, MA, USA, Methanol (CH_3OH) provided from Fisher Scientific, USA. Potassium chloride (KCl) bought from Fisher Scientific, USA.

Method

The Folch extraction was achieved as known by using the modified version, the ratio of Folch solution was used eight mL chloroform, four mL methanol, and 1.2 mg BHT. 100 mg of ground seed put into a test tube. Two mL Folch extraction solution with BHT was added to achieve a final ratio of 8:4:1.2 chloroform/ methanol/BHT into each tube (20/1 solvent/sample). ≈ 0.1 mg BHT/mL solvent or 0.01%. Samples mixed by vortex for 3 X 10 sec. Added 0.75 mL 0.9% KCl, and mixed by vortex again for 3 X 10 sec. Shaken on shaker was used for methylation a specified period of time and let sit for 1 min. If no phase separation is seen, added chloroform and 0.9% KCl 0.5 mL at a time until phase separation is seen. Briefly, centrifuge if needed. Transfer as much of the chloroform (lower) phase as possible into a weighed tube. One mL chloroform to the original tube was added and mixed by vortex vigorously again. Added this additional chloroform to the original and extracted with a 3rd mL chloroform, repeated and added the 3rd chloroform extracted to the 1st two. Blow solvent (chloroform) off with N_2 heating between 37 - 60 °C. The nitrogen stream was constantly moved therefore it quick removed the evaporating surface of the sample until all solvent portions were gone. Put under vacuum at 50 °C weighing every 10 min, until no further weight change. Recorded all the weights and the pressure (vacuum).

Protocol Bligh and Dyer method (Bligh & Dyer.,1959)

Analytical Reagent

Chloroform ($CHCl_3$) bought from Millipore, MA, USA, Methanol (CH_3OH) provided from Fisher Scientific, USA.. Potassium chloride (KCl) bought from Fisher Scientific, USA.

Method

The Bligh and Dyer extraction was performed and based on modified version, the ratios of Bligh and Dyer solution is used 4 mL chloroform, 8 mL methanol, and 1.2 mg BHT. Briefly, by mixing 1 chloroform– 2methanol with BHT (1.2.0.3, v/v/v) 100 mg of ground dry-mass seed put into test tube. Two mL of the Bligh & Dyer extraction solution and BHT added to each tube (20/1 solvent/sample). ≈ 0.1 mg BHT/mL solvent or 0.01%. Samples mixed by vortex for 3 X 10 sec, 0.75 mL 0.9% KCl was added,and mixed by

vortex again for 3 X 10 sec. Shaken on shaker used for methylation a specified period of time. Let sit for 1 min. If no phase separation is seen added chloroform and 0.9% KCl 0.5 mL at a time until phase separation is seen. Briefly centrifuge if needed. The final biphasic system is allowed to separate into two layers and transfer as much of the chloroform (lower) phase as possible into a weighed tube. One mL chloroform to the original tube was added and mixed by vortex vigorously again. Added this additional chloroform to the original and extracted with a 3rd mL chloroform, repeated and added the 3rd chloroform extracted to the 1st two. Blow solvent (chloroform) off with N_2 heating from 37 to 60 °C, then following put test tubes under vacuum at 50 °C weighing every 10 min, until no further weight change. Recorded all the weights and the pressure (vacuum).

Protocol hexane: isopropanol extraction method

Analytical Reagent

Hexane (C_6H_{14}) from Fisher Scientific, USA. Methanol (CH_3OH) provided from Fisher Scientific, USA. Potassium chloride (KCl) bought from Fisher Scientific, USA.

Method

The hexane: isopropanol extractions were achieved as identified by using the modified version, the ratio of hexane: isopropanol solution is 30 mL hexane, and 20 mL methanol, and 5 mg BHT. 100 mg of ground seed put into a test tube. Two mL of the hexane: isopropanol extraction solution and BHT to each tube (20/1 solvent/sample). ≈ 0.1 mg BHT/mL solvent or 0.01%. Mixed the samples by vortex for 3 X 10 sec, and added 0.75 mL 0.9% KCl. Mixed by vortex again for 3 X 10 sec. Shaken on shaker was used for methylation a specified period of time and let sit for 1 min. In the case of no phase separation is seen added chloroform and 0.9% KCl 0.5 mL at a time until phase separation is seen. Centrifuge briefly if needed. Transfer as much of the hexane (upper) phase as possible into a weighed tube. 1 mL hexane to the original tube was added and mixed y vortex vigorously again. Added this additional hexane to the original and extracted with a 3rd mL hexane, repeated and added the 3rd hexane extracted to the 1st two. Blow solvent (hexane) off with N_2 heating between 37 to 60 °C. Put test tubes after solvent evaporation under vacuum at 50 °C and weighed every 10 minutes, until no further weight change. Recorded all the weights and the pressure (vacuum).

Supercritical extraction method (Hot compressed hexane) protocol:

Analytical Reagent

Hexane (C_6H_{14}) from Fisher Scientific, USA. Methanol (CH_3OH) provided from Fisher Scientific, USA. Potassium chloride (KCl) bought from Fisher Scientific, USA.

Loding the Reactor

Weighed 1 g ground seeds (previously dried overnight in a vacuum oven) directly into the tared reactor vessel and added 20 mL of hexanes. Placed the gasket, mount the vessel into the support and the support onto the platform, raise the stand and align the bolts of the reactor head with the holes in the support. Finished raising the platform, screw the bolts in in a star pattern using the Allen key. Lower the platform placed the heating jacket and sealed the bottom of the vessel by placing a heating mantle between the platform and the bottom of the reactor.

Purging the Catalyst- Loaded Reactor

Make sure all black keys (and the N_2 key leading to the mass flow controller) and the back-pressure regulator are closed and that the purge hose is immersed in water in the bubbler (make sure you have a stream of water before turning on the heater). Open the N_2 cylinder and adjust pressure to 500 psi. Allow N_2 into the reactor by slowly opening the upper left black key. Allow N_2 into the condenser by slowly opening the lower right black key. Close the upper left black key to isolating the system from the N_2 cylinder. Wait for 5 minutes if the pressure stays constant they move to the next step, if not release the gas and tight the bolts and start all over again flow water through the magdrive as soon as heating is turned on. Once the system is purged, set stirring to 1000 rpm. Open the cylinder containing the desired gas, set the delivery pressure 500 psi slightly above that of the experiment and opened the appropriate key leading to the mass flow controller. Pressurized and heated the system to the desired pressure (260 psi) and temperature (240 C) following the same steps used in the previous section (changing pressures, temperatures, gas flow and reaction times as necessary). Flow water through the magdrive as soon as heating is turned on. Once the reaction time had elapsed (10 minutes), turn off the heating at the control tower, take off the heating jacket and placed it aside on the heating mantle, used forced air to cool to 27 °C by blowing air in to the reactor until it reaches 26 C. the system can be allowed to reach atmospheric pressure as it cools. Filter the solvent and the sample

using (Gelman filter paper (polypropylene filters 127 mm diameter)). Use the rotary evaporation to evaporate the solvent under vacuum (around 200 psi), after all solvent evaporated (under 70 C for hexanes) weighted the round flask at least two times until getting a consistent weight.

Direct Tranasterification method (DT) Protocol

Analytical Reagent

Chloroform (99.8%) from Millipore, MA, USA. Methanol (CH_3OH) from Fisher Scientific, USA. Potassium chloride (KCl) from Fisher Scientific, USA. Potassium carbonate (K_2CO_3) from Fisher Scientific, USA. Sulfuric acid H_2SO_4 from Fisher Scientific, USA. Isooctane (IO) from Fisher Scientific, USA. Glyceryl triheptadecanoate (C-17 TAG) from Sigma-Aldrich, USA.

Materials

Tri 17:0: Added 50 mg of tri-17:0 powder to a small glass tube (e.g. 10 x 75 mm), and added 2.5 mL of chloroform ($CHCl_3$) + 0.001% BHT and mix well (a tube ~ half full initially is ideal). Store the solution in the freezer (1 µL = 20 µg) and mark the meniscus each time opened if lower than previously marked. Added $CHCl_3$ to restore the meniscus if needed and always mixed well before used.

Method

Glass tubes (e.g. 1 cm × 10 cm) with Teflon-lined screw caps were pre-rinsed thoroughly with chloroform and dried at $103^{\circ}C$ ($\geq$ 12 h or perhaps less needed) to remove any contaminating lipid residues and water and precisely weighed. Put some ground seeds into weight dish and dried for freeze drying 72 hours at - 60°C or 3 hours at 103˚C. Ten mg seed chips added to tubes and dried for 5 hours at 103 ˚C then added tri-17:0 in $CHCl_3$ to chips at 20 µg/mg; allow $CHCl_3$ to dry (a few min.) (10 mg seed mass with 10 µL tri-17:0). Added 2 mL freshly prepared 0.001% BHT and 2.5% (v/v) H_2SO_4 in methanol CH_3OH to each tube and tightly cap. Mixed by vortex for 30 seconds, heated to 100 ˚C, and mixed by vortex after 30 min. And heated an additional 30 minutes and mixed by vortex, and 30rd minutes. Cool to room temperature and added 1 mL isooctane (IO) with 0.001% BHT, vortex and transfer 200 µL top layer after separation to GC vials and added 1 more mL isooctane and mixed. Added 1 mL 0.9% KCl (or NaCl) or K_2CO_3. If needed

for clear phase separation before taking an aliquot from upper layer. With 40 mg samples added 1.5 mL IO instead of the 1 mL above; transfer 50 µL or 5 drops IO layer to GC vials, and add 1.5 mL more isooctane for sample dilution.

Double Direct Transesterification

To modify the original protocol by reducing the level of the methyl nonadecanoate (C19-ME) concentration and to the glyceryl triheptadecanoate (C17-TAG) levels as per Griffiths et al. (2010). Also, we included 2,2-dimethoxypropane, and toluene with C19-Me and tri-17;0 and adjusted the pH to that of 14% BF_3 with concentrated HCl. According to Griffith et al. (2010) who illustrated that using two standards glyceryl triheptadecanoate (C17-TAG) that was added prior to the reaction as standard of a quantitative. They added methyl nonadecanoate (C19-ME) in the final step to solvent extraction to confirm the extraction was achieved. The last modify of Griffiths protocol was by added 2 mL of 6% K_2CO_3 or KCl in water instead of 3 mL of water, also added 3 mL toluene instead of 4 mL toluene, and increased the centrifuge form 5 to10 minute instead of 1 minute for more layer separation with transfer 1 mL instead of 0.5 mL in previous protocol.

Griffiths et al. (2010) Protocol (DDTG)

Analytical Reagents

All reagents used were of chromatography standard. Toluene (99.9%) was from VWR, USA. Chloroform (99.8%), hexane (98%), and methanol (99.9%) were from Fisher Scientific, USA. Ethanolic base (0.5N), also known as sodium methoxide (SM) prepared in the University of Kentucky, Department Plant and Soil Sciences, Dr. Hildbrand's Lab. and boron trifluoride (BF_3) was from Sigma-Aldrich, USA methanol solution (14%) was obtained from Fisher Scientific, USA. Distilled water (dH_2O) was from the University of Kentucky, Department Plant and Soil Sciences. Standards used were glyceryl triheptadecanoate (C17-TAG) and methyl nonadecanoate (C19-ME) were from Sigma-Aldrich, USA. The seeds have been provided by the Department of Plant and Soil Sciences at the University of Kentucky, Dr. Hildbrand's Lab.

Materials

2x1.5 cm Teflon-lined screw-cap test tube. 0.1% BHT stock, C-17 TAG + C-19 ME standard solution 20 mg/mL and sodium methoxide Solution.

Transesterification

The Direct Transesterification, reagents were added directly to either standards, 10 standard ground seeds of oilseed that known total oil content. Two standards were used: glyceryl triheptadecanoate (C17-TAG) and methyl nonadecanoate (C19-ME) were added prior to the reaction as a quantitative standard. A combination of base followed by acid catalysis was performed as follows: Ten mg of known dry-mass seeds were dissolved in 10 µL of toluene standard containing C-17 TAG and C-19 ME/mg sample (+ toluene to 500 µL final volume) = 10 µL 17:0 +19:0/mg sample. in glass test tubes with silicon-lined screw-cap lids. One hundred µL of 2,2-dimethoxypropane (as a water scavenger). was added. 1 mL of sodium methoxide was added and the samples mixed briefly by vortexing and Flush with argon (Ar) and seal Followed being placed in an incubator at 80°C, with shaking at 300 rpm for 10 minutes and samples were cooled for 5 min to room temperature. Adjust the pH to that of 14% BF_3 with 17 µL concentrated HCl and added one hundred µL of 2,2-dimethoxypropane (as a water scavenger). 1 mL of (Boron tri-fluoride – methanol solution) BF_3 methanol (14% BF_3) solution was added and flush with Argon (Ar) and seal. Repeating the incubation at 80°C for 10 minutes while shaking at 300 rpm and after cooling for 5 min to room temperature. 2 mL of 6% K_2CO_3 or KCl in water and 3 mL toluene were added tubes mixed by vortexing. Samples were centrifuged at 4,000 rpm for 5 -10 minutes and the upper toluene layer, containing the FAME extract, was transferred with a Pasteur pipette to vials for GC.

Qiao et al. (2015) protocol (DDTQ)

Analytical Reagents

The reagents were used in all experiments of chromatography standard. Chloroform (99.8 %) was from Millipore, MA, USA. Hexane (99.5 %), methanol (99.9 %) were from Fisher Scientific, USA, sodium hydroxide (NaOH) was prepared he University of Kentucky, Department Plant and Soil Sciences, Dr. Hildbrand's Lab, and potassium carbonate (K_2CO_3) was from Fisher Scientific, USA, and acetyl chloride (AcCl, 99 %) was from Tokyo Chemical Industry (TCI), UK. Glyceryl triheptadecanoate (C17- TAG) and

methyl nonadecanoate (C19-ME) were obtained from Sigma-Aldrich, USA. The seeds have been provided by the Department of Plant and Soil Sciences at the University of Kentucky, Dr. Hildbrand's Lab.

Materials

2x1.5 cm Teflon-lined screw-cap test tube, 0.1% BHT stock, C-17 TAG + C-19 ME standard solution 20 mg/mL. Sodium hydroxide (NaOH) 0.5 N in methanol, NaOH does not fully dissolve but a well-mixed suspension is added. Acetyl chloride (AcCl) 10% in methanol (Carefully, add AcCl slowly to swirling or stirring methanol; wear eye protection). 10 mL AcCl + 90 mL methanol.

Transesterification

The Direct Transesterification, reagents were added directly to either standards, 10 standards ground seeds of oilseed that known total oil content.Two standards were used: glyceryl triheptadecanoate (C17-TAG) and methyl nonadecanoate (C19-ME) were added prior to the reaction as a quantitative standard. A combination of base followed by acid catalysis was performed as follows: 10 mg of known dry-mass seeds were dissolved in 10 µL of toluene standard containing C-17 TAG and C-19 ME/mg sample (+ toluene to 500 µL final volume) = 10 µL 17:0 +19:0/mg sample. In glass test tubes with Teflon/rubber lined screw cap lids. One hundred µL of 2,2-dimethoxypropane (as a water scavenger) was added. One mL of freshly made 0.5 N NaOH/MeOH (suspension) were added, and the samples mixed briefly by vortexing and Flush with argon (Ar) and seal. Samples being placed in an incubator at 80°C, with shaking at 300 rpm for 20 minutes and samples were cooled for 5 min to room temperature. Adjust the pH to that of 10% AcCl in methanol with 17 µL concentrated HCl and added one hundred µL of 2,2-dimethoxypropane (as a water scavenger). 1 mL of freshly prepared AcCl/MeOH 10:100 (v/v) (prepared by slowly adding AcCl to anhydrous methanol under magnetic stirring) was added and flush with Argon (Ar) and seal. Repeating the incubation at 80°C for 20 minutes while shaking at 300 rpm and after cooling for 5 min to room temperature. 2 mL of 6% K_2CO_3 or KCl in water and 3 mL toluene were added tubes mixed by vortexing. Samples were centrifuged at 4,000 rpm for 5 -10 minutes and the upper toluene layer, containing the FAME extract, was transferred with a Pasteur pipette to vials for GC.

Bead Beating Extraction protocol (Sitepu et al., 2012):

Bead Beating Extraction Protocol 1

Analytical Reagents

Chloroform was from Millipore, MA, USA. Methanol (CH_3OH) from Fisher Scientific, USA. Potassium chloride (KCl) from Fisher Scientific, USA. Potassium carbonate (K_2CO_3) from Fisher Scientific, USA. Sulfuric acid H_2SO_4 from Fisher Scientific, USA. Isooctane (IO) from Fisher Scientific, USA. Glyceryl triheptadecanoate (C-17 TAG) from Sigma-Aldrich, USA. Methyl nonadecanoate (C-19 ME) from Sigma-Aldrich, USA. (SM) prepared in the University of Kentucky, Department Plant and Soil Sciences, Dr. Hildbrand's Lab. Toluene (C_6H_5-CH_3) from VWR, USA. zirconia beads BioSpec from Product (BSP), USA. 2 mL screw cap tubes from Fisher Scientific, USA. sodium methoxide (SM) prepared in the University of Kentucky, Department Plant and Soil Sciences, Dr. Hildbrand's Lab.

Materials

0.1% BHT stock, C-17 TAG + C-19 ME standard solution 20 mg/mL. $CHCl_3$ in CH_3OH (2:1) + 0.001% BHT. Freshly prepared 2.5% (v/v) H_2SO_4 in CH_3OH + 0.001% BHT.

Method Extraction 1

10 mg of known dry mass seeds to 2 mL screw cap tubes. And added 10 µL of toluene standard containing C-17 TAG and C-19 ME/mg sample as the direct transesterification, reagent. 1 mL chloroform $CHCl_3$ in methanol CH_3OH (2:1) and 0.001% BHT were added. Followed with put 5 zirconia beads to tubes. Tubes were homogenized in an MP Bio Fastprep®-24 homogenizers and ran soybean seed program. Sonication for 15 minutes at room temperature and transferred tube contents to 12x75 mm glass tubes after weighing tubes. 1 mL chloroform $CHCl_3$ to the 2 mL tubes with beads was added and shake for a period of time. Transfer as much of the $CHCl_3$ as possible into 10 or 12x75 mm glass tubes and 1 mL 6% K_2CO_3, was added. Vortex was used and centrifuge for 5 minutes if needed. Transfer as much of the lower $CHCl_3$ phase as possible into dried and weighed screw-top glass tubes, following (Folch et al. 1956) by added 1 mL chloroform to the original tube, vortex vigorously again. Added this additional chloroform to the original and extracted with a 3rd mL chloroform, repeat and add the 3rd chloroform

extract to the 1st two. Evaporated the $CHCl_3$ with N_2 and heating to 50˚C and weigh again. (gravimetric method following Folch et al. (1956).

After measured the weigh added 2 mL freshly prepared and 2.5% (v/v) H_2SO_4 in H_3OH-0.001% BHT to each tube and tightly cap. (Process as per modified Li et al. (2006) protocol). The samples mixed briefly by vortexing for 30 s, heat to 100˚C for 10 minutes, and vortexed after 5 minutes cool to room temp.1 mL isooctane (IO) with 0.001% BHT was added and vortexed. Transferred 200 µL top layer after separation to vials for GC and 1 more mL isooctane was added and mixed. If needed for clear phase separation before taking an aliquot from upper layer added 1 mL 6% K_2CO_3.

Bead Beating Extraction Protocol 2

Materials

0.1% BHT stock, C-17 TAG + C-19 ME standard solution 20 mg/mL, and standard sodium methoxide Solution

Method Extraction 2

10 mg of known dry-mass seeds to 2 mL screw cap tubes and 10 µL of toluene standard containing C-17 TAG & C-19 ME/mg sample was added. 0.5 mL sodium methoxide and 5 zirconia beads to tubes were added. Put tubes in MP extractor and ran soybean seed program. Sonication for 15mins in the room Temp and transfered tube contents to 12X75 mm glass tube. 1 mL isooctane (IO) with 0.001% BHT, to tubes with beads, was added and vortexed. Transfer isooctane (IO) with 0.001% BHT into 10 or 12x75 mm glass tubes. Then transfer 200 µL top layer after separation to vials for GC and 1 more mL isooctane was added and mixed. If needed for clear phase separation before taking an aliquot from upper layer added 1 mL 6% K_2CO_3.

Nile red/fluorescence protocol (Sitepu et al., 2012)

Nile red/fluorescence protocol 1

The improved yeast protocol for lipid quantification described by (Sitepu et al., 2012)

Analytical Reagents

Acetone from VWR, USA. Dimethyl sulfoxide (DMSO) from Sigma-Aldrich, USA. Nile Red ($C_{20}H_{18}N_2O_2$) from ACROS Organics, USA. Black 96-well micro-plate

with clear flat bottom from VWR, USA. Viewseal from research products international (RPI), USA.

Materials

Nile read acetone and dimethyl sulfoxide (DMSO). Black 96-well microplates with clear flat bottom and seal with Viewseal. Nile red stock 0.05 mg/mL in acetone

Methods

1 and 5 mg of ground seed material were added to wells of a black 96-well micro-plate with the clear flat bottom. To each well added 25 µL dimethyl sulfoxide (DMSO) and 2, 10 and 25 µL 0.05 mg/ml Nile Red and added 23, 15 and 0 µL acetone respectively (final concentration of 1, 2 and 5 µg/mL Nile red). Mixed well with seed powder and seal microplate wells with Viewseal. Initial absorbance reading was at 600 nm; initial fluorescence excitation at 530/25, emission at 590/35; and kinetic reading for 20 min with 60 sec intervals. The optic position was set to top 50%. Take kinetic readings for 20 min with 60 sec intervals with shaking for 30 sec.

Nile Red/ fluorescence Protocol 2

Analytical Reagents

Nile red stock 0.05 mg/mL in acetone

Materials

Nile read acetone. Black 96-well microplates with clear flat bottom and seal with Viewseal.

Methods

0, 1, 10, 25, and 50 µg soybean oil in acetone (Soybean oil standard made 10mg/10 mL acetone) were added to wells of a black 96-well micro-plate with the clear flat bottom. 10, 25 and 100 µL of Nile Red (0.05 mg/ml in acetone). Mixed well with seed powder and seal microplate wells with Viewseal. Initial absorbance reading was at 600 nm; initial fluorescence excitation at 530/25, emission at 590/35; and kinetic reading for 20 min with 60 sec intervals. The optic position was set to top 50%. Take kinetic readings for 20 min with 60 sec intervals with shaking for 30 sec.

Gas chromatography (GC)for Chromatograph analysis.

GC vials were then run on a Varian CP-3800 Gas Chromatograph using a 25m x 0.25 mm ID fused silica column with a Varian (chrompack) CP=Select CB for FAME, with a film thickness of 0.25 um. The temperature program ran from 90°C to 250°C with 25°C ramp for a total of an 8 minute run time with a constant column flow mode of 0.9 mL/min utilizing a splitless injection. Quantification was performed by using a flame ionization detector and peaks quantified using Star Chromatography Workstation Version 6.00, with peak area being used to calculate relative percentages of FAMEs.

RESULTS AND DISCUSSION

Soxhlet method:

Results

Percentages of oil recovered from a duplicate with 5 g chia, G8 variety seeds, it was ground by burr mill machine, with acetone (Ac) and petroleum ether (PE) [Appendix Chapter 2 Table 2-A](). The cycle time of two solvents, Ac and PE was measured. PE solvent was faster than Ac, with 7 minutes for each cycle compared to Ac, with approximately 15 minutes per cycle at 160°C. The percentage of oil recovered was 29.6% with PE and 30.2% with Ac. The temperature was recorded 160°C as the average temperature for all units. The second experiment [Appendix Chapter 2 Table 2-A]() percentages of oil recovered from a duplicate with 5 g, ground seeds of chia, G8 variety with Ac and PE. The cycle time for PE was 9 minutes, for Ac was 18 minutes, and the percentage oil recovered were 30.1% with PE and 30.7% with Ac at 150°C. The temperature recorded, 150°C, was average temperature for all units.

The third experiment, [Appendix Chapter 2 Table 2-A]() percentages of oil recovered from a duplicate with 5 g, ground seeds of soybean, Jack variety with Ac and PE. The percentages of total oil recovered were higher for soybean, Jack with PE solvent than with Ac. The PE organic solvent was recorded a cycle time of 10 minutes, but Ac was recorded a time approximately 18 minutes for each cycle under 120°C. The percentage oil recovered was 23.9% with PE and 28.6% with Ac. The average temperature for all units was 120°C. The fourth experiment [Appendix Chapter 2 Table 2-A]() with soybean, Jack variety and percentages of total oil recovered from a duplicate with 5 g, ground seeds of soybean, Jack with Ac and PE. In which, a cycle time for PE was 9 minutes, and Ac was 18 minutes. Percentages of oil recovered were 25.6% with PE and 24.1% with Ac at a temperature of 120°C. The average temperature for all units was 124°C (Figure 4).
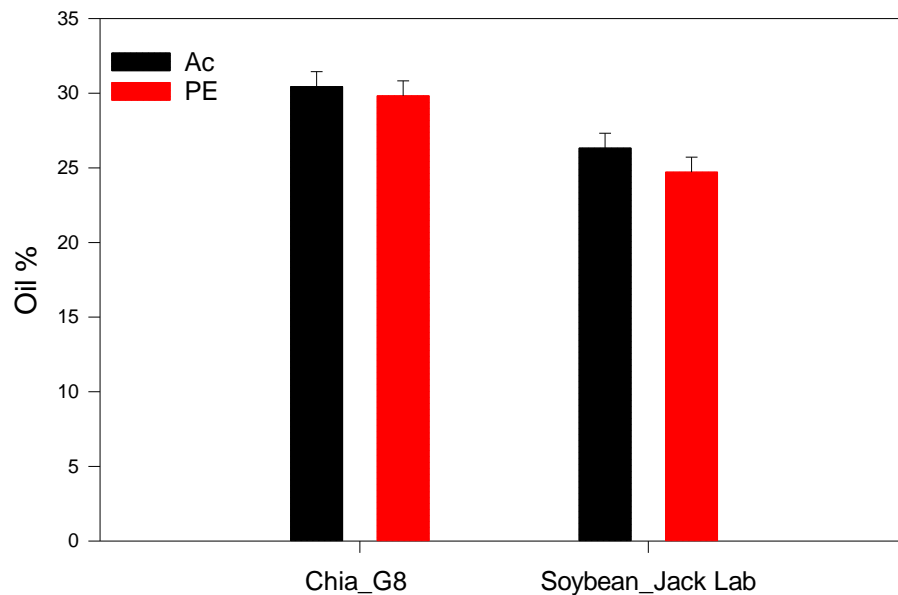
Figure 4: Percentages of total oil recovered by the Soxhlet method from chia_G8 and soybean_Jack lab using acetone (Ac) and petroleum ether (PE) solvents.

Percentages of oil recovered by two different solvents (PE and Ac) were measured. SAS output by the GLM procedure showed there is not a significant interaction (P=0.63) between samples and solvents. There is not a statistically significant difference (P=0.2881) between PE and Ac solvents. There is a statistically significant difference (P=0.0006) between varieties Appendix Chapter 2 Table 2-B

Using Medium Moisture Content (MMC) and Low Moisture Content (LMC) seeds with Soxhlet method.

The Soxhlet method was run a duplicate of ground seeds materials for three varieties, chia_G8, and soybean JACK R3 and VGD1-2 R3, and 5 g of ground seeds with MMC%. Extraction was conducted with PE and Ac. Also, the Soxhlet method was run with the three varieties with LMC%, and each run used triplicate with 1.5 g of ground seeds. Extraction was conducted with only PE. Soxhlet cycles were recorded, and percentages of oil recovered were measured (Figure 5).
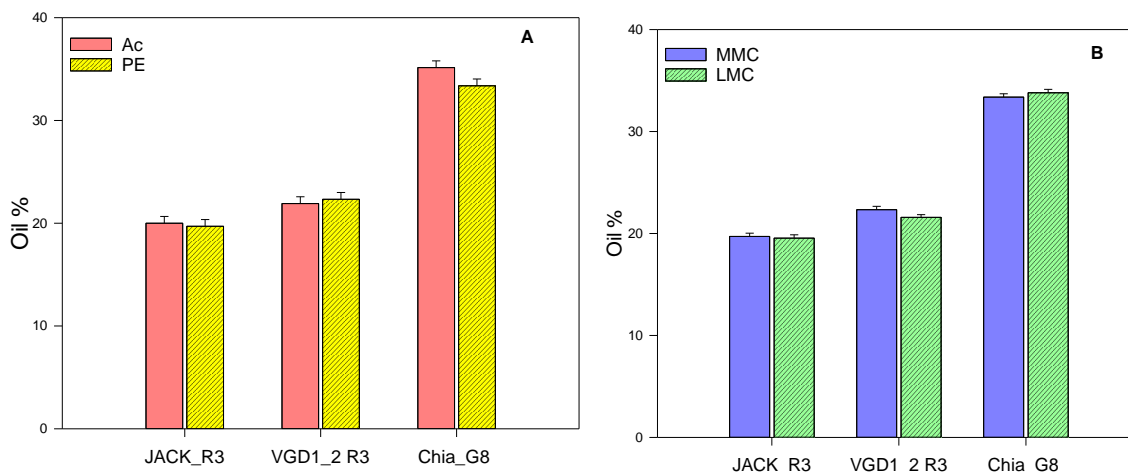
Figure 5 A-B: Percentages of total oil recovered by Soxhlet with three varieties (JACK R3, VGD1-2 R3, chia_G8) with acetone (Ac) and petroleum ether (PE) and medium moisture content (MMC) and low moisture content (LMC).

The Soxhlet method ran with medium moisture content (MMC > 10%) by two solvents acetone (Ac) and petroleum ether (PE). The SAS output by the GLM Procedure showed there is no interaction between samples and solvents ($P = 0.26$). Percentages of oil recovered by the Soxhlet method with MMC seeds is not a statistically significant difference ($P = 0.1$) between PE and Ac solvents with three samples. There is a statistically significant difference ($P < .0001$) between varieties (JACK R3, VGD1-2 R3 and Chia_G8) Appendix Chapter 2 Table 3-A.

The Soxhlet ran with different moisture contents of seeds mass low moisture content (LMC) less than 4.0% and medium moisture content (MMC) above 10% in this experiment was used one an organic solvent petroleum ether (PE). SAS output by the GLM procedure showed there is no interaction between samples and moisture ($P = 0.97$). Percentages of oil recovered by the Soxhlet method with MMC seeds is not a statistically significant difference ($P = 0.32$) between MMC and LMC with three samples. There is a statistically significant difference ($P < .0001$) between samples (JACK R3, VGD1-2 R3 and Chia_G8) Appendix Chapter 2 Table 3-B

Discussion

Temperature could be the one the most important factors that affect the recovery of oil. The temperature around the flasks was adjusted and the speed of cycle times was

recorded. Usually, high temperatures provide improved extraction efficiencies (Wang & Weller, 2006). In the Soxhlet extraction process, the solvents are recovered during evaporation and the temperature can accelerate a solvent evaporate and cycle time. The evaporation and boiling point for Ac and PE is different, the Ac boiling point is 36-60°C and PE is 56°C. The temperatures of evaporation and extraction have an important consequence on the final products' quality (Mamidipally & Liu, 2004). The faster cycle time was recorded, with 15 minutes by Ac and 7 minutes by PE at 160°C comparing with 120 and 150°C.

Total lipid might be extracted efficiently with the Soxhlet method using acetone (Ac) as the solvent than petroleum ether (PE). It was reported Ac solvent was the most effective in the extraction of total lipids and bioactive components, especially phenolic compounds and carotenoids. The oil obtained by extraction with Ac was additionally characterized by the highest oxidative stability. This information may be of value to producers for obtaining chia oil that is more stable and has better nutritional and nutraceutical properties (Dąbrowski et al., 2016). The results (Figures 5-A) showed, there is not a statistically significant difference (P= 0.1) between PE and Ac solvents with three samples.

The effect of dry weight and moisture content of soybean flours via Soxhlet extraction method was described in the literature review by Canessa and Snyder. (1991). The percentages of oil extraction can be increased by increasing moisture contents of the samples might be beacuse increased phospholipid extraction (Canessa & Snyder, 1991). The Soxhlet experiment with two different moisture contents (LMC < 4.0 %) that provided from freeze dryer (FD), and MMC >10% that obtained from seeds room (SR). Percentages of oil recovered with two different moisture content (LMC) and (MMC) showed not statistically significant difference (P= 0.32) between MMC and LMC with three samples (JACK R3, VGD1-2 R3 and Chia_G8).

Folch, Bligh and Dyer, and hexane-isopropanol (HIP):
Results

Three techniques used to extract total oil content from two oil crops, chia_G8, and soybean_Jack lab. 100 mg ground seeds mass added and ran all samples by used Folch,

Bligh & Dyer, and hexane-isopropanol (HIP) methods with seeds moisture less than 10%. The total oil in the test tubes weighed, and the oil extraction was measured. (Figure. 6) Appendix Chapter 2 Table 4-A
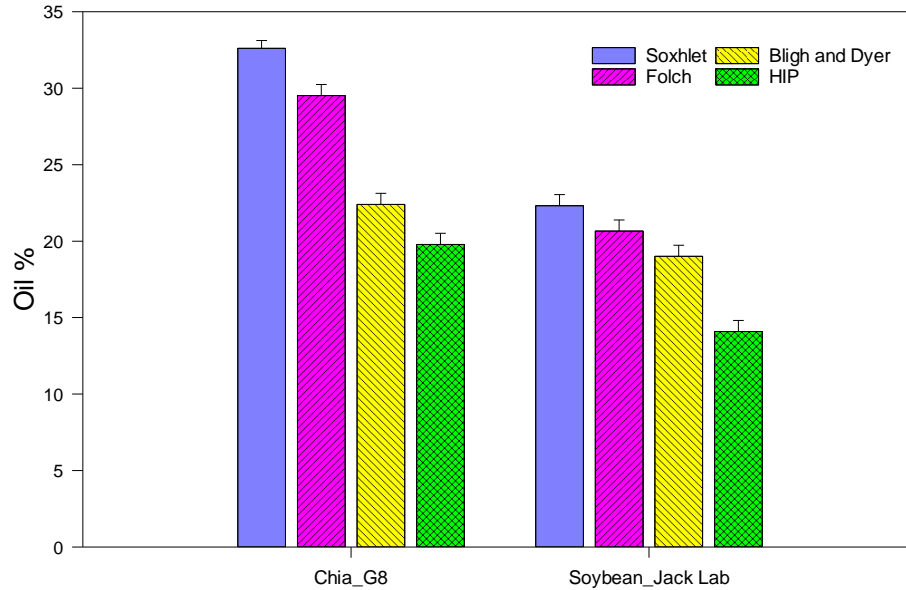
Figure 6-A: Total percentage of oil extracted among three techniques (Folch, Bligh and Dyer and HIP compared with Soxhlet as a standard) with chia_G8 and soybean_Jack lab.

Figure 6-A shows the effect of different levels of methods depends on what level of varieties is present. SAS output by the GLM procedure showed, there is a statistically significant interaction (P = 0.0003) between methods (Soxhlet, Folch, Bligh and Dyer, and hexane: isopropanol (HIP)) and samples (Chia_G8 and Soybean_Jack lab).There is a statistically significant difference (P <0.001) between methods, and there is a statistically significant difference (P <0.001) between samples.

In the SAS output by using contrast between the Soxhlet method as a control with Folch, Bligh and Dyer, and HIP methods. There is a statistically significant difference (P = 0.001) between the Soxhlet method and Bligh and Dyer method. There is not a statistically significant difference (P = 0.0844) between Soxhlet method and Folch method. There is a statistically significant difference (P <0.001) between the Soxhlet and HIP method Appendix Chapter 2 Table 4-B.

Overall, The Folch technique was the better technique can used for testing effect moisture contents on these three gravimetric methods and for comparing with the Soxhlet method that used as a standard in the present study (Figure 6-B).
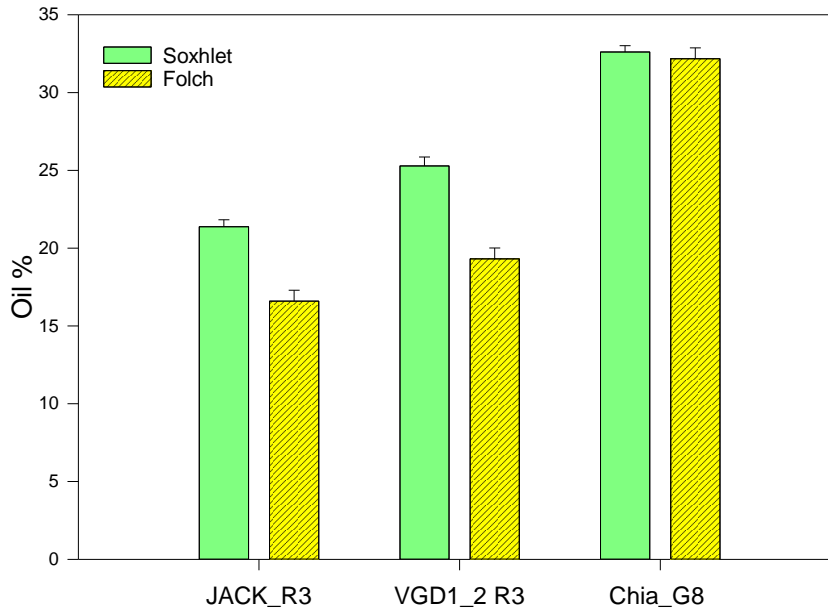
Figure 6-B: Total percentage of oil extracted between Folch method with MMC (>10 %). and compared with the Soxhlet as a standard, with three varieties Chia_G8, JACK_R3 and VGD1-2 R3.

Figure 6-B shows the percentages of oil extraction by Folch technique with medium moisture content (MMC) compared with the Soxhlet method. SAS output by the GLM procedure showed, there is a statistically significant interaction (P = 0.0009) between methods (Soxhlet and Folch) and samples (JACK_R3, VGD1-2 R3 and Chia_G8).There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples. In the SAS output by using the contrast between the Soxhlet method as a control and the Folch method. There is not a statistically significant difference (P = 0.1552) between the Soxhlet method and the Folch method Appendix Chapter 2 Table 4-C.

Discussion

The gravimetric methods reported is the best accurate techniques used to measure lipid content due to its ability to measure total lipids weight directly (Han et al., 2011). However, the gravimetric methods did not handle the problem of accuracy due to the gravimetric method does not permit analysis of compositional except if unless associated with mass spectrometry (MS). The gravimetric methods need a big amount of biomass and there are different factors can effect on accuracies such as the type balance that using to measure weight mass efficiently and the lowest amount recovered of dry lipid is 10 mg (Hounslow et al., 2017). The Soxhlet method determines the higher percentage of oil extraction. The Folch procedure would be considered due to it requires much shorter time than the Soxhlet method for extraction oil. In usual, the Folch method needs around 1 hour to extract oil while Soxhlet needed 12 hours (Elnajjar et al., 2017).

The results (Figure 6-A) approved, there is not a statistically significant difference (P = 0.1552) between the Soxhlet method and the Folch method. The Bligh and Dyer and HIP methods provided significant difference (P <0.001) than the Soxhlet with lower oil quantification.

Supercritical extraction method (Hot compressed hexane (HCH)):

Results

Supercritical hexane extraction has not been applied to lipid quantification of oilseeds but likely might be superior, as was found with algae and similar protocols for extruded products (Shin et al., 2014; Strange et al., 1997). HCH ran with three bias samples JACK_R3, VGD 1-2 R3 and Chia G8 to extract total oil content. One gram ground seeds added and ran all samples a triplicate by using HCH method (Figure 7).
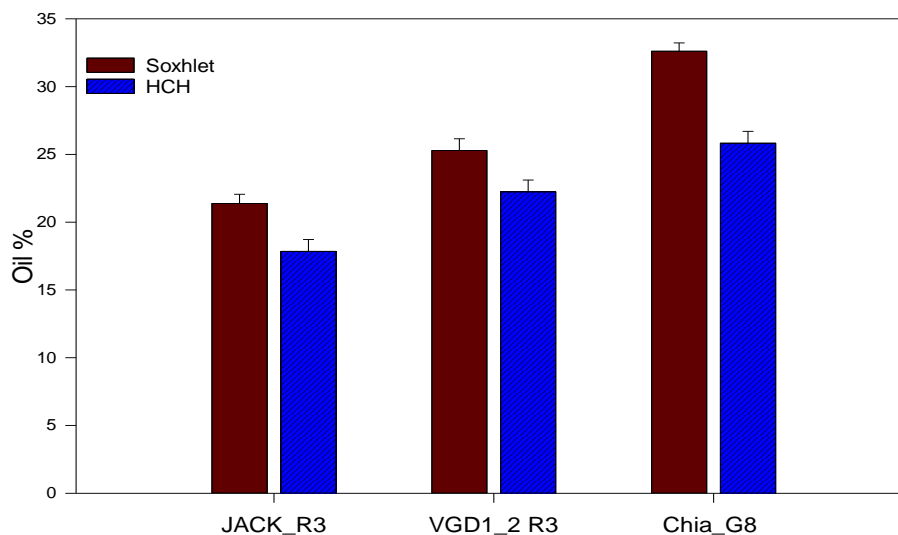
Figure 7: Hot compressed hexane (HCH) with JACK_R3, VGD1_2 R3 and Chia_G8 compared with the Soxhlet method.

Percentage oil of supercritical fluid extraction or Hot compressed hexane (HCH) with 1 gram seeds mass of three varieties (JACK_R3, VGD1_2 R3 and Chia_G8.). The SAS output by the GLM procedure showed, there is not a statistically significant interaction (P = 0.0589) between methods (Soxhlet and HCH) and samples (JACK_R3, VGD1_2 R3 and Chia_G8).There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples Appendix Chapter 2 Table 5.

Discussion

The results of Hot compressed hexane (HCH) method provided significant difference (P = <0.001) lower yield of oil extraction with all three varieties of bias samples compared with the Soxhlet method. It is reported the high percentages of moisture content in the samples can be a restriction to supercritical fluid extraction. According to Mercer and Armenta. (2011) reported with micro-algae that the high level of moisture content in the samples, which tend to acts as a barrier against dissemination of oil out of the cell because the moisture prevented dispersal of CO2 inside the samples (Mercer & Armenta, 2011).

Results

        The direct transesterification method (DT) follows Li et al. (2006); Zhang et al. (2009) qualified as fast and the seed amount considered is minimum (Li et al., 2006). In this experiment, we used 10 mg ground seeds mass from three varieties, JACK_R3, VGD1_2 R3 and Chia_G8.
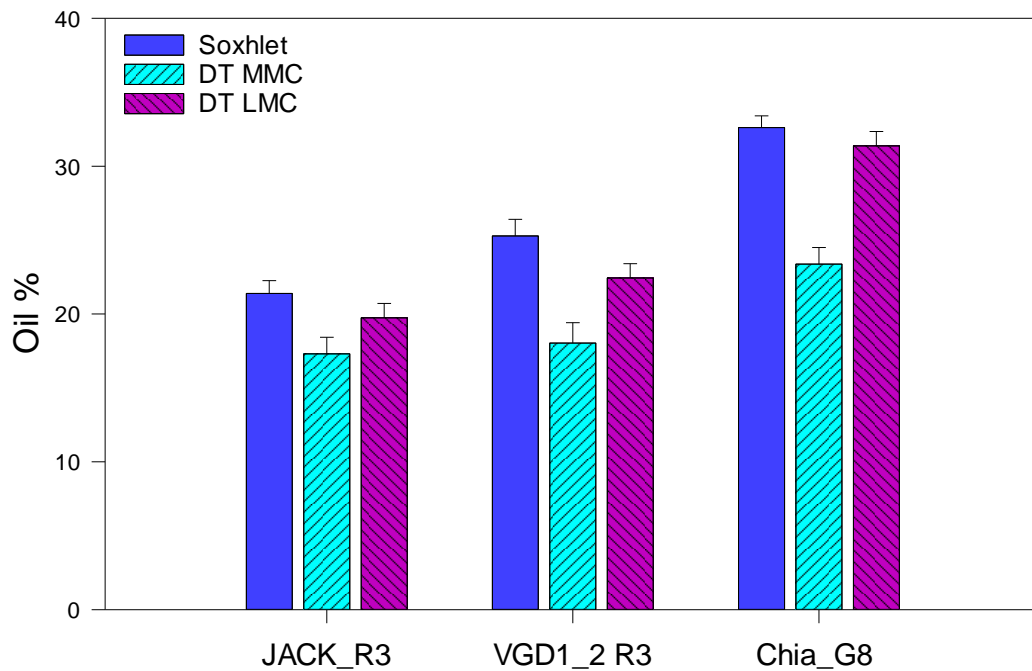
Figure 8 Direct Transesterification (DT) method and Soxhlet method as a standard with MMC and LMC and three varieties JACK_R3, VGD1_2 R3 and Chia_G8.

        The direct transesterification technique was employed with 30 minutes incubate time under temperature 100°C with different moisture content, medium moisture content (MMC above 10%) and low moisture content (LMC less 4%) with the Soxhlet method as a standard (Figure 8) and Appendix Chapter 2 Table 6-A. The SAS output by the GLM procedure showed, there is not a statistically significant interaction (P = 0.0887) between methods (Soxhlet, Direct Transesterification (DT) with LMC and MMC) and samples (JACK_R3, VGD1_2 R3 and Chia_G8).There is a statistically significant difference (P

<0.001) between methods and there is a statistically significant difference (P <0.001) between samples. The SAS output by using the contrast between the Soxhlet method as a control with DT_LMC and DT_MMC. There is a statistically significant difference (P = 0.035) between the Soxhlet method and DT method with LMC. There is a statistically significant difference (P <0.001) between the Soxhlet method and DT method with MMC Appendix Chapter 2 Table 6-B.

Discussion

Total mean of oil extracted from the three varieties were higher with LMC (less than 4%) than MMC (above 10%) with 30- minute incubation time. Lemões et al., 2016 reported the DT for microalgae provided higher fatty acid methyl esters (FAMEs) produced in dry biomass. (Lemões et al., 2016). According to Hoarau et al. (2016) DT for wet microalgal biomass produced more FAMEs than Folch (77–93 %) and Bligh and Dyer (19–63 %) methods. Increased FAMEs production by direct methanolysis is attributed to the increased ability to penetrate the cell and the fewer number of steps in this process. The FAMEs produced by direct methanolysis of wet biomass was lower than dry biomass. Notwithstanding the biomass moisture content, dry biomass provided more oil yield than wet biomass (Hoarau et al., 2016). Comparing this result of DT method with the standard method (Soxhlet) was achieved. These results is a statistically significant difference between the Soxhlet method and DT method with different moisture content (MMC and LMC). The DT provided lower oil recovered than the Soxhlet method 27.02% avreage between samples. The DT_LMC provided 24.5% avreage of oil extraction between samples and DT_MMC obtained 19.8% average of oil extraction between samples.

DT technique gave precision of oil extraction between samples but the percentages of oil extraction was lower than the Soxhlet method and this technique is not the faster technique can be used to extract oil from seeds. However, according to Frigo-Vaz and Wang. (2014) the direct acid methylation is fast process, high-purity and might complete during two hours period comparing with the Soxhlet method (Frigo-Vaz & Wang, 2014).

Double Direct Transesterification- Griffiths et al. (2010) and Qiao et al. (2015) method.

Results

The Double Direct Transesterification Griffiths (DDTG) and Double Direct Transesterification Qiao protocols can show accurate results with small seed samples compare with Soxhlet method. For evaluated and developed Griffiths protocol (DDTG), we used two different techniques. The first technique, stir bar technique was used in the original paper of Griffihs et al. (2010). The second technique, we used sonication technique. The double direct transesterification (DDT) protocol uses sodium methoxide ($NaCH_30$) and 14% BF_3 (Boron tri-fluoride) methanol solution.

The double direct transesterification-Qiao (DDTQ) protocol, is similar to (Griffiths et al., 2010), but uses a progressive combination of alkaline catalysts methanol (MeOH) and acid catalysts Acetyl chloride (AcCl) that were found to improve the extraction yield of fatty acids in *Phaeodactylum tricornutum*. The Qiao et al (2015) claimed their combinations is superior for extracting the total lipid yield from an alga using a 2-step acid and base is equivalent to AOAC 991.39 (Qiao et al., 2015).

In the present study, we had evaluated Griffiths protocol with 10 bias samples of soybean including JACK_R3, VGD1_2 R3 and G8 of chia. In the beginning, we tested the various amount of ground seeds from two oil crops that include (Chia_G8 and Soybean_Jack). The ground seeds amount tested 100 mg, 50 mg, 20mg, 10mg chia_G8 and soybean_Jack. We ran all samples in a triplicate by used DDTG protocol and the result in Appendix Chapter 2 Table7-A. The results showed there is not a statistically significant difference (P = 0.11) between seeds amount (100 mg, 50 mg, 20mg, 10mg) and techniques (sonication and stir bar) with both samples (Chia_G8 and Soybean_Jack) in Appendix Chapter 2 Table 7-B.

Due to the results is not a significant difference in both techniques stir bar and sonication between differents seeds amount, we selected 10 mg amount of seed can use to compare between techniques. We ran a triplicate of two samples (chia_G8 and soybean_Jack) with 10 mg ground seeds amount (burr mill grind machine) with two techniques sonication and stir bar the results in Appendix Chapter 2 Table 8. The results showed low oil recovered in both techniques (stir bar and sonication). To modify the protocol by combined tri-17:0 and methyl-19:0 and adjusted the protocol via change

standard solution 20 mg/mL of C-17 TAG + C-19 ME. Then repeated a triplicate by using the amount of seeds mass 10 mg form regular soybean, JACK_R3 (medium oil percent) and high oil line soybean, , VGD1_2 R3 (high oil percent) using stir bar and sonication, as Appendix Chapter 2 Table 9-A. The results showed, there is not a statistically significant interaction (P = 0.21) between methods (DDTG with sonication, DDTG with stir bar and Soxhlet as a control) and samples (JACK_R3 and VGD1_2 R3). There is not a statistically significant difference (P = 0.1283) between methods and there is a statistically significant difference (P <0.001) between samples Appendix Chapter 2 Table 9-B.

Another factor can affect the oil extraction from oilseed is incubate time, we ran DDTG in a triplicate two soybean varieties, JACK_R3 and VGD1_2 R3 by comparing different incubate time at 80°C with 20 minutes as original protocol, 40 minutes and 10 minutes with both techniques sonication and stir bar Appendix Chapter 2 Table 10-A. The results by SAS output showed, there is not a statistically significant interaction (P = 0.14) between methods (sonication and stir bar) and incubate time (10, 20 ,40 minutes), but there is a statistically significant difference (P =0.0004) between incubate time. Mean and Scheffe's test for oil percent showed there are not significantly different between 10 and 20 minutes incubate times in group A and 40 minutes is significantly different group B Appendix Chapter 2 Table 10-B.

After we set there are not significantly different between 10 and 20 minutes incubate times. We ran DDTG protocol for comparing between sonication and stir bar with three varieties of bias samples (JACK_R3, VGD1_2 R3 and Chia-G8). In this experiment used incubate time 10 minutes at 80°C Appendix Chapter 2 Table 11-A.
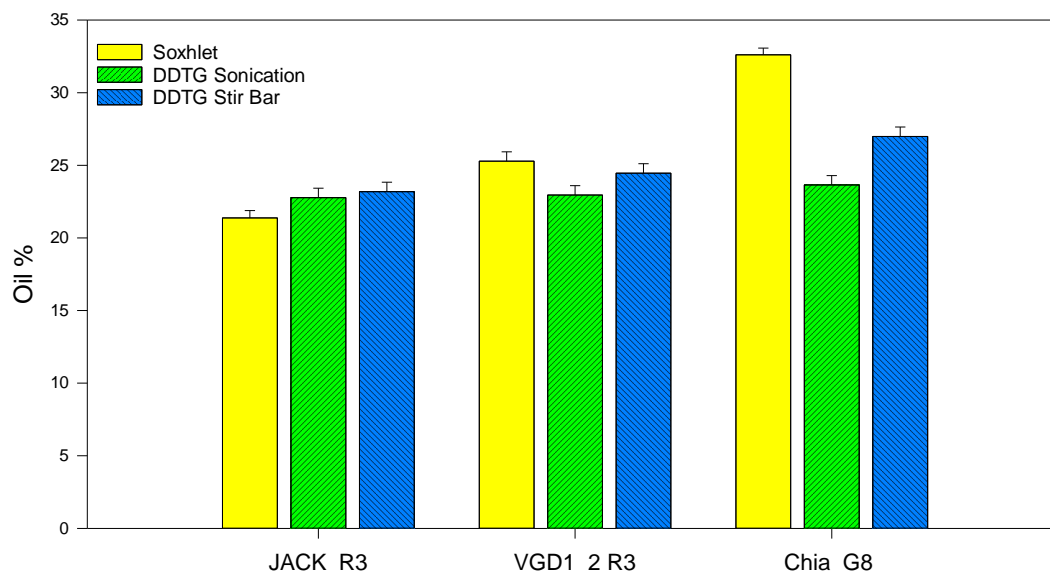
Figure 9-A: The Double Direct Transesterification Griffiths (DDTG) by Sonication and Stir Bar mixing techniques with the Soxhlet method

The SAS output by the GLM procedure showed, there is a statistically significant interaction (P <0.001) between methods (Soxhlet, double direct transesterification Griffiths (DDTG) with sonication and stir bar techniques) and samples (JACK_R3, VGD1_2 R3 and Chia_G8).There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples. The SAS output by using the contrast between the Soxhlet method as a control with DDTG_Sonication and DDTG_Stir bar. There is a statistically significant difference (P = 0.003) between the Soxhlet method and DDTG_Stir bar. There is a statistically significant difference (P = 0.004) between the Soxhlet method and DDTG_Sonication technique.

The results appeared there is a statistically significant difference (P <.0001) between stir bar and sonication and the highest mean of oil extraction with stir bar technique for all three bias samples Appendix Chapter 2 Table 11-B. The results proved that the DDTG with stir bar mixing was as good or better than with sonication.

The DDTG method by stir bar mixing ran with Chia_G8 in different incubate times 10 and 20 and compared with the Soxhlet as a control Appendix Chapter 2 Table 12-A.

The SAS output by the GLM procedure showed, there is a statistically significant interaction (P = 0.0002) between methods (Soxhlet, double direct transesterification

Griffiths (DDTG) with 10 minutes and 20 minutes incubate time). The SAS output by using the contrast between the Soxhlet method as a control with DDTG_10 minutes and DDTG_20 minutes. There is a statistically significant difference (P = 0.0006) between the Soxhlet method and DDTG_10 minutes. There is a statistically significant difference (P = 0.0002) between the Soxhlet method and DDTG_20 minutes. There is not a statistically significant difference (P = 0.4780) between the DDTG_20 and 10 minutes incubate time Appendix Chapter 2 Table 12-B. The results provided there is not significantly different between 10 minutes and 20 minutes incubate time, one of the objectives of the present study find faster methods to extraction total lipid from oilseed, we accepted the 10 minutes could be a better incubate time for this protocol.

After we set the best protocol can be used with oilseeds. The DDTG protocol ran with a triplicate of all 11 bias samples of soybean and chia Appendix Chapter 2 Table 13-A.
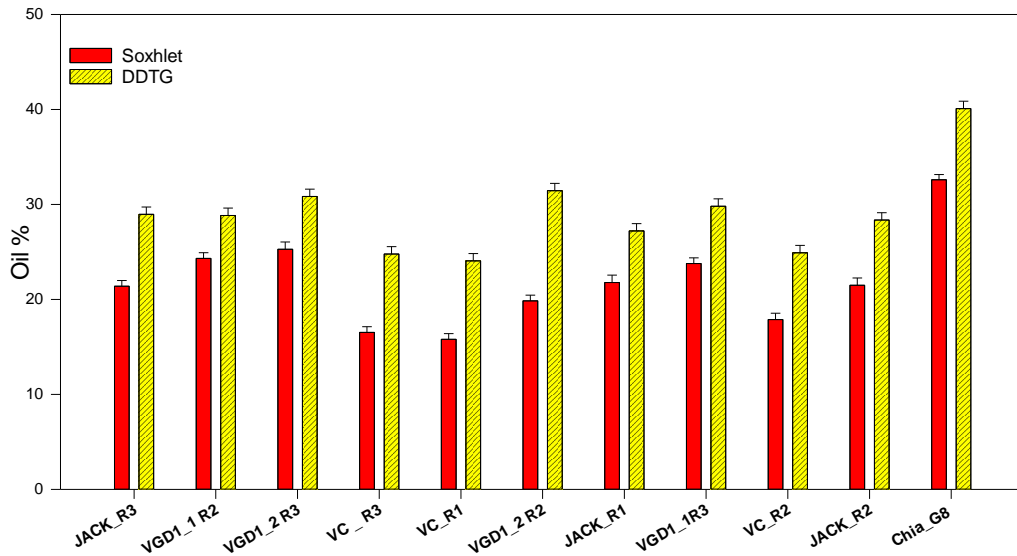
Figure 9-B: Percentages of oil extraction between the Double Direct Transesterification Griffiths (DDTG) method and the Soxhlet method with 11 Bias Samples

The SAS output by the GLM procedure showed, there is a statistically significant interaction (P =0.0004) between methods (Soxhlet and double direct transesterification Griffiths (DDTG)) and samples (JACK_R3, VGD1_1 R2, VGD1_2 R3, VC _ R3, VC_R1, VGD1_2 R2, JACK_R1, VGD1_1R3, VC_R2, JACK_R2, and Chia_G8). There is a

statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples. The SAS output by using the contrast between the Soxhlet method as a control with DDTG method. There is a statistically significant difference (P <0.001) between the Soxhlet method and DDTG method Appendix Chapter 2 Table 13-B.
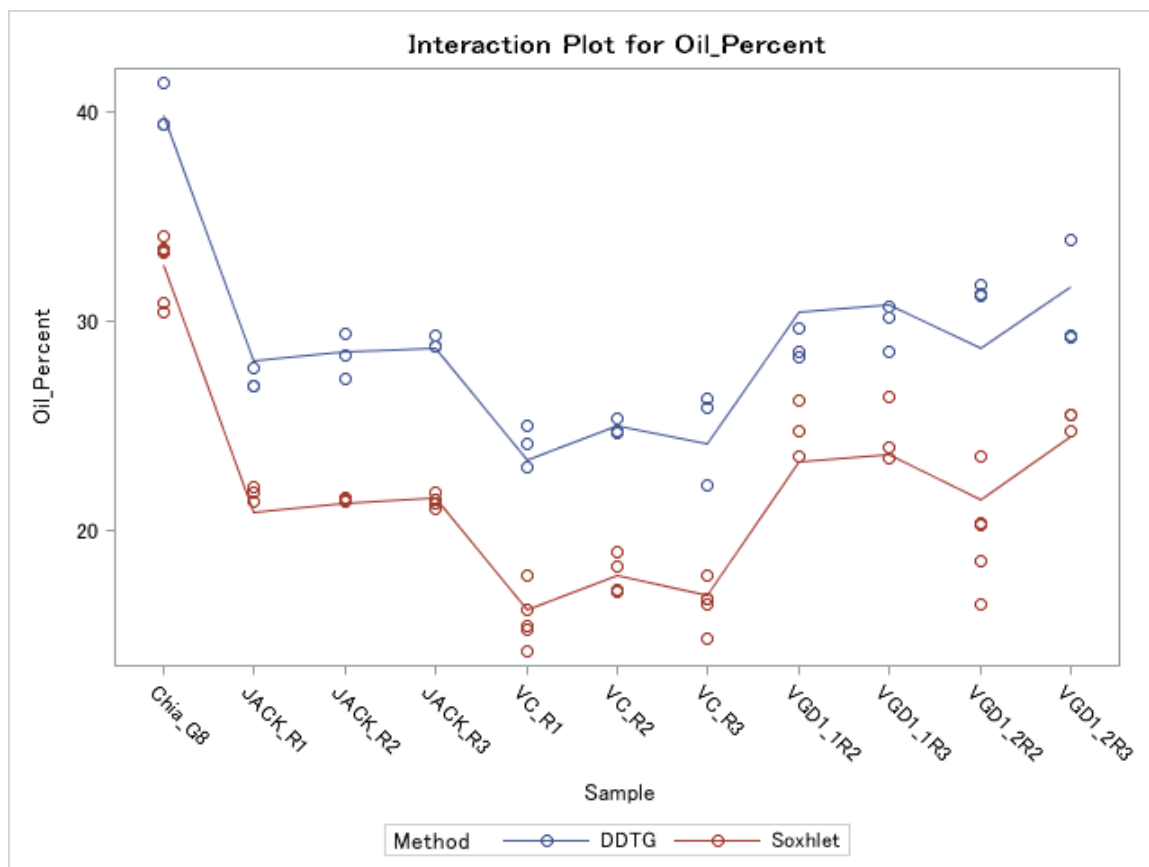
Figure 9-C: Interaction Plot between the Double Direct Transesterification-Griffiths (DDTG) method and the Soxhlet method for 11 bias samples

The Double Direct Transesterification-Qiao (DDTQ):

This protocol ran with 11 bias samples involved in this study that cover the range of oilseed. The double direct transesterification Qiao (DDTQ) protocol ran with 10 mg ground seeds. The seed was ground by burr mill grind machine and was verified in a triplicate with three different varieties of oilseed (JACK_R3, VGD1_2 R3 and Chia_G8) Appendix Chapter 2 Table 14 and repeated in a triplicate with three same bias samples (JACK_R3, VGD1_2 R3 and Chia_G8) to reduce a standard error percent and confirmed the previous results as Appendix Chapter 2 Table 15.

After we ran the DDTQ protocol many time and we set the best protocol. The DDTQ was ran a triplicate with 11 bias samples (10 soybean and Chia_G8) Appendix Chapter 2 Table 16-A. Ten mg ground seeds by burr mill and then ground by coffee grind machine for making seeds mass has small particle size like powder.
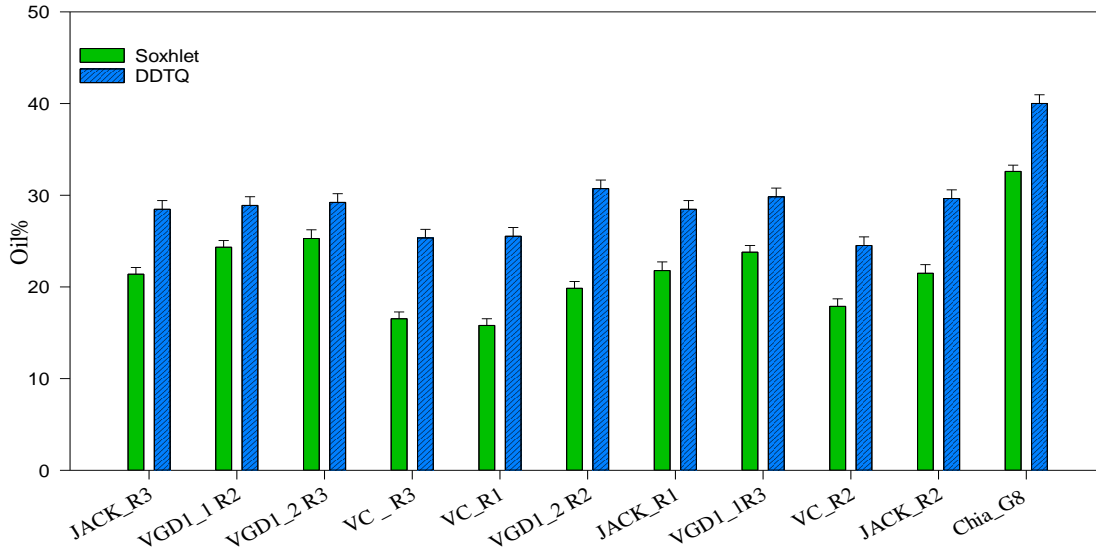
Figure 10-A: Percentages of oil extraction between the Double Direct Transesterification Qiao (DDTQ) method and the Soxhlet method with 11 Bias Samples

The SAS output for 11 bias samples by the GLM procedure showed, there is a statistically significant interaction (P =0.005) between methods (Soxhlet, double direct transesterification Qiao (DDTQ)) and samples (JACK_R3, VGD1_1 R2, VGD1_2 R3, VC _ R3, VC_R1, VGD1_2 R2, JACK_R1, VGD1_1R3, VC_R2, JACK_R2, and Chia_G8). There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples. In the SAS output by using the contrast between the Soxhlet method as a control with DDTQ method. There is a statistically significant difference (P <0.001) between the Soxhlet method and DDTQ method Appendix Chapter 2 Table 16-B.
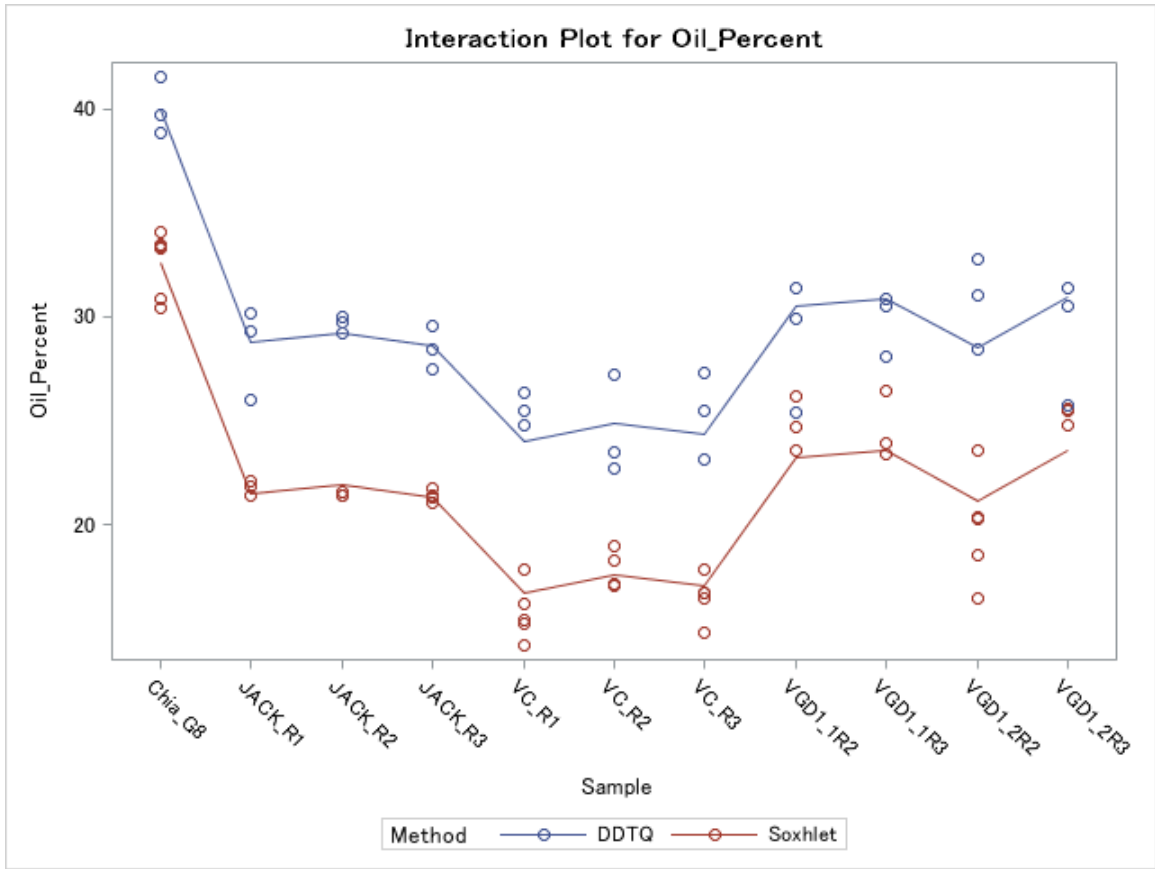
Figure 10-B: Interaction plot between the Double Direct Transesterification-Qiao (DDTQ) method and the Soxhlet method for 11 bias samples.
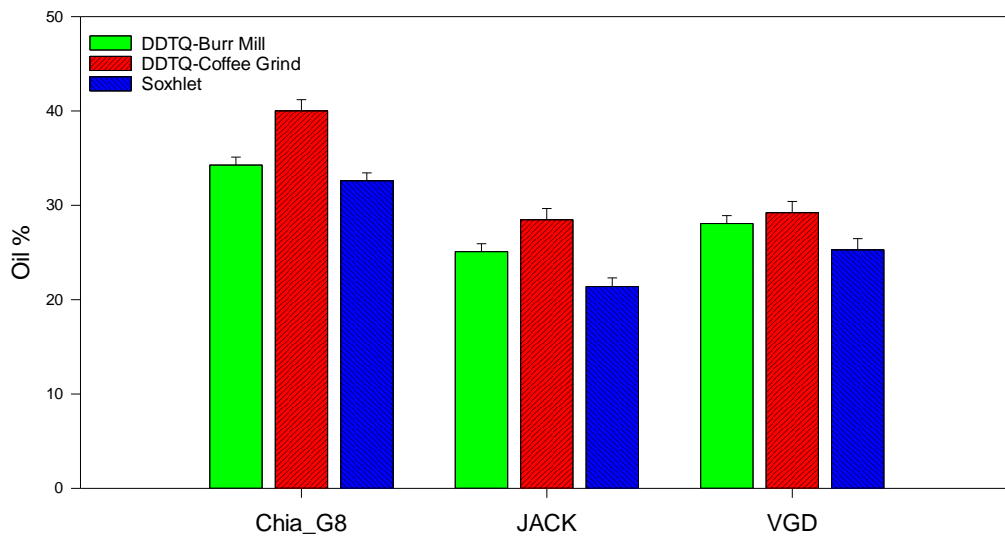
Figure 10-C: Percentages of oil extraction between DDTQ method with normal particle size seeds and small particle size seeds and the Soxhlet method with JACK_R3, VGD1_2 R3 and Chia_G8.

The normal partical size is the bias samples seeds ground by burr mill grind machine and smal partizle size or the fine ground seed was given by burr mill grind machine first and more ground by coffee grinds machine second (like powder). The fine ground seeds material tested with DDTG method with 11 bias samples in Table 16 in Appendix Chapter 2 and normal ground or normal particle size data was shown in Table 14 and 15 in Appendix Chapter 2. For comparison between different particle size by using burr mill grind machine (normal particle size) and coffee grinds machine (small partical size like powder)

The SAS output by the GLM procedure showed, there is not a statistically significant interaction (P=0.22) between methods (Soxhlet, double direct transesterification Qiao DDTQ with a different type of particle size) and samples (JACK_R3, VGD1_2 R3, and Chia_G8). There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples. In the SAS output by using the contrast between the Soxhlet method as a control with DDTQ with burr mill machine and coffee grind machine. There is a statistically significant difference (P =0.001) between the Soxhlet method and DDTQ with burr mill machine. There is a statistically significant difference (P <.0001) between the Soxhlet method and DDTQ with coffee grind machine. There is a statistically significant difference (P =0.0003) between the DDTQ with burr mill machine and DDTQ with coffee grind machine Appendix Chapter 2 Table 16-C. The mean of three bias samples and Scheffe's test for oil percent showed the percentages of oil recovered (32.57%) with coffee grind machine and 29.15% with burr mill machine.

Discussion

The results appeared that the DDTG method via the stir bar mixing technique was as good or better than with sonication. It is not significantly different between seed amounts (10, 20, 50, and 100 mg) and there is not significantly different between 10 minutes and 20 minutes incubate time. The best protocol of DDTG can use with stir bar mixing with less

amount of seeds 10 mg and 10 minutes incubate time compared to the Soxhlet method as control, there is a significant difference between the Soxhlet and DDTG method. The DDTG method provided high quantification of oil yield and precision results (high oil extraction) and efficient with small seed samples and faster and easier than gravimetrical methods.

The double direct transesterification- Qiao (DDTQ) method provided high yield with small particle size provided by burr mill and coffee grind machine than normal particle size used burr mill machine. The DDTQ protocol obtained the high quantity of oil with similar accurate to DDTG method and efficient as the DDTG but safer.

The different between (DDTG) and (DDTQ) protocol, the DDTQ protocol used safer combined with two catalysts, Sodium hydroxide (NaOH) in methanol (MeOH) (alkaline catalysts) and Acetyl chloride (AcCl) in methanol (MeOH) (acid catalysts), but DDTG added other two catalysts sodium methoxide (base catalysts) and BF3 methanol (14% BF3) solution (acidic catalysts) sequentially to quantify the fatty acid composition and confirm the transesterification competency. However, the incubate times was 10 minutes with DDTG protocol and 20 minutes with DDTQ protocol. Therefore the DDTG protocol faster (20 minutes) than DDTQ.

Bead Beating Extraction follows Sitepu et al. (2012).
Results and Discussion

Bead beating extraction (BBE) technique that produces direct mechanical damage to cells depends on the high speed with beads. This method modified with two protocols and tried with main three bias samples including JACK_R3, VGD1_2 R3, and Chia_G8. The first technique considered by following Folch et al. (1957) method with added chloroform CHCl3 and methanol CH3OH (2:1) and GC analyses with Direct Transesterification (DT) for following (Li et al., 2006; Zhang et al., 2009) protocol using two standard tri-17:0/19:0 ME. The second protocol for regular GC with sodium methoxide.

The first protocol ran a triplicate of three main oilseeds in present study JACK_R3, VGD1_2 R3 and Chia_G8. Ten mg ground seeds (burr mill machine) were added.
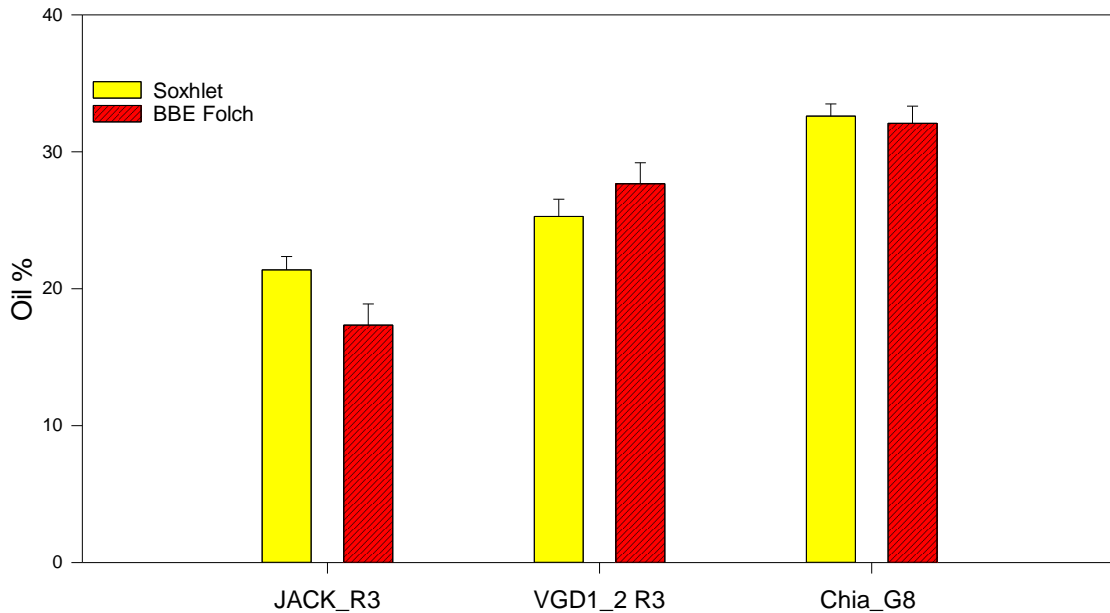
Figure 11-A: The Bead beating extraction (BBE) first technique follow Folch et al. (1957) and the Soxhlet method with three bias samples.

The figure 11-A shows BBE following Folch et al. (1957) procedure and solvent compared to the Soxhlet method with three bias samples (JACK_R3, VGD1_2 R3 and Chia_G8) Appendix Chapter 2 Table 17-A.

The SAS output by the GLM procedure showed, there is not a statistically significant interaction (P = 0.0913) between methods (Soxhlet, BBE_Folch) and samples (JACK_R3, VGD1_2 R3 and Chia_G8).There is not a statistically significant difference (P= 0.6781) between methods and there is a statistically significant difference (P <0.001) between samples. In the SAS output by using the contrast between the Soxhlet method as a control with BBE-Folch. There is not a statistically significant difference (P = 0.4514) between the Soxhlet method and BBE_Folch Appendix Chapter 2 Table 17-B. These results were not accurate due to the standard error was more than 1% for three main bias samples. the Tukey test showed there is not a significant difference between the Soxhlet and BBE-Folch.

However, the Soxhlet method provided 27.0% oil mean and 26.6% gave by BBE-Folch
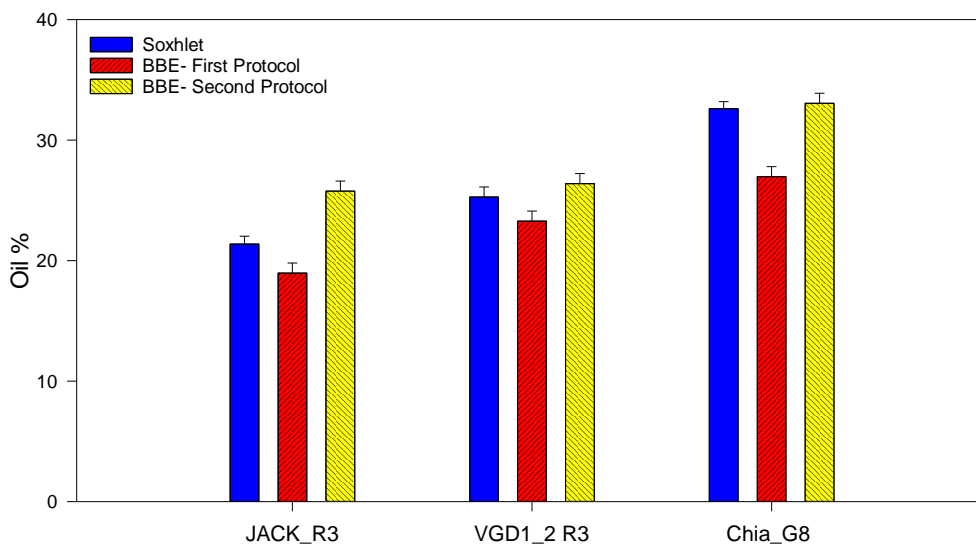
Figure 11-B: The Bead beating extraction the first protocol with Direct Transesterification (DT), the second protocol with regular GC using sodium methoxide and the Soxhlet method with three bias samples.

The BBE technique following (Li et al., 2006; Zhang et al., 2009) Appendix Chapter 2 Table18-A, and regular GC with solvent of sodium methoxide Appendix Chapter 2 Table18-B, ran a triplicate of three bias samples (JACK_R3, VGD1_2 R3, and Chia_G8). Ten mg ground seeds (burr mill machine) were used for evaluated percentage of oil extraction for these protocols. In this experiment as we indicated before using two standards tri-17:0/19:0 ME.

The SAS output by the GLM procedure showed, there is a statistically significant interaction (P = 0.0204) between methods (Soxhlet and Bead Beating Extraction (BBE) with the first protocol and second Protocol) and samples (JACK_R3, VGD1_2 R3, and Chia_G8). There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples.

In the SAS output by using the contrast between the Soxhlet method as a control with BBE_First protocol and BBE_Second protocol. There is a statistically significant difference (P = 0.015) between the Soxhlet method and BBE_Second protocol. There is a statistically significant difference (P <0.001) between the Soxhlet method and BBE_First protocol. To compare between BBE first and second protocol, There is a statistically

significant difference (P <0.001) between the BBE_Second protocol and BBE_First protocol <u>Appendix Chapter 2 Table18-C</u>. The Scheffe's test for mean of oil percent showed, there is not a significantly different between BBE_Second protocol (28.4%) and the Soxhlet method (27.0%) group A and BBE_First protocol (23.0%) group B. Therefore, the first protocol provides less oil quantity than second protocol between three bias samples.

Generally, the second technique was better than first technique for percentages of oil extraction. Therefore the second technique repeated after added new step was using sonication for 15 minutes at room temperature. The BBE second technique ran with sonication and without sonication of the three main bias samples (JACK_R3, VGD1_2 R3, and Chia_G8) in the <u>Appendix Chapter 2 Table 19-A.</u>
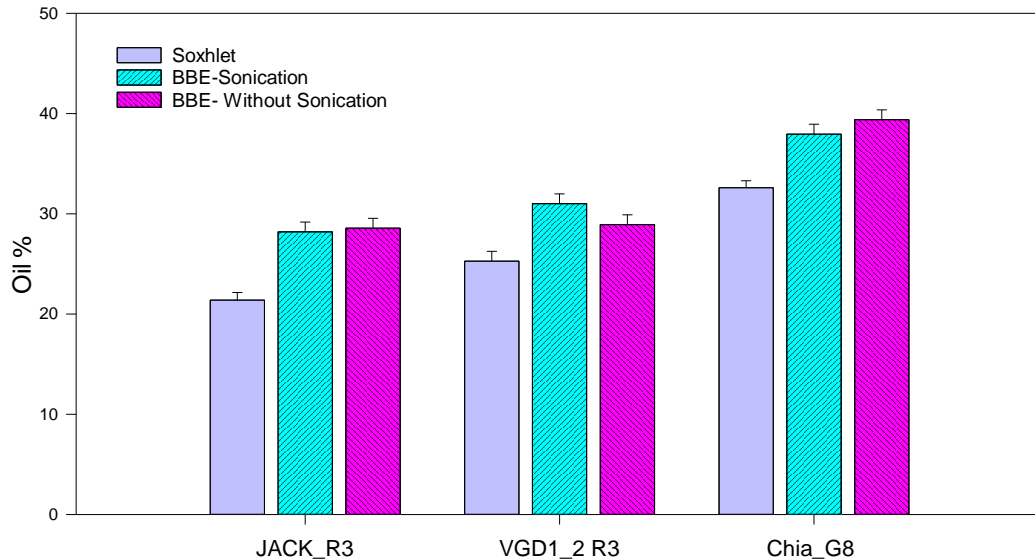
Figure 11-C. The bead beating extraction second technique regular GC by using sodium methoxide and the Soxhlet method

The SAS output by the GLM procedure showed, there is not a statistically significant interaction (P = 0.2693) between methods (Soxhlet and and Bead Beating Extraction (BBE) with Sonication and without Sonication) and samples (JACK_R3, VGD1_2 R3, and Chia_G8).There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples. The SAS output by using contrast between the Soxhlet method as a control with. There is a statistically significant difference (P <0.001) between the Soxhlet method and

BBE with Sonication. There is a statistically significant difference (P <0.001) between the Soxhlet method and BBE without Sonication [Appendix Chapter 2 Table 19-B.](#)

The Tukey test showed there are no significant differences between BBE with sonication (32.4%) and BBE without sonication (32.3%) group A and the Soxhlet method (27.0%) group B. The bead beating extraction with sonication provided the same or better results than not-sonication depends on a standard error that was lower after added sonication technique in the protocol [Appendix Chapter 2 Table 19-A.](#) The results found the better technique can be used with bead beating extraction is a second protocol with sonication technique that increased percentages of oil extraction between bias samples comparing the Soxhlet method.

Repeated BBE second protocol with sonication 15 minutes at room temperature and homogenized tubes twice times in an MP Bio Fastprep®-24 homogenizers with soybean seed program, the results in the [Appendix Chapter 2 Table 20-A](#). The results found high standard error via homogenized tubes twice times in an MP Bio Fastprep®-24 homogenizers with soybean seed program.

The SAS output by the GLM procedure showed, there is not a statistically significant interaction (P = 0.2317) between methods (Soxhlet and Bead Beating Extraction (BBE) with homogenized by soybean seed program one times and homogenized by soybean seed program twice times) and samples (JACK_R3, VGD1_2 R3, and Chia_G8).There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples. The SAS output by using the contrast between the Soxhlet method as a control, there is a statistically significant difference (P <0.001) between the Soxhlet method and BBE homogenized by soybean seed program one times. There is a statistically significant difference (P <0.001) between the Soxhlet method and BBE homogenized by soybean seed program twice times. There is not a statistically significant difference (P=0.8665) between BBE homogenized by soybean seed program one times and BBE homogenizers by soybean seed program twice times [Appendix Chapter 2 Table 20-B](#).

The results proved the BBE with regular GC using sodium methoxide has high accurcy results than first protocol. The BBE second protocl ran a triplicated with 11 bias samples for comparing with Soxhlet method [Appendix Chapter 2 Table 21-A](#)
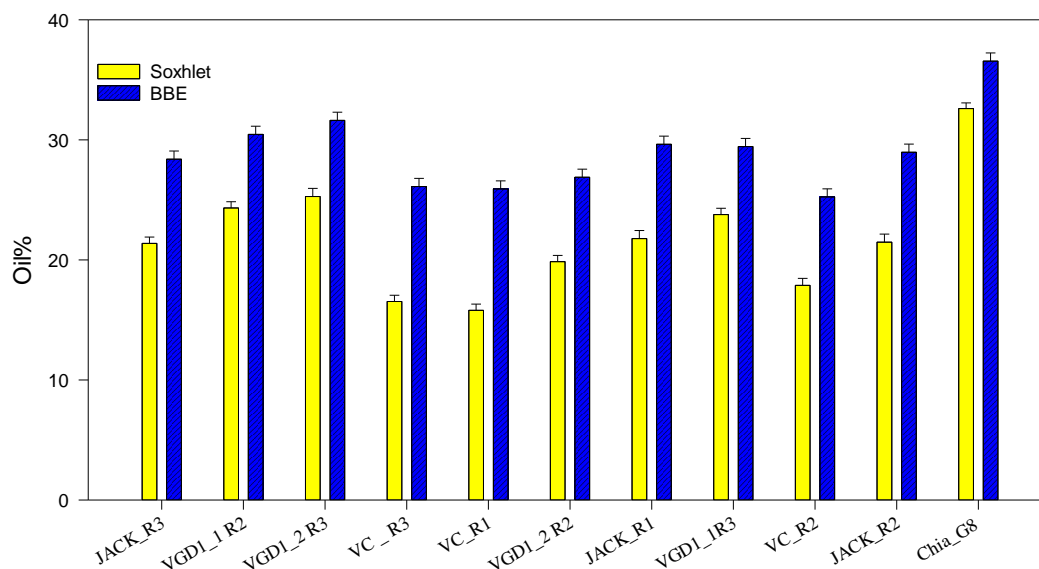
Figure 11-D: Percentages of oil extraction between the Bead Beating Extraction (BBE) method and the Soxhlet method with 11 Bias Samples

The SAS output by the GLM procedure showed, there is a statistically significant interaction (P <0.001) between methods (Soxhlet and bead beating extraction (BBE)) and samples (JACK_R3, VGD1_1 R2, VGD1_2 R3, VC _ R3, VC_R1, VGD1_2 R2, JACK_R1, VGD1_1R3, VC_R2, JACK_R2, and Chia_G8). There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples.

In the SAS output by using the contrast between the Soxhlet method as a control with DDTQ method. There is a statistically significant difference (P <0.001) between the Soxhlet method and DDTQ method Appendix Chapter 2 Table 21-B.
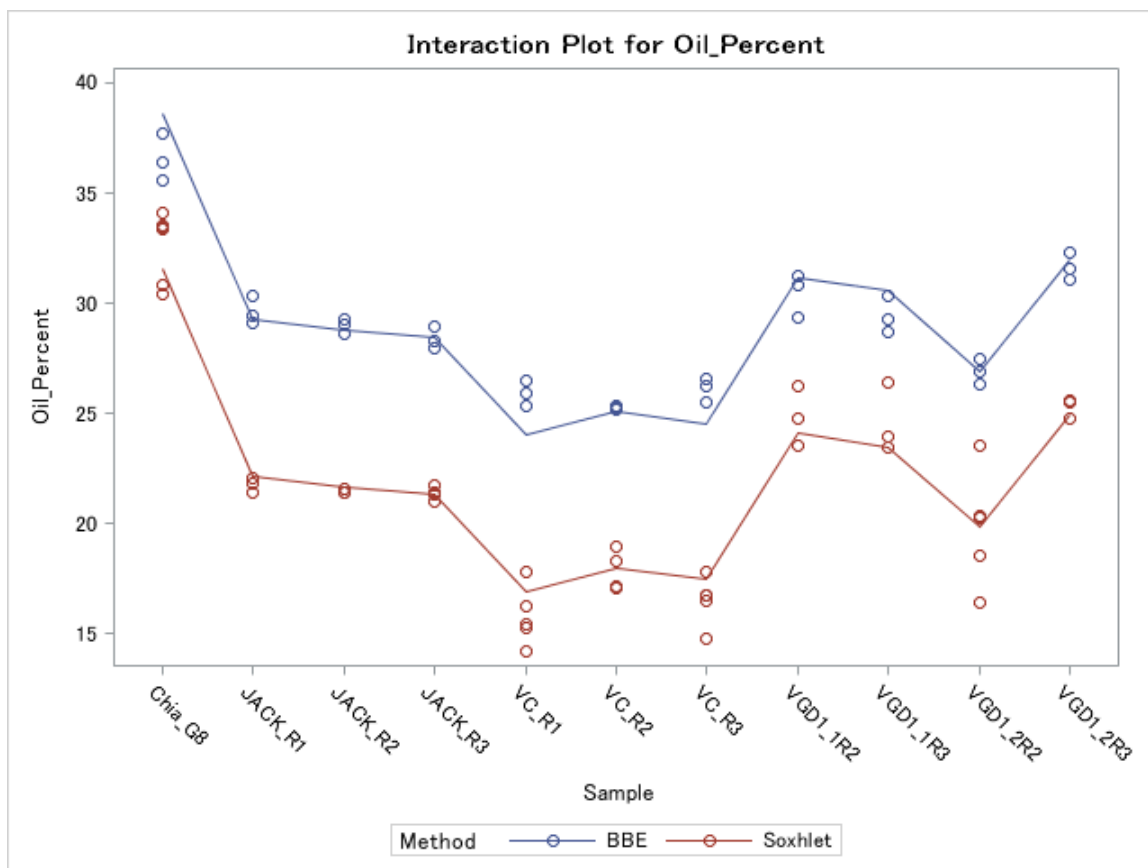
Figure 11-E: Interaction between the Bead Beating Extraction (BBE) method and the Soxhlet method with 11 Bias Samples

Discussion

The Bead Beating Extraction (BBE) was approved by following Folch et al. (1957) procedure and the results were not accuracy. The main issue with this technique might be the process was not complete of measuring the samples weight due to the samples did not stable adequately after evaporation under N2 flowed in the tubes. The small amount of biomass (10 mg) might cause big standard error comparing with (100 mg) used with Folch method. To compare between first protocol by DT method and second protocol by using regular GC. There is a statistically significant difference (P <0.001) between the BBE_Second protocol and BBE_First protocol. The mean of oil percent showed for three main bias samples provided (28.4%) higher yield with BBE_Second protocol than BBE_First protocol (23.0%). There is not significantly different between BBE second protocol and the Soxhlet method. Addition to, the first protocol was required a long time

for finished all the steps and was complicated compared with the second protocol. This protocol has many steps, talk long time to get the final results that should intercept with the main purpose of this research to find the most efficient method for oil extraction from soybean and chia.

The second technique of BBE that had utilized and found comparable oil ratios using 10 mg ground seeds. The BBE second protocol by Tukey test showed there are no significant differences between BBE with sonication (32.4%) and BBE without sonication (32.3%). The BBE with sonication provided less standard error between replications that was lower after added sonication technique in the protocol. The BBE provided high oil quantification comparing with the Soxhlet method (as control). The BBE method with sodium methoxide exhibited precision results of oil extraction and it is the easier and cheaper and faster technique can use of all lipid quantification methods that can be approved the objective of this study.

Near Infrared Reflectance Spectroscopy (NIRS)
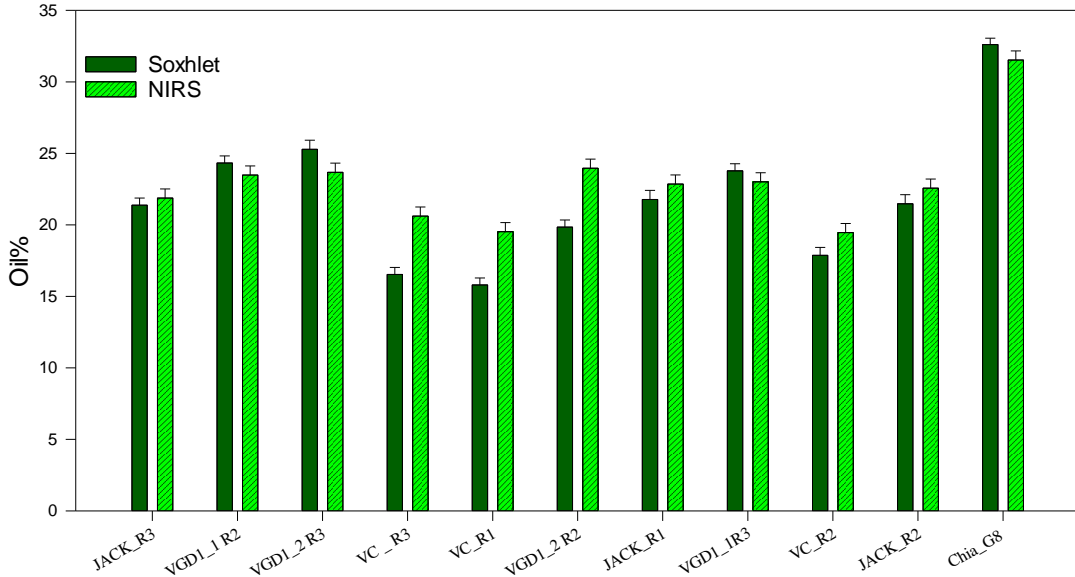
Figure 12-A:Percentages of oil determination between the Near Infrared Reflectance Spectroscopy (NIRS) method and the Soxhlet method with 11 Bias Samples

The SAS output by the GLM procedure showed, there is a statistically significant interaction (P =0.005) between methods (Soxhlet and Near Infrared Reflectance

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Spectroscopy (NIRS)) and samples (JACK_R3, VGD1_1 R2, VGD1_2 R3, VC _ R3, VC_R1, VGD1_2 R2, JACK_R1, VGD1_1R3, VC_R2, JACK_R2, and Chia_G8). There is a statistically significant difference (P = 0.0003) between methods and there is a statistically significant difference (P <0.001) between samples. The SAS output by using contrast between the Soxhlet method as a control with NIRS method. There is a statistically significant difference (P = 0.003) between the Soxhlet method and NIRS method Appendix Chapter 2 Table 22.
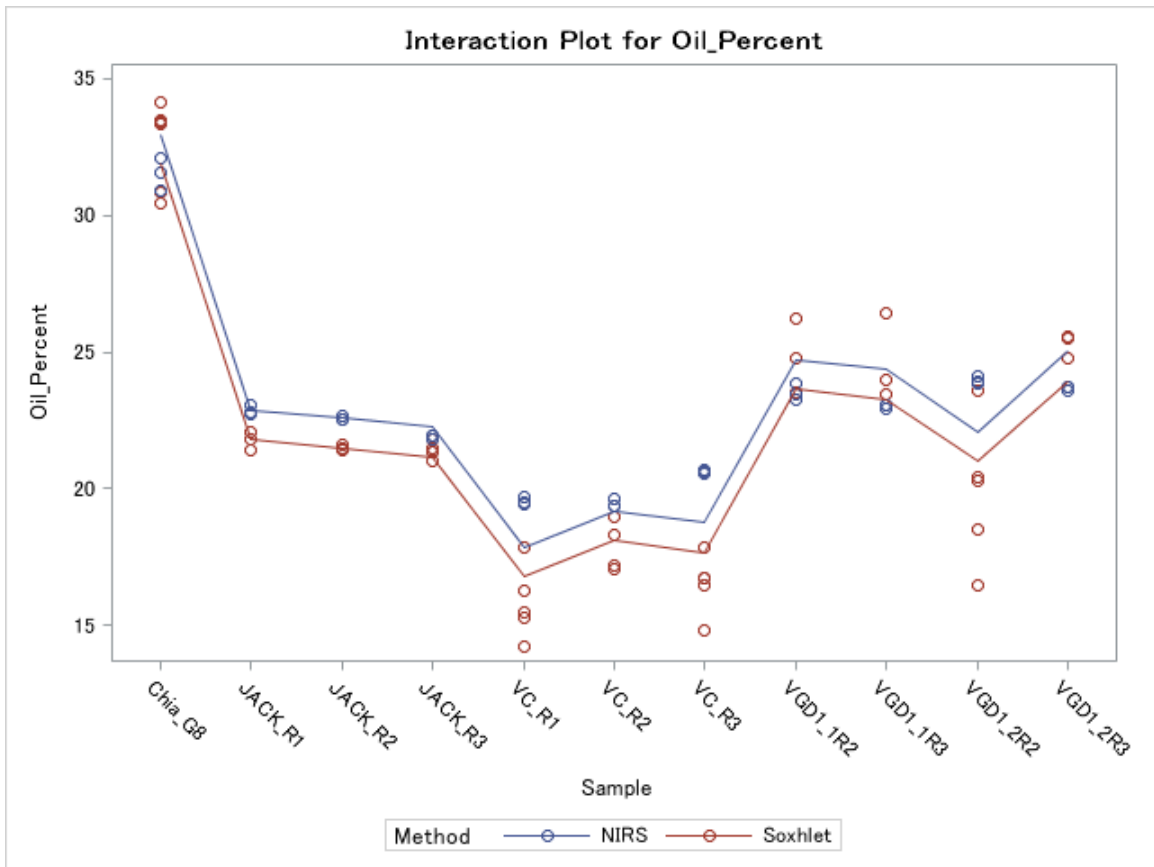
Figure 12-B:Interaction between between the Near Infrared Reflectance Spectroscopy (NIRS) method and the Soxhlet method with 11 Bias Samples

Discussion

There is a statistically significant difference (P = 0.003) between the Soxhlet method and NIRS method. The higher oil was recorded by NIRS (23.0%) group A than the Soxhlet method (22.0%) group B for 11 bias samples involved in this study.

Nile red fluorescence method (Sitepu et al., 2012)

Results

Fluorometry does need for lipids to be extracted; only the fluorometric dye needs to penetrate the material well. The nile red fluorescence was tried with a triplicate, and this experiment was performed on main three bias samples including JACK_R3, VGD1_2 R3, and Chia_G8. technique Nile red fluorescence ran with two different seeds mass (1 and 5 milligrams) and three different Nile red fluorescence amount (2, 10 and 25 µL) that adjusted via acetone (23, 15 and 0 µL) respectively. The Nile red fluorescence was used by shaking and without shaking. High oil provided with 25 µL Nile Red.
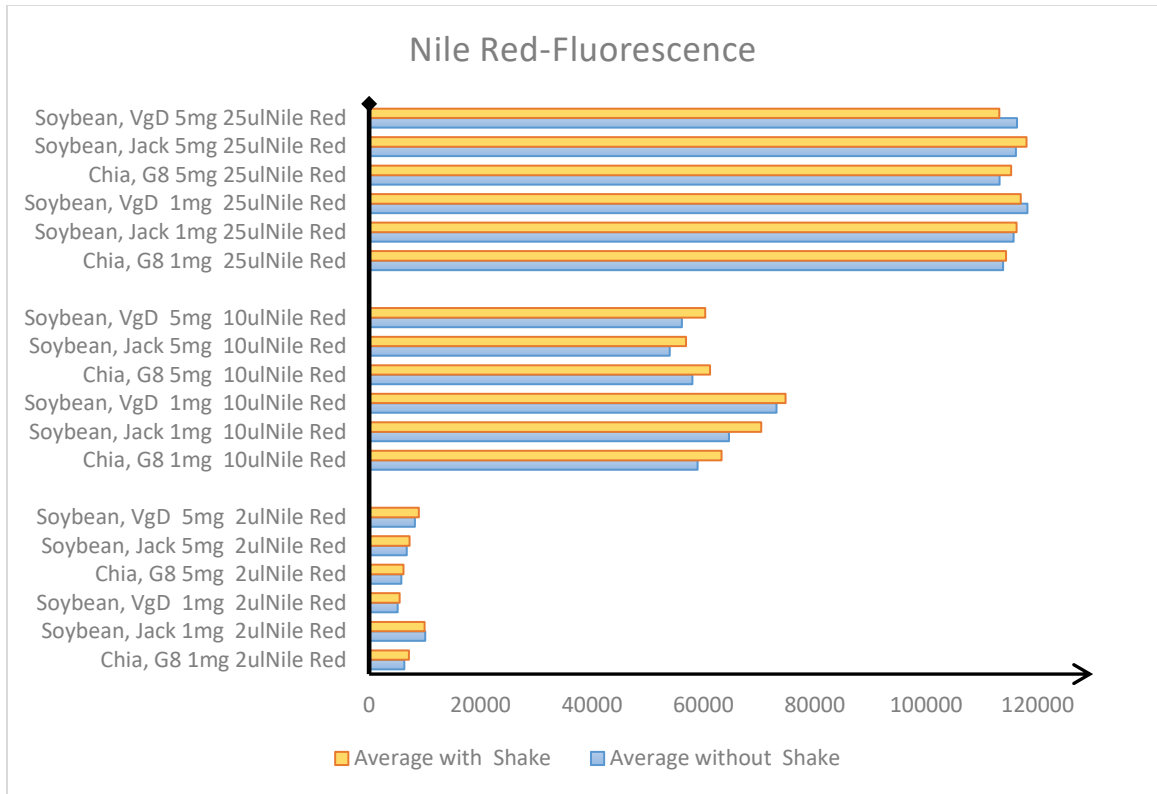
Figure 13: Nile Red Fluorescence with and without shaking

The figure shows the Nile red fluorescence was used by shaking and without shaking. The high number was provided with 25 µL Nile Red with shaking.

Second experiment

The Nile red fluorescence ran with 0, 1, 10, 25, and 50 µg soybean oil in acetone 10, 25 and 100 µL of Nile Red (0.05 mg/ml in acetone were added. The results of the second experiment was not clear Appendix Chapter 2 Table 23.

Discussiont

This technique had used with yeast and considered for the fluorimetric microplate protocol to modify this protocol with oilseeds by added 25 microliter dimethyl sulfoxide (DMSO) and added 0.05 mg/ml Nile Red amount was adjusted with acetone. A major factor affecting lipid quantification is the barrier cell walls present for lipid removal. Fluorometry does need for lipids to be extracted, only the fluorometric dye needs to penetrate the material well. However, this technique did not provide clear results. There were no significant variations between bias samples high to low.

CONCLUSIONS:

The study evaluated 10 different methods with the oilseeds soybean and chia, some of them have been known for oil extraction from seeds and others were not applied before to oil seeds. The main aim of the present study was to find one or more techniques that are fast, safe and inexpensive with a small amount of ground seeds. The Soxhlet method is the standard method used in this study. The Soxhlet method used petroleum ether (PE) and acetone(Ac), to determine if the solvent polarity and water solubility could affect the quantity of oil extraction. There is not a statistically significant difference between PE and Ac solvents. No significant differences for Soxhlet oil extraction method were found between medium moisture content (above 10%) and low moisture content (less than 4%). The Folch technique provided higher percentages of oil extraction than Blight and Dyer and hexane-isopropanol techniques with two different varieties. There is not a statistically significant difference (P =0.0844) between Soxhlet method and Folch method, but lower oil quantification than Soxhlet method. Supercritical fluid extraction (HCH) method provided lower yield of oil extraction with all three varieties of bias samples compared with the Soxhlet method. A Direct Transesterification (DT) method with LMC and MMC provided a statistically significant difference than the Soxhlet method. The DT with LMC

provided higher yield than DT with MMC between samples but lower than the Soxhlet method.

The Double Direct Transesterification Griffiths (DDTG) method gave more accurate results with stir mixing than sonication with 10 mg seeds amount and 10 minutes incubate time. There is a significant difference between the Soxhlet and DDTG method and high oil quantification found with DDTG. The Double Direct Transesterification - Qiao (DDTQ) method gave simillar oil percentages to DDTG method. There is a significant difference between the Soxhlet and DDTQ method and higher oil recovered with DDTQ.

The Bead Beating Extraction (BBE) method showed, there is a statistically significant difference (P <0.001) between the BBE second protocol with sodium methoxide and BBE first protocol followed by Folch method and a Direct Transesterification method and high percentages of oil with sodium methoxide than the other two BBT techniques. There is a statistically significant difference (P <0.001) between the BBE second protocol and the Soxhlet method. The BBE provided high oil quantification comparing with the Soxhlet method. Near Infrared Reflectance Spectroscopy (NIRS) method showed, there is a statistically significant difference (P = 0.003) between the Soxhlet method and NIRS method. The last part of current study was with Nile red and fluorescence technique. This technique throughput without the need for lipid extraction. However this technique did not provided accurate results.

Four methods ran with 11 bias sample to compared with the Soxhlet method that have high oil quantification than Soxhlet. The SAS output showes comparisons significant at the 0.05 level are indicated by (***) between Soxhlet as control with four others methods (Double Direct Transesterification Griffiths (DDTG), Double Direct Transesterification Qiao (DDTQ), Bead Beating Extraction (BBE), and Near Infrared Reflectance Spectroscopy (NIRS)) Table 7. There is a statically significant difference between the Soxhlet and others methods.

Table 7: Comparisons significant at the 0.05 level are indicated by *******.

Comparisons significant at the 0.05 level are indicated by ***.				
Treatment Comparison	**Difference Between Means**	**Simultaneous 95% Confidence Limits**		
DDTQ - Soxhlet	7.1792	6.2149	8.1434	***
DDTG - Soxhlet	7.0603	6.0960	8.0245	***
BBE - Soxhlet	7.0525	6.0883	8.0167	***
NIRS - Soxhlet	0.9889	0.0247	1.9532	***

The SAS output by the GLM procedure showed, there is a statistically significant interaction (P <0.001) between (Figure 14-A) methods (Soxhlet, DDTG, DDTQ, BBE, and NIRS) and samples (JACK_R3, VGD1_1 R2, VGD1_2 R3, VC _ R3, VC_R1, VGD1_2 R2, JACK_R1, VGD1_1R3, VC_R2, JACK_R2, and Chia_G8). There is a statistically significant difference (P <0.001) between methods and there is a statistically significant difference (P <0.001) between samples Appendix Chapter 2 Table 24
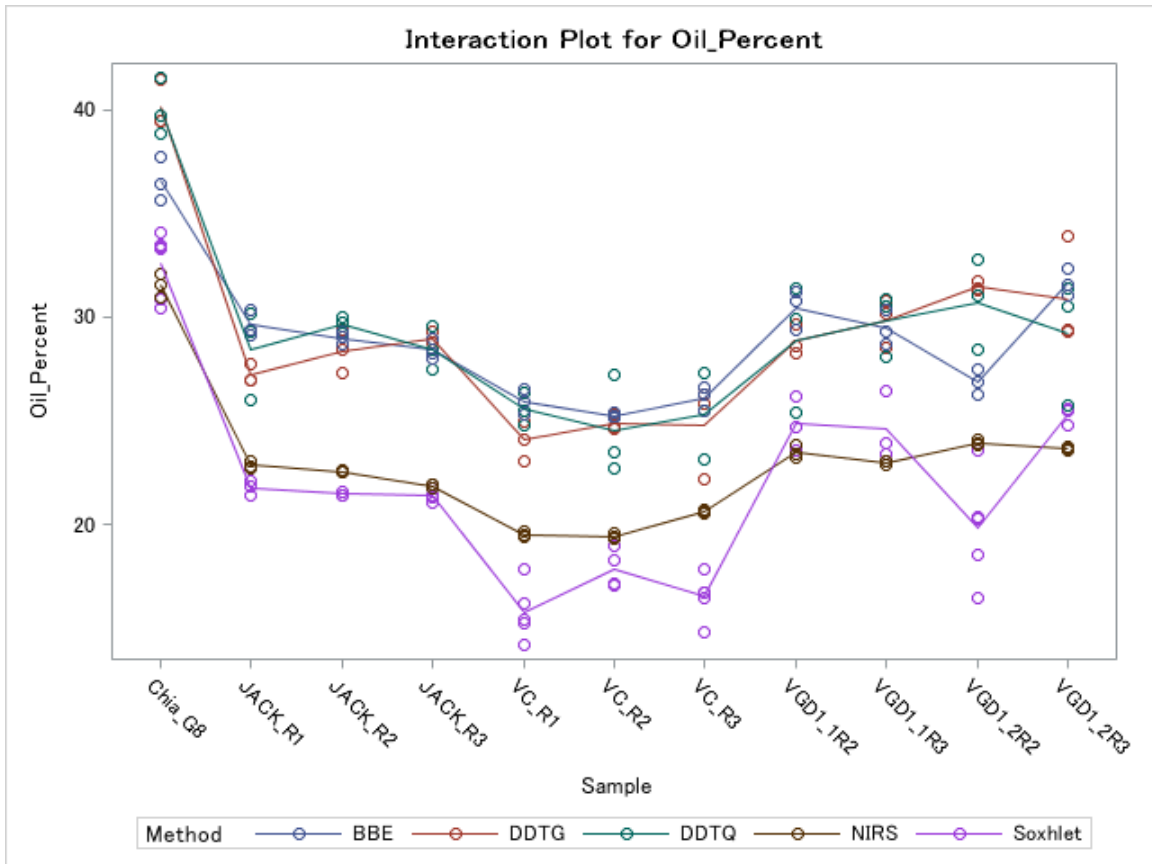
Figure 14-A: interaction between five method (Soxhlet, DDTG, DDTQ, NIRS, and BBE) and 11 bias samples.

In the SAS output by using the contrast between the Soxhlet method as a control with DDTG, DDTQ, NIRS, and BBE methods. There is a statistically significant difference (P <0.001) between the Soxhlet method and DDTG method, there is a statistically significant difference (P <0.001) between the Soxhlet method and DDTQ method, There is a statistically significant difference (P <0.001) between the Soxhlet method and BBE method, and There is a statistically significant difference (P=0.0019) between the Soxhlet method and NIRS method Appendix Chapter 2 Table 24.
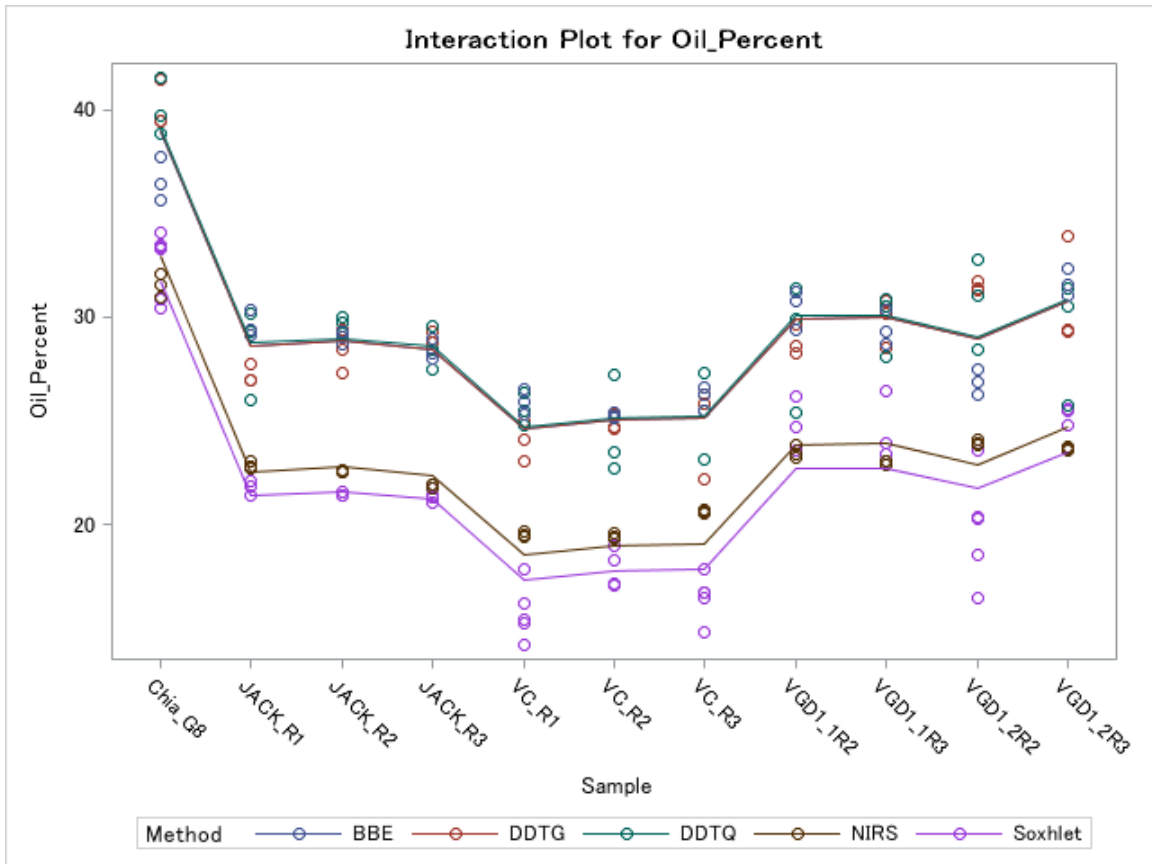
Figure 14-B :The interaction Plot and the contrast between five methods (Soxhlet, DDTG, DDTQ, NIRS, and BBE)

The contrast provided precision on oil extraction compared with the Soxhlet method and mean of oil extraction with DDTG, DDTQ, BBE, and NIRS were higher than the Soxhlet. The Tukey test showed there are not significant different between DDTG, DDTQ and BBE group A, the NIRS group B and the Soxhlet group C.
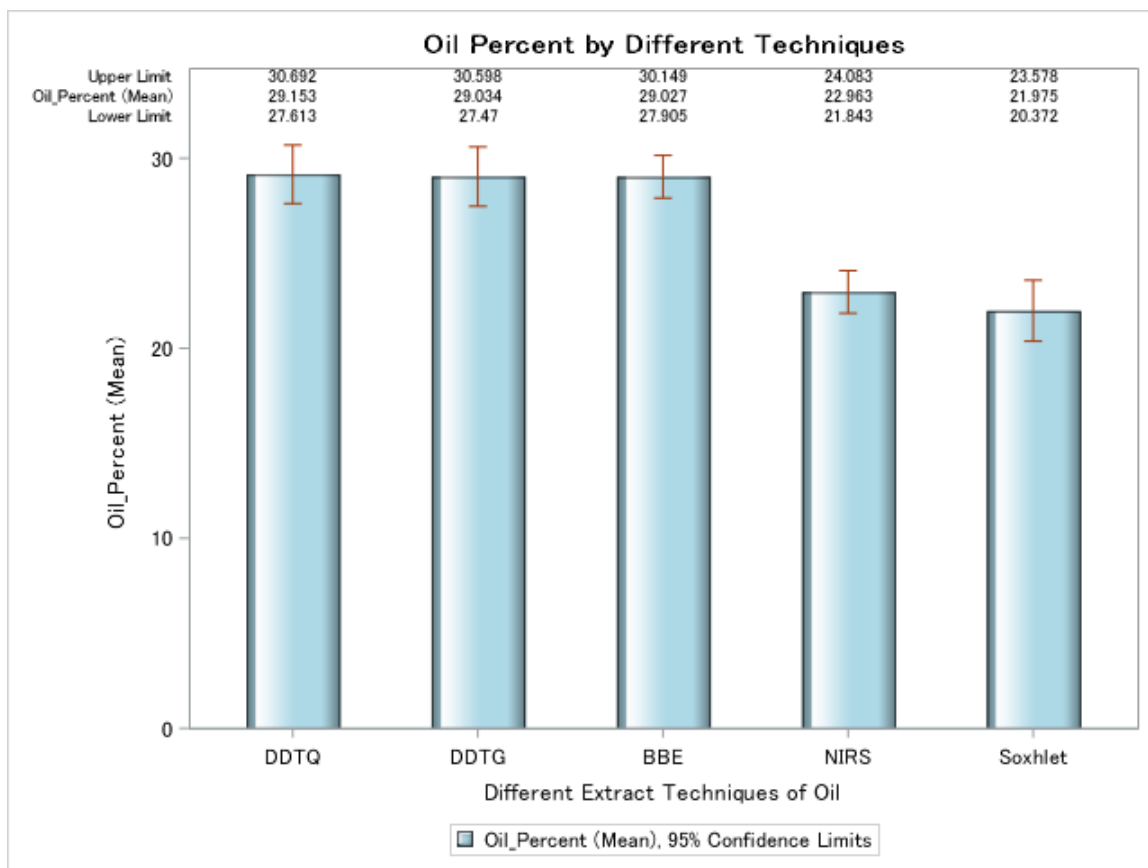
Figure 14-C: Percentages of oil extraction between five methods (DDTQ, DDTG, BBE, NIRS, and Soxhlet) with 11 bias samples.

The DDTG, DDTQ, and BBE provided high quantification oil extraction compared with the Soxhlet method, and there are no significantly different between these methods. The BBE displayed the best precision results for all oil quantification methods (Figure 14-C) comparing with Soxhlet. The BBE method with sodium methoxide is high efficiency, and the easier, safer, cheaper and faster technique can use all oil quantification methods that can be approved the purpose of this study.
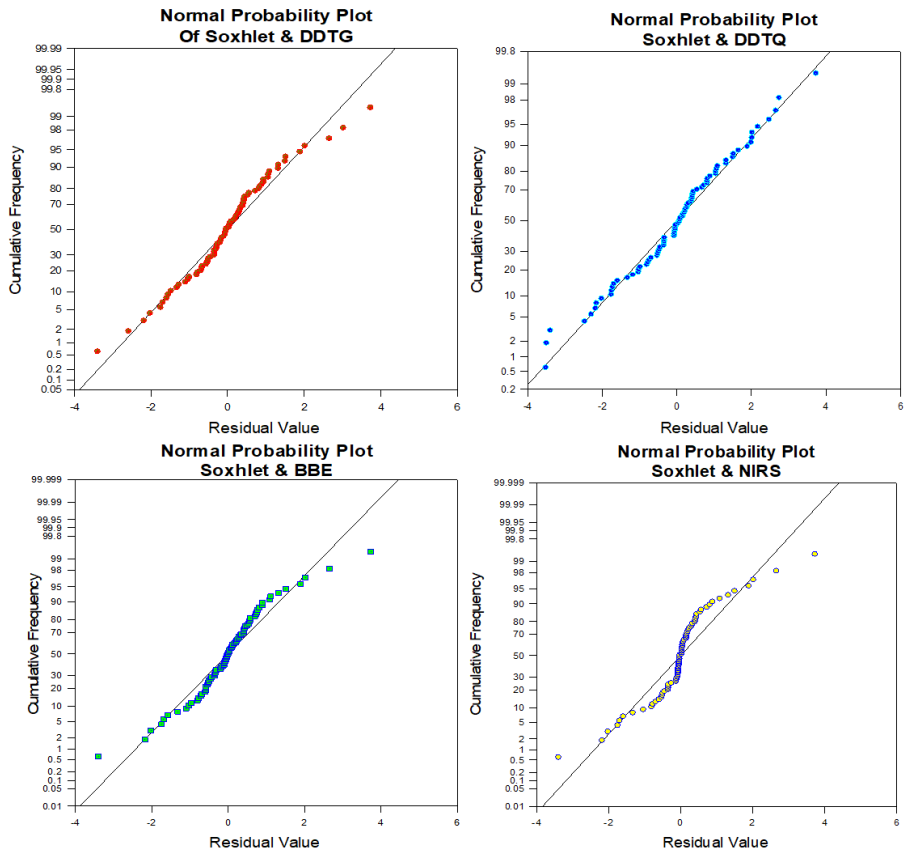
Figure 14-D: Residual value of DDTG, DDTQ, BBE, and NIRS with the Soxhlet method

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CHAPTER 3: Characterization of Oil and Protein in Mutant Chia (*Salvia hispanica L.*) Seeds

INTRODUCTION:

Chia (*Salvia hispanica* L) is a member of the *Lamiaceae* or *mint* family, is an annual summer herb in Kentucky, and is one of the main traditional foods of Central America (Ayerza and Coates, 2004). Chia is especially fascinating because it is grown to produce oil and protein for consumption and industry (Ayerza, 1995). The chia plant is an important crop in countries such as the USA, Argentina, Chile, and Italy. However the climate conditions make it difficult in these countries to plant chia seeds, and improved agronomic practices are needed to adapt chia in these agriculture zones (Coates, 1996; Coates, 2011). The first new chia varieties, specifically in Lexington KY, were developed by mutation, ethyl methane sulfonate (EMS) and gamma radiation (Jamboonsri, 2010). The G8 variety of chia seeds was created from mutagenesis by Gamma radiation by Dr. Phillips and Dr. Hildbrand at the University of Kentucky. The original seeds were "Pinta". It is an early flowering wild type in Mexico. The wild type Pinta were mutagenized with gamma rays (Gy) to produc early flowering plants.

The objective of this study was to investigate the oil, protein quality and yield Kg/ha altered by genotypic components and environmental conditions during the growing season in different locations. Growing chia under different climate conditions could influence protein and oil content and seed yield. According to Ayerza and Coates, (2004) growing chia in different locations caused significant differences in oil and protein content (Ayerza and Coates, 2004). Location can affect the composition of the chia seeds, apparently due to one or more environmental factors, such as temperature, light, soil type and available nutrients (Ayerza, 1995). Seed oil and protein levels are significantly correlated with temperature. Numerous studies claim that the ecosystem has a strong influence on the protein content of chia seeds, as has been reported for many other crops (Johann Vollmann et al., 2007). There is a positive correlation between protein content and temperature in oilseed crops like soybean (Coates, 2011; Kumar et al., 2006; Thomas et al., 2003). The varying protein content in chia seeds among varieties was also reported for numerous other crops including sorghum (Saeed et al., 1987) and soybean (J Vollmann et al., 2000), as well as chia (Ayerza and Coates, 2004). These variations for other crops, such

as soybeans and sorghum, have similarily demonstrated that changes in protein components are impacted by the environment (Saeed et al., 1987; J Vollmann et al., 2000). Increasing oil content could affect the nutritional value of chia as a source of oil for human health (Medic et al., 2014). The remnant of the chia plant after oil extraction might be used as a protein source for animal or human consumption (Coates, 2009) when the chia is not planted for its protein content. Chia may be the best source of beneficial soluble fiber (Ayerza and Coates, 2004; Cahill, 2003).

HYPOTHESIS

- Measure heritability in chia plants with a range of levels of oil and protein content and seed yield.
- Compare differences between two locations for oil and protein percentages in chia.

MATERIALS

A population of 180 M3 mutation individual chia plants was harvested in October 2015 from which forty M4 chia genotypes were planted (Appendix Chapter 3 Table1) based on seed composition characteristics, with six plants representing each characteristic:

Lower or higher for:

Seed oil content
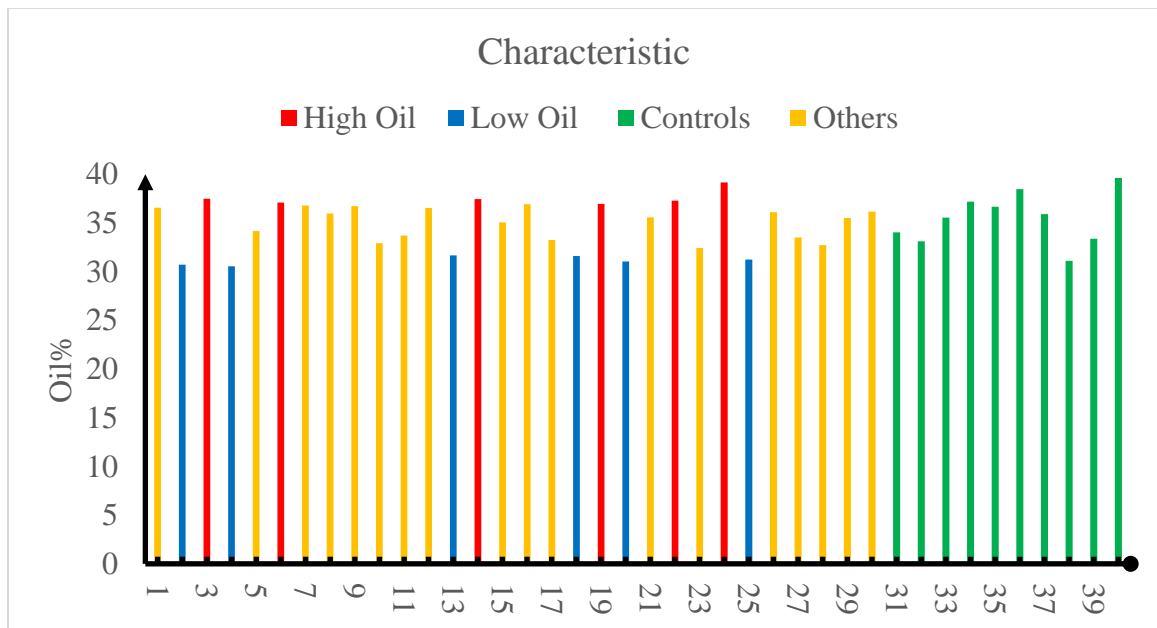
Figure 15-A: Six characteristics of a mutation individual plant that selected of high or low percentage of oil content.

Figure 15-A shows the level of oil percentages in forty plants., six high oil percent plants (marked with red color) and six low oil percent plants (marked with blue color) were selected. Ten plants (marked with green color) were selected as controls and the rest (marked with orange color) were selected for other characteristics.

Seed protein content
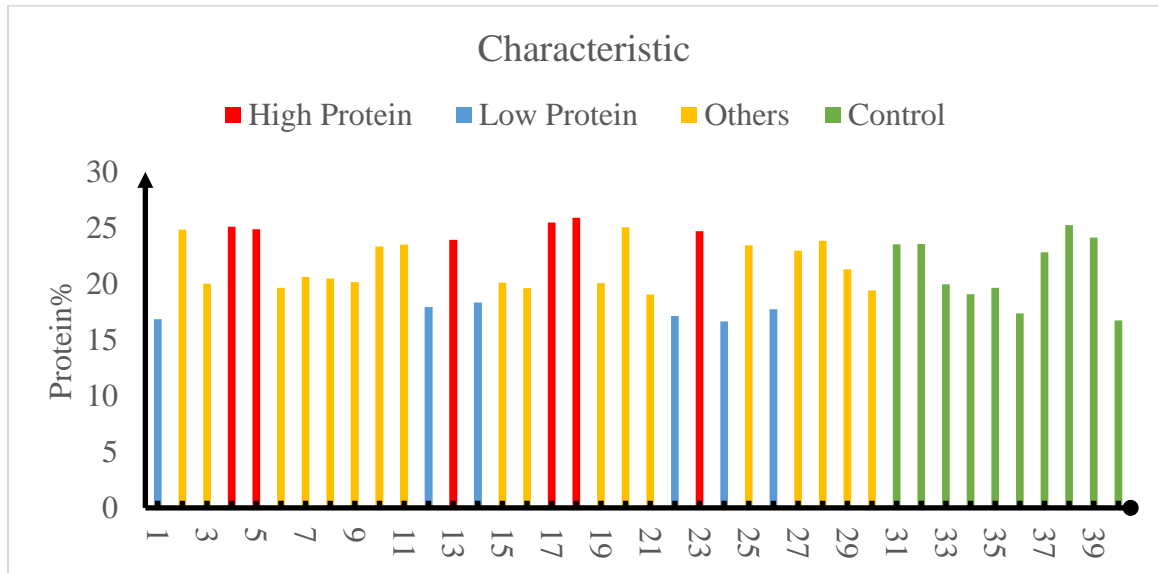
Figure 15-B. Six characteristics of a mutation individual plant that selected of high or low percentage of protein content.

Figure 15-B shows the level of protein percentages in forty plants, six high protein percent plants (marked with red color) and six low protein percent plants (marked with blue color) were selected. Ten plants (marked with green color) were selected as controls and the rest (marked with orange color) were selected for other characteristics.

Seed density
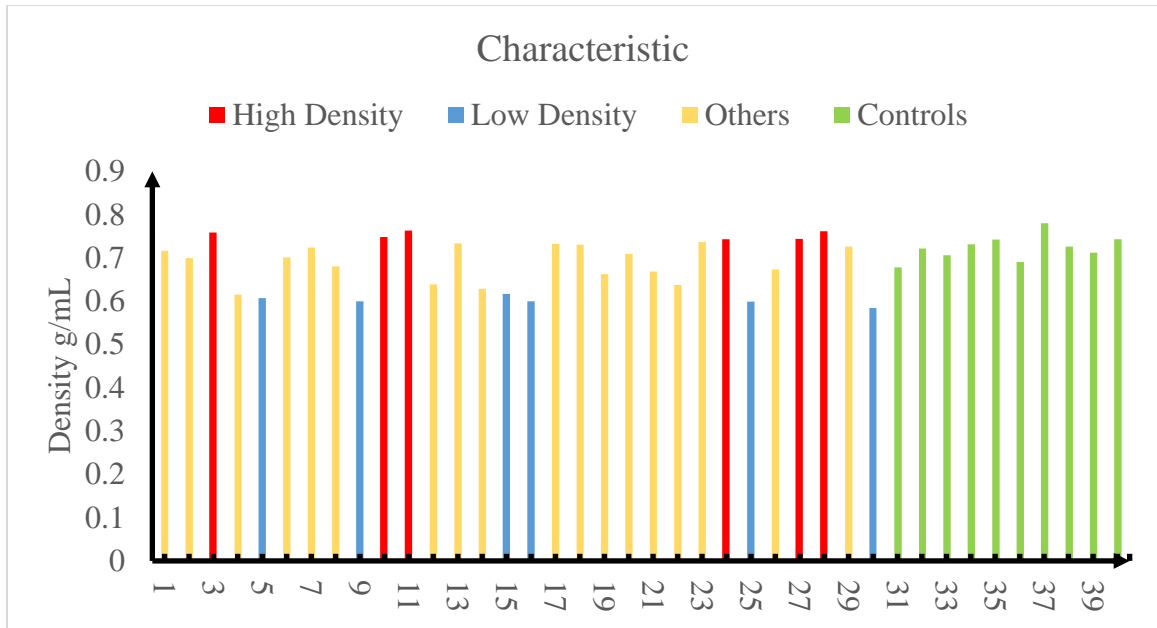
Figure 15- C: Six characteristics of a mutation individual plant that selected of high or low percentage of density g/mL.

Figure 15-C shows the level of density g/mL in forty plants, six high density plants (marked with red color) and six low density plants (marked with blue color) were selected. Ten plants (marked with green color) were selected as controls and the rest (marked with orange color) were selected for other characteristics.

Seed yield
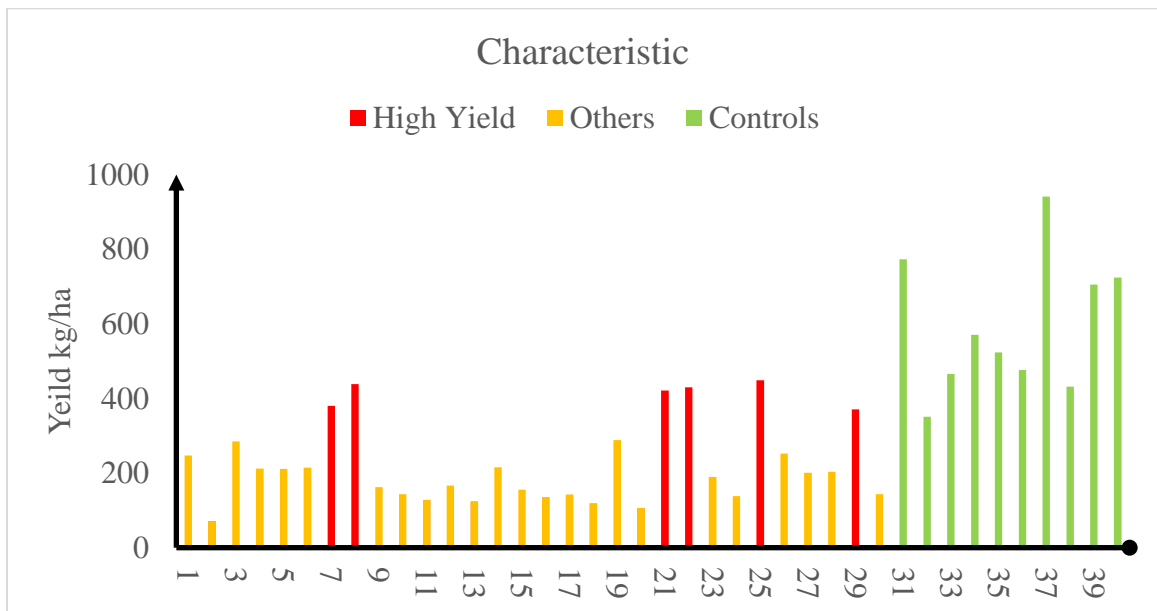
Figure 15. D: Six characteristics of a mutation individual plant that selected of high yield kg/ ha

Figure 15-D shows the level of yield kg/ha in forty plants, six high yield plants (marked with red color) were selected. Ten plants (marked with green color) were selected as controls and the rest (marked with orange color) were selected for other characteristics.

METHODS:

This study was conducted in the field and lab. The seeds have been provided by the Department of Plant and Soil Sciences at the University of Kentucky. A population of 180 individual chia plants was harvested in October 2015 from a nursery of mutant lines from which 40 were selected and sown in two locations in Kentucky, USA. The oil and protein contents of the 180 mutant single chia plants were screened using NIRS (Near Infrared Spectroscopy), and the volume and weight of each single plant were measured. Fatty acid composition was determined by a GC-FID automated gas chromatograph. Forty plants were selected based on characteristics, with six plants representing each characteristic: lower and higher in oil, protein, and density and higher yield. Two locations have been chosen in Kentucky to cultivate chia plants. One location was the field of the experimental station at Spindletop Farm in Lexington, KY (38°07'43.0"N 84°29'44.4"W, 282 m above sea level). The other was the field of Robinson Forest substation farm (Quicksand Farm) near Jackson, KY (37°31'56.6"N 83°20'46.5"W, 226 m above sea level). The soil type of Spindletop Farm is a Maury silt loam (Yang et al., 2013). The soil type of Robinson Forest substation farm is a Pope silt loam (Fike et al., 2006; Makris, 2003).

A randomized complete block design (RCBD) was carried out in field trials with two replications for both locations, in 7.62-meter long lines with 0.91- meter spaces between rows. Planting dates were May 26, 2016 in Spindletop and Jun 2, 2016 in Quicksand. Harvesting dates were October 24, 2016 in Spindletop field, and October 31, 2016 in Quicksand field. Two separate one meter lengths were harvested by hand for each replicate. Within each location where the chia was grown, a total of 160 lines were harvested. In each site, the samples were collected, cleaned by a threshing machine and sent for analysis to the laboratory in the Plant and Soil Department at the University of Kentucky. All 320 M5 seeds collected were measured for oil and protein content by NIRS.

Weight and volume were measured for these samples and weights of 1000 seeds were measured for each sample. The data was collected and statistical analysis was performed with SAS® software at the Plant and Soil Department.

Agronomic Characteristics

Chia (*Salvia hispanica* L.) is an annual summer crop in Kentucky. The growth period of chia is between 3 to 5 months depending on the ranges where chia can be grown. In Argentina, Bolivia, and Mexico, the chia seeds are planted in 6-8 meter lenght, and the row spaces were 0.9 meter. No irrigation system has been approved for chia. Herbicide was used at Spindletop Farm because big weeds were a main problem for the chia plant, but at Quicksand Farm, weeds had no effect on yield production. No diseases or pests were recorded in both locations. The main problem at Quicksand Farm was plant lodging that was recorded due to the stem lengths being 1.5 to 1.8 meter, compared with stem lengths at Spindletop Farm, which were between 1 to 1.2 meter. No ants were recorded in the two sites which was reported as the biggest problem in Argentina, Columbia, and Bolivia. Chia plants were harvested by hand. Not all varieties mature at the same time. We needed to wait for all the seeds to dry, and that increased the risk of losing seeds from varieties that matured early to wind and rain. The spikes of the flowers mature and dry before the side branches that stay green for a while. Chia plants grew better at Quicksand than Spindletop due to higher nutrients and nitrogen levels in the soil at Quicksand than at Spindletop and Spindletop had competition with weeds.

Laboratory Analysis

One hundred eighty M3 mutant single plants were harvested and sent for laboratory analysis at the University of Kentucky. All the plants were cleaned by hand and oil and protein content were determined by using a Near Infrared Reflectance Spectroscopy (NIRS) machine. NIRS measured triplicate of each line and provided the percentage of oil and protein with zero moisture content. The moisture content was measured by using a force dry oven at 103°C for 48 hours. The weight of seeds for each single plant was recorded, and volume of the seeds was measured. The density g/mL of seeds was determined by dividing seed weigh by seed volume. Oil composition was determined by GC-FID. Forty M3 plants were selected depending on characteristics as explained in the

methods section. M4 seeds were planted in May and June 2016 and harvested in October 2016. Three hundred twenty M5 bulk seeds were sent to the laboratory and oil and protein content were screened by NIRS and seed weight was measured. The volume of the each sample was measured,1000 seeds weight was recorded, the seed density g/mL was divided, and the moisture content was recorded. The same varieties were planted in both locations from M5 progeny seeds on June 1, 2017 at Spindletop Farm and on June 2, 2017 at Quicksand Farm. Four replications were carred out in the field with 10 feet length and 3 feet spaces between the rows.

Seed oil composition

Method

Fatty acids composition was determined by regular GC protocol. A test tube and vial were prepared and five seeds were put in the test tube and ground. Five hundred µL sodium methoxide was added to the test tube and then it was shaken for 10 minutes. One mL isooctane with 0.001% BHT was mixed in the test tube by shaking for 3-5 minutes. Two hundred µL of the upper layer was transferd to GC vials.

Gas chromatography (GC)

GC vials were then run on a Varian CP-3800 Gas Chromatograph using a 25m x 0.25 mm ID fused silica column with a Varian (chrompack) CP=Select CB for FAME, with a film thickness of 0.25 um. The temperature program ran from 90 C to 250 C with 25 C ramp for a total of an 8 minute run time with a constant column flow mode of 0.9 mL/min utilizing a splitless injection. Quantification was performed by using a flame ionization detector and peaks quantified using Star Chromatography Workstation Version 6.00, with peak area being used to calculate relative percentages of FAMEs.

Statistical analysis

The statistical analysis was performed using SAS software [Proc GLM; SAS version 9.4 (Cary Institute, 2014)]. The research question was, how do parental information (covariate) and the variety of plants (treatment) explain various responses (oil, protein, yield, etc.)?. The study design was a RCBD with two locations, two blocks per location, and 40 varieties (treatments), and the covariate of parental heritability was measured.

Analysis of covariance (ANCOVA) was used to adjust or control for differences among the groups under study based covariate, which is a confound variable to identify if a factor statistically reduces the effects of other independent variables (S. B. Green & Salkind, 2010). In the current study, parental information was involved as a covariate (oil, protein, yield, etc.) to evaluate whether progeny means (response variable such as oil, protein, yield, etc.) on the dependent variable are the same or significantly different across several factors (treatment, blocks per location). The ANCOVA measured the heritability of covariant (parental information) on the response variable (progeny oil, protein, yield, etc.) to determine if factors (40 treatment, location, replication per location) have any effect on the response variable.

Environmental factors

Temperature °C
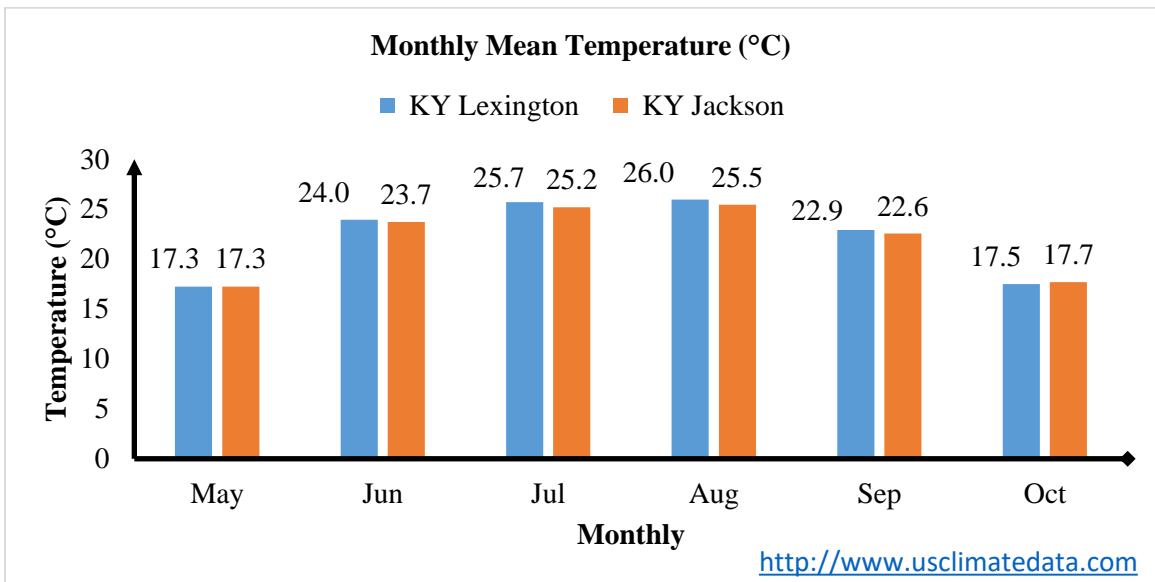
Figure 16-A: Average of Monthly Temperature °C from May to October 2016.

The average temperature at Spindletop Farm in Lexington was warmer than at Quicksand Farm near Jackson. The mean temperature during the growth periods of the chia seed was 22.2°C at Spindletop, and the mean temperature at Quicksand was 22°C.

Precipitation

Monthly Mean Precipitation
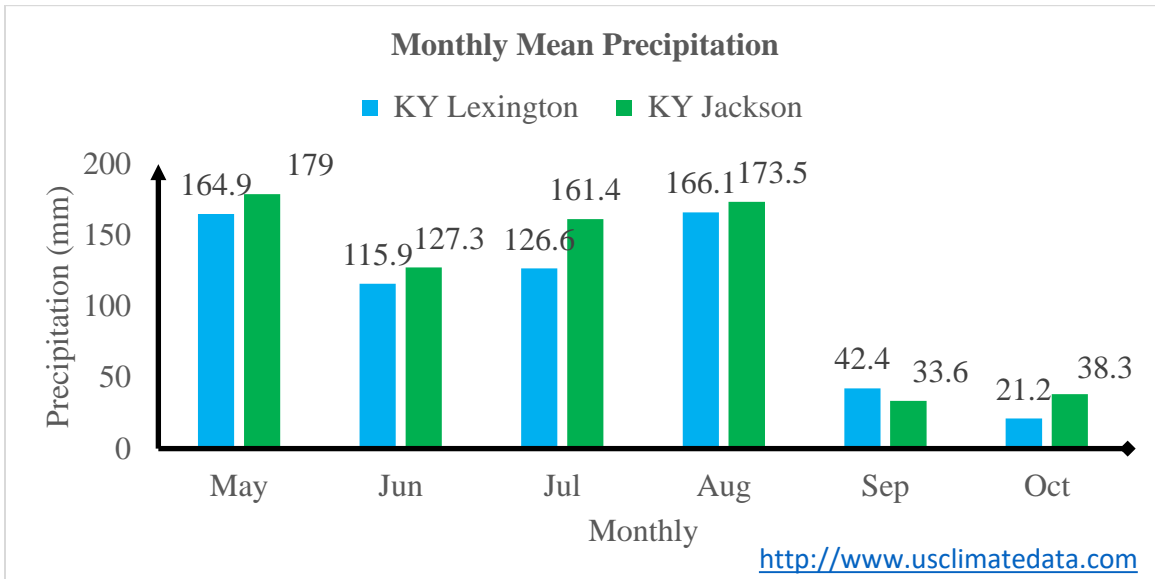
Figure 16-B: Average of monthly precipitation from May to October 2016.

The average precipitation at Quicksand Farm in Lexington was greater than at Spindletop Farm near Jackson, especially for June, July, and August. The flooding happened in the experiment area at Quicksand in July. The mean precipitation during the growing season was 106.2 mm at Spindletop Farm, and the average precipitation was 118.9 mm at Quicksand Farm.

Soil Type:

The soil type at Spindletop Farm is a Maury silt loam, classified as fine-silty, semi-active, mixed, mesic, and Typic Paleudalf (Yang et al., 2013). The Pope silt loam soil is a light brown silt loam on the surface. The underlain at 6 – 8 inches is yellowish-brown or yellow silt loam. The soil at depths ranging from15 – 20 inches is mottled yellow and gray heavy silt loam. (Baldwin et al., 1938). The soil type at Robinson Forest Substation Farm is a Pope silt loam, classified as fine or coarse-loamy, mixed, active, mesic, thermic, and Fluventic Dystrochrept (Fike et al., 2006; Makris, 2003). On the surface, the Maury silt loam is a rich-brown, mellow silt loam. The underlain at 12-15 inches is yellowish brown to buff- colored friable silty clay loam. The soil at depths ranging from 15 to 28 inches is buff-colored or slightly reddish yellow, moderately friable silty clay (Baldwin et al., 1938).

Flowering date

The planting date at Spindletop Farm on May 26, 2016 was earlier than at Quicksand Farm on June 2, 2016 and the flowering date was earlier at Spindletop than at Quicksand. The plants started flowering in August, but these plants exhibited different flowering ranges and mostly flowerd 70 days after the sowing date. Generally, the flowering time was faster at Spindletop than Quicksand because the average temperature at Spindletop was higher by 0.5°C in July and August (Figure 13-B). It was observed that one replicate line for treatment number 56 obtained an early flowering date at the end of July that was after 57 days after sowing at Spindletop Farm. This line was the fastest flowering line for the present experiment. The percentages of flowering dates for both locations were recorded in Appendix Chapter 3 Table 2

RESULTS

Agronomic results

The growing period from planting date to harvest time at Spindletop Farm and Quicksand Farm was 152 days. Quicksand had a higher seed yield than Spindletop, and the mean of grain yield at Spindletop was 152 kg/ha and at Quicksand was 302 kg/ha. No significant ($P > 0.05$) differences in seed yield between the sites were found. There was a significant difference in seed yield produced among treatments with P-value <.0001. An average of 1000-seed weights was significantly ($P < 0.05$) different between varieties. Spindletop had higher seed weight of 1.04 g per1000 seeds and Quicksand varieties had 0.96 g per 1000 seeds. There was significant (P<0.05) difference between seeds weight of the progeny from both sites. The Seed weight of each location was not significantly different (P- value = 0.0725). The Spindletop site provided lower seed weight at 13.98 g than the Quicksand at 27.67 g per meter length.

Seed composition

NIRS screening of the seed components exhibited the existence of oil and protein in the seed from two locations. Seeds content are displayed in the statistical output section. Different protein contents were found between selections from Spindletop and Quicksand. The percentage of oil from parents of chia seeds (34.9%) grown at Spindletop in 2015. All

samples in parents exceeded the average oil percentage (30.5%) produced by seeds grown at Spindletop and Quicksand in 2016. Protein analyses were significantly different between parents and progeny seeds (P- value <.0001). Overall, significant (P < 0.05) differences between locations were detected. Spindletop seeds had a significantly (P< 0.05) higher protein content (24.3%) than seeds from Quicksand site (22.4%), but selections show no significant (P< 0.05) differences in protein content between replications by locations. Oil content was significantly (P< 0.05) different for all of the comparative analysis performed between parents and progeny. Significant (P < 0.05) differences between locations were noticed. Spindletop site provided 29.8% and Quicksand obtained 30.8% oil content among treatments within selections. In general, there was no significant difference in percentages of oil content between replications by location due to P-value = 0.6, which is larger than 0.05 significant value.

DISCUSSION

Agronomic Study

Seed yields exhibited a large variation between selections and sites (P < 0.05). Quicksand had a higher average seed yield than Spindletop. The 302 kg/ha progeny yield at Quicksand was similar to the 317 kg/ha yield recorded for parents of the same varieties. Neither of the sites had a significantly (P<0.05) higher seed production than the other site, with Spindletop providing 152 kg/ha and Quicksand obtaining 302 kg/ha yields. Significant (P<0.05) difference in 1000-seed weights was found between two sites. This might be related to the environment effect of seed mass. The SAS output results showed that environmental variation between sites had less effect on seed weight because there was no significant difference between locations (P = 0.07). Ayerza (2009) reported the growing period of plants could be lengthened with increased elevation (Ayerza, 2009). However, the present study found the length of the growing period was 152 days at both locations and elevation did not show any effect on the growing period at both sites. According to Coates variances in seed yields among varieties might be a result of a mixture of factors including environmental, genetics, seeding dates, row spacing, agronomic practices and their interactions (Coates, 1996). There was significant difference in seeds weight, 1000 seeds weight, and seeds yield among the varieties. The genotype factor did not appear to have large effect among replications, locations, and treatments. The length of the growing periods from planting to harvesting was 152 days at both sites (at Spindletop Fram from May 26, 2016, to October 24, 2016 and Quicksand Farm from June 1, 2016, to October 31, 2016). Row spacing was 0.91 meter for all experiments, and agronomic practices (fertilizer and herbicide) were the same at both locations. All these factors had no effect on seeds yield. However, the environmental factors, such as temperature, rainfall, and soil type, could appear to have an effect on seeds yields. Ayerza and Coates (2009) reported The high temperatures could result in a reduction in yield, decreased seed set and rate of photosynthesis (Ayerza and Coates, 2009). Spindletop Farm was warmer than Quicksand by 0.5°C in July and August (Figure 14-B). Avrage rainfall (Figure 14-A) was greaterat Quicksand than Spindletop due to flooding that was recorded in the experiment plots at Quicksand farm in late July and the beginning of August 2016. Difference between mean temperature was 0.2°C and between mean perceptaion was 12.7 mm during the growing

season. The diffrence between the averages might affect the yield production because the Quicksand had higher yield with lower temperature during the growing period.

The soil type at Spindletop was Maury silt loam, and at Quicksand was Pope silt loam. Chia was reported to grow better in sandy soil, which is well-drained soils with moderate salinity. The sandy soil has pH ranging from 6 to 8.5, which is favored by chia plants (Yeboah et al., 2014). Different soil types could impact the seeds yield because they have diffrent level of nutrients such as nitrogen. According to Bochicchio et al. (2015) added organic fertilization (nitrogen) did not improve the seeds yield and the lodging incidence was higher (Bochicchio et al., 2015). Makris (2003) reported the Pope and Maury soils have a similar particle size and the distribution of silt, clay, and sand is 81%, 13%, and 6% respectively for both soil types. The Maury soil had higher pH and higher levels of phosphatic limestone than the Pope soil. However, the Pope soil had greater organic matter (OM) content of 5.5% than the Maury with 4.5% (Makris, 2003). The soil type and nitrogen availability could impact the yield produced. At Quicksand, there was more vegetative growth and taller stem growth of between 1.5-2 meter length. At Spindletop stem growth was approximately 1-meter length.

Seed composition:

Overall the parents and progeny selections showed significant (P<0.05) difference in protein content. Progeny protein content within each site was 5 and 15% more than the parent's protein content. Significant (P< 0.05) differences were detected between Spindletop Farm and Quicksand Farm and Spindletop seed had the higher protein content of 24.35%, than at Quicksand of 22.42% and at both locations progeny protein were higher than parent protein by 21.35%. Chia seeds among varieties showed a range of protein content between 28.5% to 20.7%. These variations in protein content were reported with numerous crops including sorghum (Saeed et al., 1987), soybean (J Vollmann et al., 2000) and chia (Ayerza and Coates, 2004). The variances might be an outcome of genetics alone or interaction of genetics x environment. Higher temperatures could significantly affect chia seed content as was verified for other seed oil crops such as soybean (Thomas et al., 2003). The higher content of protein detected might be relevant to the temperature difference between locations. The recorded mean temperature over the growth periods of

the crop cycle was 22.2°C at Spindletop, which was slightly higher than Quicksand at 22.0°C. The temperature was 0.5% higher in August and September at Spindletop. It was reported that the protein of chia seed increased as the temperature increased and altitude decreased (Ayerza and Coates, 2004). However, Spindletop has a higher altitude than Quicksand but the recoreded temperature was warmer. Coates (2011) reported that in wide-ranging chia plants, protein content tended to reduce as altitude rose. (Coates, 2011). In the present study Spindletop Farm had higher protein content, higher altitude, and higher temperature than Quicksand. In general, these differences in environmental factors probably had not affected protein content. The elevation of both sites was different. Spindletop is 282 m above sea level, and Quicksand is 226 m above sea level.It is reported that ecosystem has a strong influence on the protein content of chia seeds and many other crops (Saeed et al., 1987; J Vollmann et al., 2000). There is a positive impact between protein content and temperature in oilseed crops like soybean (Coates, 2011; Kumar et al., 2006; Thomas et al., 2003). Although the negative relationship between altitude and temperature is often mollified by some other factors, usually air temperature declines 1°C per 160 meter increase (Miller, 1975). The negative relationship between protein content and elevation might be explained by the cause-effect relationship (Coates, 2011). Elevation above sea level was negatively related to protein content and positively related to oil content. The growing cycle length was reported to have a positive relationship with elevation (Ayerza, 2009). However, in the present study, the latitude was similar between locations and the growing cycle length was the same in both locations (152 days). Therefore, the protein contents were not affected by elevation. The difference in protein content cannot be fully explained by the environment conditions (Ayerza and Coates, 2004). Although Quicksand had higher rainfall and lower temperature, Spindletop appears better suited for the production of high-protein chia.

Oil content showed significant ($P<0.05$) difference between parents and progeny. The parents had 34.9% higher oil than progeny in both locations. The progeny seeds provided 29.8% oil content at Spindletop and 30.8% at Quicksand. The cool temperature can affect increased oil content by incrased unsaturated fatty acids and reduced protein content for chia and other oilseeds. The different seeds oil contents between locations were probably because of the impact of one or more factors of the environment. The seed protein

and oil quantity and quality might be influence by temperature, light, soil type, and nutrition (Ayerza, 2009). The seeds at both locations and among varieties varied in oil content. The oil content of progeny seeds on both sites ranged from 27.03 to 35.59% , which was lower than the range of parents oil content from 30.54 to 39.63% among varieties that were planted in 2015 at Spindletop Farm. The different oil contents are probably associated with environment conditions, as reported for other crops. Some reportes estimated the oil content could be affected by temperatures. High temperatures reduce oil content, while low temperatures increase oil content (Ayerza, 2001; Ayerza and Coates, 2009; Cherry et al., 1985; Yaniv et al., 1995). However, in this study there was no significant correlation between oil content and temperature. Commonly, there is an inverse relationship between temperature, altitude and oil content. As the altitude decreases, temperature increases (Ayerza, 2010; Thomas et al., 2003). The percentage of oil content of some crops, including sorghum, soybean, chia, and others, tended to decrease as temperature increased (Ayerza, 2009; Boschin et al., 2007). Other studies established that the chemical component of chia oil is affected by different factors like the fertility of the soil, climatic change and environment conditions. The seed composition was investigated for other oilseed crops and it has a direct relationship with soil pH. The seed composition influence could have contributed oil content and protein simultaneously with other factors. Location generally affected chia's protein and oil contents during the growing period and reduced seed yields. Generally, oil and protein contents had small variations between the replications at each location. The current study has been extended by two years at both locations to confirm the results and to further evaluate the level of heritability of oil and protein content and yield.

LIMITATION:

In the current study, there were some limitations that affected seeds yield and oil and protein content. The Spindletop Farm had many weeds that competed with the plants. This had a negative effect on seed yield, and the plants exhibited short stems and less vegetative growth. Another factor that had a negative impact on yield at Spindletop Farm was high wind during seed maturation. The chia yield was also affected by shattering and some seeds were lost after maturity because not all varieties mature at the same time. We

needed to wait for all the seeds to dry, and that increased the risk of losing seeds from varieties that matured early due to wind and rain. The head of the flower matures and dries before the side branches, which stay green for a while. The period between when the head matures and when the rest of the plant matures is considered a significant risk for losing the yield due to high wind, rainfall, frost and other environmental effects. Quicksand Farm's yield faced other factors, such as flooding during the growing season in the experiment area at the end of July and beginning of August during the vegetative stage of the chia plant growth. The other factor at Quicksand was lodging due to plant stem length, high wind and heavy rainfall. The long stem length was recorded as more than 1.5 meters, which could be due to the increased level of nitrogen. This can increase vegetative growth and lead to crowding of the plants.

CONCLUSION

The location can impact photoperiod, protein and oil content, and yield kg/ha of chia genotypes. The M5 progeny seeds from plants grown at Spindletop and Quicksand contained significantly ($P < 0.05$) more protein than M4 seeds from the parents. There were significant differences ($P < 0.05$) between locations and higher oil content for progeny seeds at Quicksand than at Spindletop. There was no significant difference between M4 parents seeds and M5 progeny seeds and higher yield kg/ha at Quicksand than at Spindletop. Location affected the content of the chia seeds, probably due to environmental factors, such as temperature, light, soil type and available nutrients. The environment condition data cannot fully explain the difference in protein and oil contents. However, chia at Spindletop appears better suited for the production of high protein, but higher oil content was found at Quicksand. Chia seeds produced at both sites provided higher protein and lower oil content than seeds from parents. The impact of genotype is more apparent on seed yield than protein and oil content. The data in this study does not match other research that claimed high temperatures decreased oil content and increased protein. Location mainly affected protein and oil contents of chia seeds and did not effect the length of chia's growing period or seed yield. This study found large differences in protein content at Spindletop and higher oil content at Quicksand for progeny. These differences may be due to environmental differences between the two locations . Temperature seemed to be a weak influence on oil quality, and there was no significant relationship between elevation and protein content.

The present study will be completed after adding one more year of planting at both locations. The extension of the study can be essential to verify the outcome and to understand the biochemical principles for this experiment.

STATISTICAL OUTPUT

Progeny oil% in two locations and parents oil%
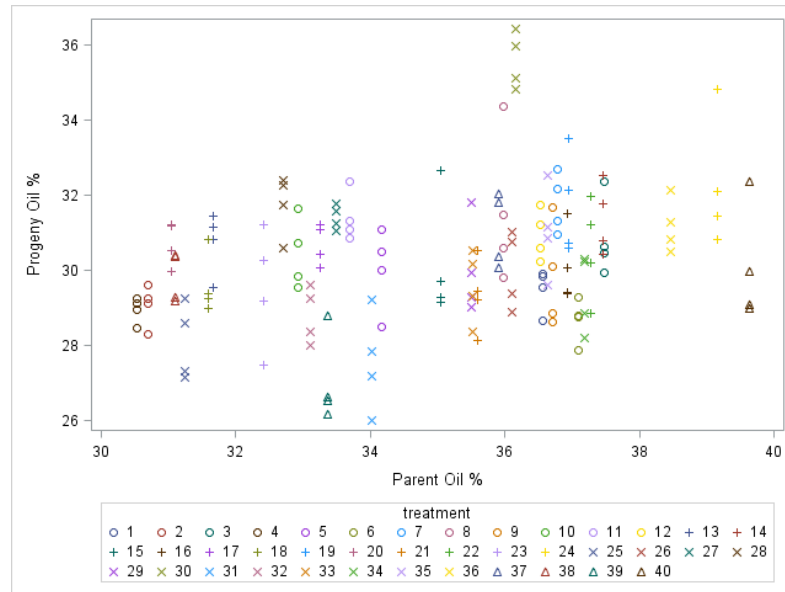
Figure 17 -A: scatter diagram between progeny and parents oil%.

Consider the scatter plot above, which shows a correlation between the mean of progeny oil percent in both locations (Spindletop and quicksand) and mean of parents' oil percent among forty treatments.

Table 8-A : The GLM Procedure Dependent Variable: Progeny Oil %

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	374.48	8.92	11.17	<.0001
Error	117	93.38	0.80		
Corrected Total	159	467.86			

R-Square	Coeff Var	Root MSE	Mean of Progeny oil%
0.80	2.95	0.89	30.31

Table 8-A (continued).

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Parents oil%	1	38.12	38.12	47.76	<.0001
Location	1	42.17	42.17	102.60	0.01
Replication(location)	2	0.82	0.41	0.52	0.60
Treatment	38	293.37	7.72	9.67	<.0001

The GLM table via SAS output shows:

1. There were significant differences in percentages of oil content between parents and progeny P- value <.0001 less than 0.05 and R-square = 0.8, that means the model explains 80% the variability of the response data around its mean.
2. There was not significantly different in percentages of oil content between replication by location due to P-value = 0.6, larger than 0.05 significant value.
3. There were significant differences in percentages of oil content among treatments.
4. There were significant differences in percentages of oil content between locations, as we can see in boxplot below:
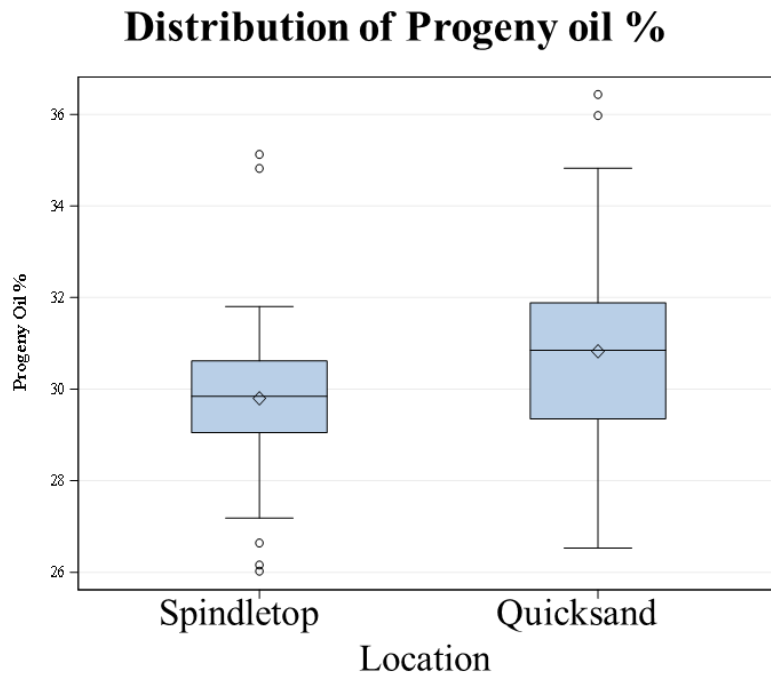
Figure 17 -B: Distribution of progeny oil% between locations.

- Percentages of oil contents between locations p-value = 0.01 less than 0.05. The mean of percentages of oil contents for progeny seeds at Spindletop farm provided 29.8; this percentage was less than Quicksand farm that provided 30.8 (was higher).
- The progeny seeds from plants grown in Spindletop and Quicksand contained significantly ($P < 0.05$) less percentages of oil than did seeds from parents.

Table 8-B: Mean of Progeny and Parents Oil%

Level of location	N	Progeny oil%		Parents oil%	
		Mean	Std Dev	Mean	Std Dev
Spindletop	80	29.80	1.50	34.94	2.47
Quicksand	80	30.83	1.77	34.94	2.47

.
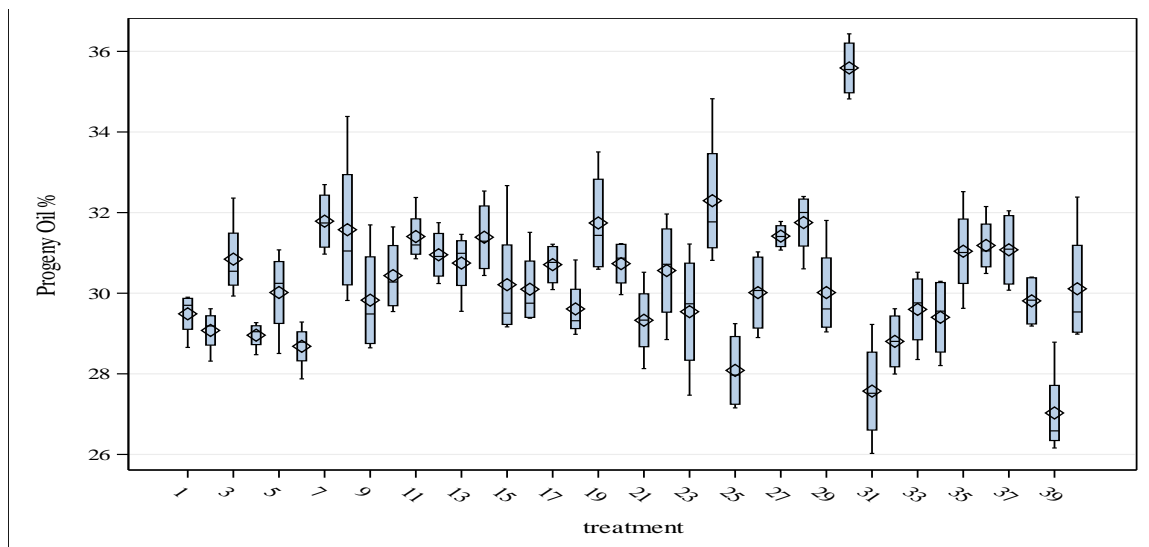
Figure 17-C: Distribution mean of progeny oil% seeds among treatments

- The distribution of progeny oil seeds among treatments significantly different in percentages of oil seeds content among treatments, p- value <.0001 less than (0.05). The table below shows the amount of progeny oil% content in seeds.

Progeny protein% in two locations and parents protein%
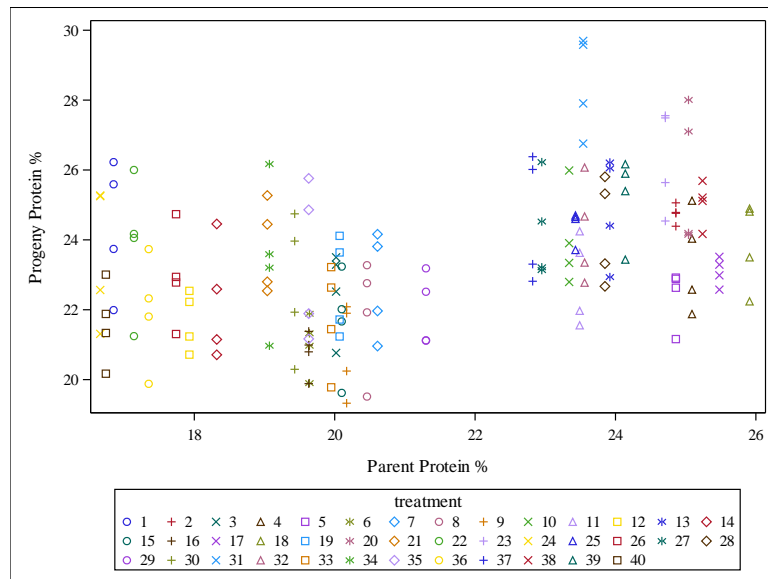
Figure 18-A : scatter diagram between progeny and parents' protein%.

The scatter plot above, which shows a correlation between the mean of progeny protein percent in both locations (Spindletop and quicksand) and mean of parents' protein percent among forty treatments.

Table 9-A : The GLM Procedure Dependent Variable Progeny Protein %

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	549.64	13.09	15.03	<.0001
Error	117	101.90	0.87		
Corrected Total	159	651.55			

R-Square	Coeff Var	Root MSE	Mean of progeny protein%
0.843596	3.99	0.93	23.39

Table 9-A (continued).

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Parents Protein%	1	109.90	109.90	126.18	<.0001
Location	1	148.71	148.71	154.69	0.0064
Replication (location)	2	1.92	0.96	1.10	0.335
treatment	38	289.11	7.61	8.74	<.0001

The GLM table via SAS output shows:

1. There were significant differences in percentages of protein content between parents and progeny P- value <.0001 less than 0.05 and R-square = 0.84, that means the model explains 84% the variability of the response data around its mean.

2. There were not significantly different in percentages of protein content between replication by location due to P-value = 0.33, larger than 0.05 significant value.

3. There were significant differences in percentages of protein content among treatments, P-value <.0001.

4. There were significant differences in percentages of protein content between locations, P-value = 0.006 as we can see in boxplot below:
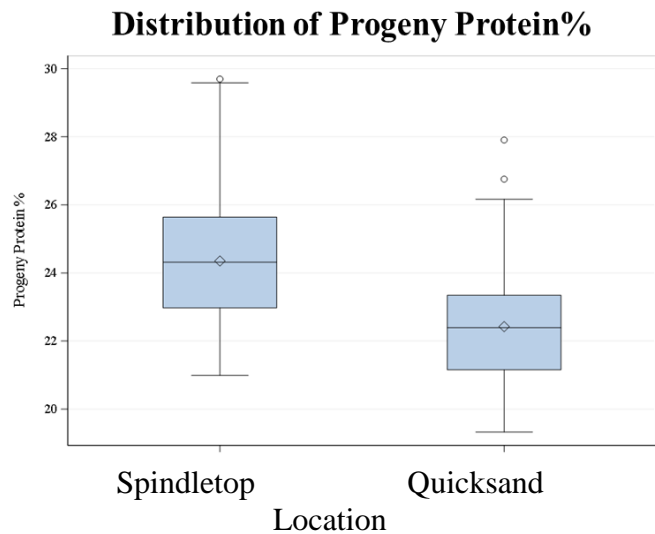
Figure 18-B: Distribution of progeny protein% between locations.

- Percentages of protein contents between locations P-value= 0.0064 were less than 0.05. The progeny mean of protein% contents seeds at Spindletop farm provided 24.4% and an average of protein% content seeds at Quicksand farm provided 22.4% (was less).

- The progeny seeds from plants grown in Spindletop and Quicksand contained significantly ($P < 0.05$) more amount of protein% than did seeds from parents.

Table 9-B: Mean Progeny and Parents Protein %

Level of location	N	Progeny Protein %		Parents protein %	
		Mean	Std Dev	Mean	Std Dev
Spindletop	80	24.35	1.81	21.35	2.88
Quicksand	80	22.42	1.76	21.35	2.88
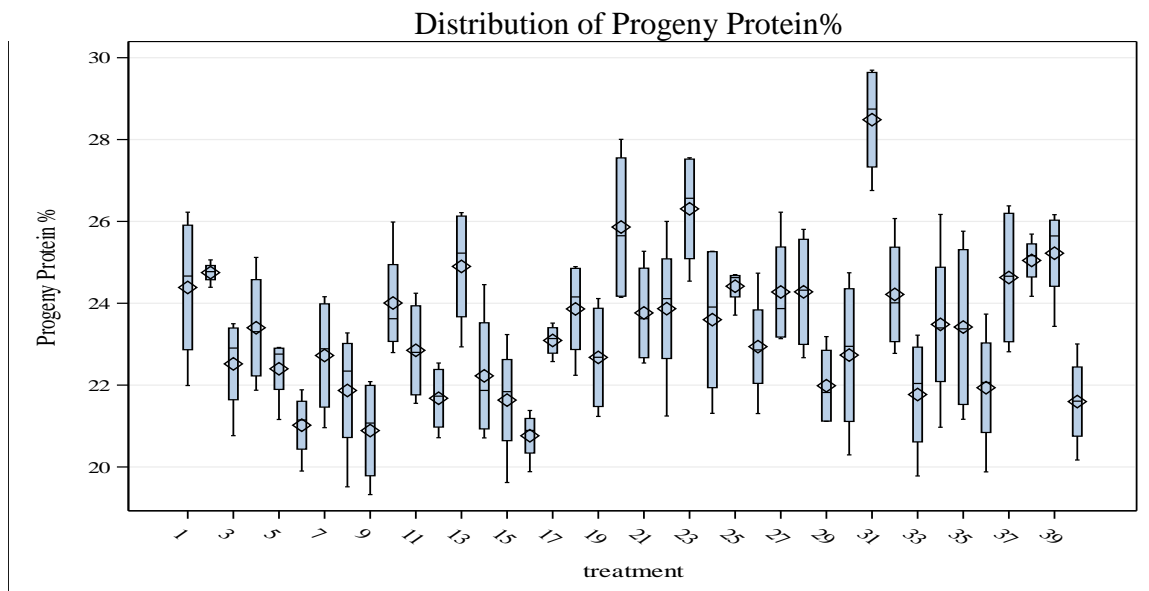
Figure 18-C: Distribution of mean of progeny protein % seeds among treatments.

- The Distribution of progeny protein seeds among treatments were significant differences in percentages of protein seeds content among treatments, p- value = <.0001 less than P< 0.05. The table below shows the amount of progeny protein% content in seeds.

Progeny yield kg/ha in two locations and parents yield kg/ha
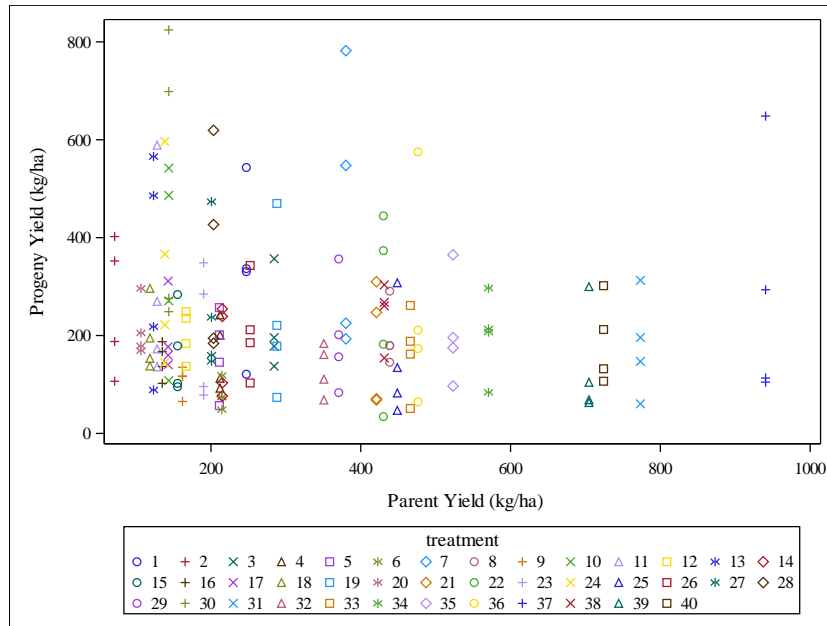
Figure 19-A: scatter diagram between progeny and parents' yield kg/ha

- The scatter plot above, which shows a correlation between the mean of progeny yield kg/ha in both locations (Spindletop and quicksand) and mean of parents' yield kg/ha among forty treatments.

Table 10-A: The GLM Procedure Dependent Variable Progeny yield kg/ha

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	2259088.43	53787.82	4.38	<.0001
Error	117	1436394.56	12276.88		
Corrected Total	159	3695483.00			

R-Square	Coeff Var	Root MSE	Mean of Progeny kg/ha
0.61	48.65	110.80	227.77

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Parent yield kg/ ha	1	30387.103	30387.10	2.48	0.1184
Location	1	896999.51	896999.50	12.31	0.0725
Replication (location)	2	145731.77	72865.89	5.94	0.0035
Treatment	38	1185970.05	31209.74	2.54	<.0001

The GLM table via SAS output shows:

1. There were not significantly different in yield produced between parents and progeny P- value = 0.12 more than 0.05 and R-square = 0.61, that means the model explains 61% the variability of the response data around its mean.There was significantly different in yield produced between replication by location due to P-value = 0.0035, less than 0.05 significant value.

2. There were significant different in yield produced among treatments, P-value <.0001.

3. There was not significantly different in yield produced between locations, p-value = 0.0725 slightly close to 0.05 significant value. As we can see in boxplot below:
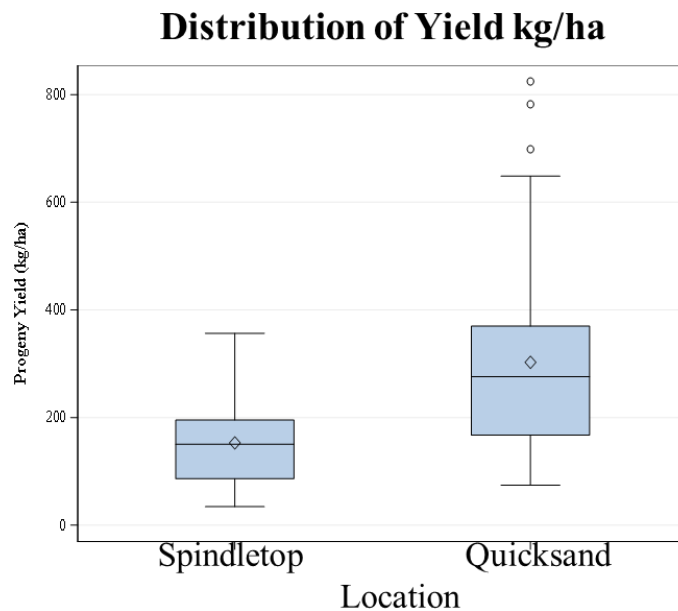
Figure 19-B: Distribution of progeny yield kg/ ha between locations.

- Amount yield produced between locations p-value = 0.0725 more than 0.05 significant value. The mean of yield kg/ha for progeny seeds at Spindletop farm provided 152.9 kg/ha and at Quicksand farm that provided 302.6. The plant produced at Quicksand higher yield approximately 50% more yield than Spindletop.

- The progeny seeds from plants grown in Spindletop and Quicksand contained not significantly different due to P- value = 0.12, more than 0.05. Progeny seeds produced in both location was less than seeds yield from parents with at 317.6 kg/ ha.

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- But on other case progeny mean in Quicksand was close to mean of parents with 15 kg/ha differences.

Table 10-B : Mean of Progeny and Parents Yield kg/ha

Level of location	N	Progeny Yield kg/ha		Parents Yield kg/ha	
		Mean	Std Dev	Mean	Std Dev
Spindletop	80	152.89	73.79	317.63	206.63
Quicksand	80	302.64	173.15	317.63	206.63
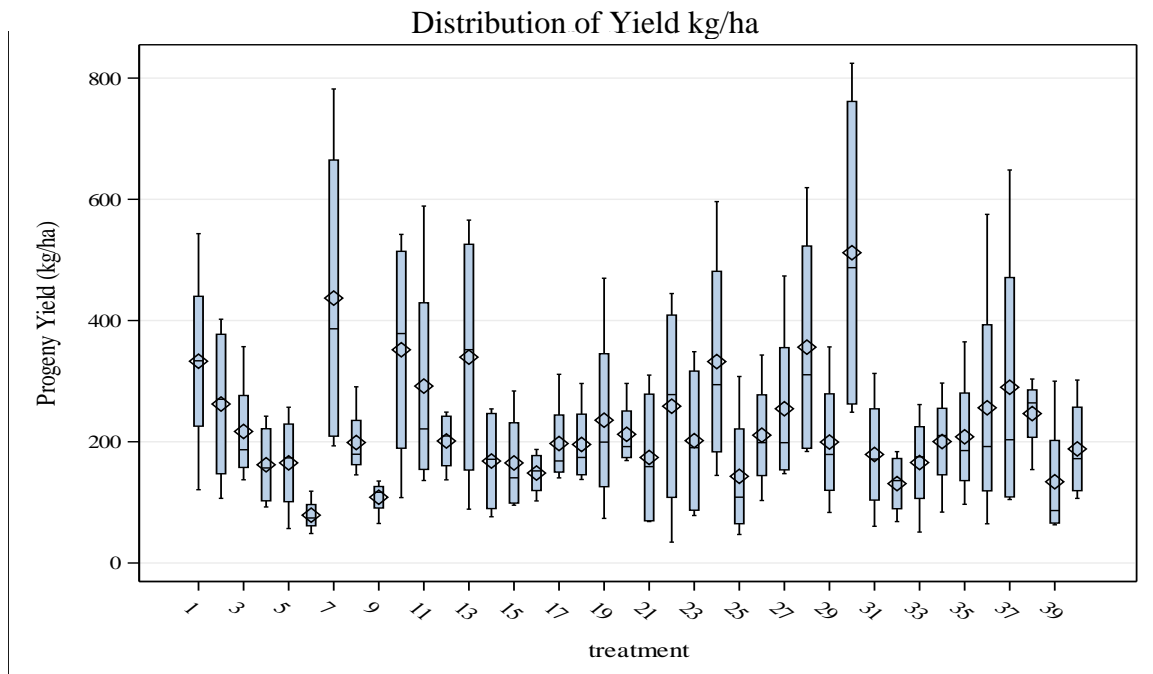
Figure 19-C: Distribution mean of progeny yield among treatments.

- The Distribution of progeny yield among treatments significantly different among treatments, p- value <.0001 less than (0.05).

Progeny 1000 seed weight (g) in two locations

Table 11-A: The GLM Procedure Dependent Variable: 1000 seed weight (g) (WKS)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	0.87	0.021	9.56	<.0001
Error	117	0.25	0.002		
Corrected Total	159	1.13			

R-Square	Coeff Var	Root MSE	WKS Mean
0.774353	4.66	0.05	0.99

Source	DF	Type I SS	Mean Square	F Value	Pr > F
location	1	0.224	0.224	166.08	0.006
rep(location)	2	0.003	0.001	0.62	0.539
treatment	39	0.645	0.017	7.62	<.0001

The GLM table via SAS output shows:

1. R-square of 1000 seed weight (g)= 0.77, that means the model explains 77% the variability of the response data around its mean..

2. There was not significantly different in 1000 seed weight (g) between replication by location due to P-value = 0.5, larger than 0.05 significant value.

3. There were significant differences in 1000 seed weight (g) among treatments. The P-value <.0001 less than 0.05 significant value.

4. There were significant differences in 1000 seed weight (g) between locations, as we can see in boxplot below:
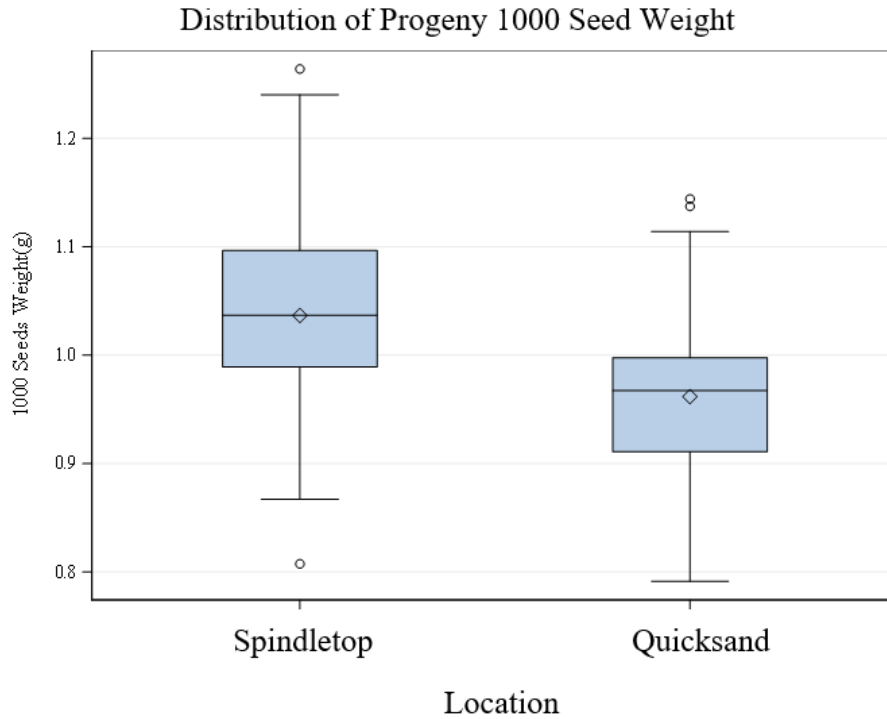
Figure 20: Distribution of progeny 1000 seeds weight (g) between locations.

- Amount of 1000 seed weight (g) between locations p-value = 0.006 less than 0.05 significant value. The mean of 1000 seed weight (g) for progeny seeds at Spindletop farm provided 1.03 g and at Quicksand farm that provided 0.9. The plant produced amount of 1000 seed weight (g) at Quicksand less than Spindletop.

Table 11-B : Mean of Progeny and Parents for 1000-Seed Weight g

Level of Location	N	1000 Seed Weight (g)	
		Mean	Std Dev
Spindletop	80	1.04	0.08
Quicksand	80	0.96	0.07

CHAPTER 4: Evaluating Heritability of Omega-3 Content in Chia Seeds Among Segregating Progeny from High x Normal Segregating Population.

In *Salvia hispanica* L., numerous alternatives in qualitative characteristics, such as the color of the seed coat, stem pigmentation, and shattering, have previously been changed with a crossing between varieties. Effects of segregating generations of chia plant from crosses between different varieties could be changes in components of oil composition, mainly α-linolenic (omega -3), acid content. The composition of chia oil is mainly α-linolenic, linoleic, oleic, stearic and palmitic acids. Predominantly, α-linolenic acid ranges from 54.2% to 64.2% of the oil content (Ayerza and Coates, 2004). Chia seeds consist of 39% oil, which has the highest known content of α-linolenic acid, up to 68% (Ayerza, 1995) compared with 57% in flax seeds. Chia seeds have a long shelf life compared to other linolenic (omega -3) sources such as flaxseed or marine products since they don't show any of the problems associated with other sources such as fishy flavor, animal weight loss and digestive problems (Ayerza and Coates, 2004).

We expect the outcome of the current research to show the heritability in the fatty acid composition after crossing among a mutagenize variety in our area with other varieties growing in different reigns and various growth conditions known to have a high ratio of alpha-linolenic acid. This study was conducted in the field, greenhouse, and lab. The seeds have been provided by Dr. Tim Phillips Department of Plant and Soil Science at the University of Kentucky. The F1 parent (sbc) seed (s-long day, b-white flowers, c-white seed) came from the crossing of the G8 plant mother (sBC, long day, blue flowers, charcoal seed) grown in Kentucky (short length period) and Salba plant father (Sbc, short day, white flowers, white seed) produced in Peru (Long length period). F2 single plant came from crossing between mother F1 (sbc) and father (Mi Costenita) high omega 3 variety that planted in a greenhouse at the Spindletop Farm. 210 F2 seeds were planted in the greenhouse with the mother (sbc) as a control and were transplanted into the field at Spindletop Farm. From 210 plants, 52 flowered and rest did not produce flowers; 37% had white flowers, and 63% had blue flowers. From these 52 flowering plants, 19 were selected and planted in the greenhouse with six replications, eleven high α- linolenic acid and eight low α- linolenic acid, along with three verities planted in the same greenhouse include (Salba X G8 2016), G8 2015 and Salba 2015 as a control. The grandparents were planted

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in the summer of 2015 in the greenhouse, and F1 seeds were planted in end of May and transplanted in the last week of June 2016 and harvested on November 27, 2016. Subsample F2 seeds with three controls (grandparent and parents) were planted in the greenhouse on January 20, 2017. F2 and F3 subsample seeds were measured for oil and protein content characteristics determined using NIRS. Fatty acid composition was determined by GC-FID analysis. The data collected will be subjected to Statistical Analysis System (SAS). In what way is the fatty acids profile, mainly alpha-linolenic acid (omega-3) fatty acids, altered by segregating generations and hand-crossing method.

APPENDIX CHAPTER 2

Table 1-A: The GLM procedure using contrast with three levels (High, Medium, Low)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	410.70	31.59	16.83	<.0001
Error	25	46.94	1.88		
Corrected Total	38	457.64			

R-Square	Coeff Var	Root MSE	Oil_Percent Mean
0.897432	6.74	1.37	20.34

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Replication	4	4.91	1.23	0.65	0.63
Sample	9	357.0937929	39.68	21.13	<.0001

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
High vs Low	1	325.22	325.22	173.21	<.0001
Mid vs Low	1	143.82	143.82	76.60	<.0001
Mid vs High	1	74.38	74.38	39.62	<.0001

Parameter	Estimate	Standard Error	95% Confidence Limits	
High vs Low	24.53	1.86	20.69	28.37
Mid vs Low	18.56	2.12	14.19	22.93
Mid vs High	15.39	2.44	10.35	20.42

Table 1-B: Mean of 10 bias samples with the same letter are not significantly different.

Tukey Grouping		Mean	N	Sample
	A	25.283	3	VGD1_2R3
B	A	24.840	3	VGD1_1R2
B	A	24.607	3	VGD1_1R3
B	C	21.777	3	JACK_R1
B	C	21.483	3	JACK_R2

Table 1-B (continued)

Tukey Grouping		Mean	N	Sample
B	C	21.382	5	JACK_R3
D	C	19.848	5	VGD1_2R2
D	E	17.875	4	VC_R2
D	E	16.526	5	VC_R3
	E	15.794	5	VC_R1

Table 2-A: The percentages of oil recovered from chia, G8, and soybean, Jack seeds with different solvents.

Sample	Solvent type	Moisture %	Cycle/ min	% oil Recovered	S.E.	Temp . °C
Chia, G8	Acetone	9.3	15	30.2	2.0	160
Chia, G8	Petroleum Ether	9.3	7	29.6	0.8	160
Chia, G8	Acetone	9.3	18	30.7	2.0	150
Chia, G8	Petroleum Ether	9.3	9	30.1	0.8	150
Soybean, Jack	Acetone	9.3	18	28.6	0.9	120
Soybean, Jack	Petroleum Ether	9.3	10	23.9	0.5	120
Soybean, Jack	Acetone	9.3	18	24.1	0.5	120
Soybean, Jack	Petroleum Ether	9.3	9	25.6	1.3	120

Table 2-B: The SAS output by The GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.656282	7.16	1.99	27.83

Source	DF	Type III SS	Mean Square	F Value	Pr> F
Sample	1	85.10	85.10	21.43	0.0006
Solvent	1	4.91	4.90	1.24	0.28
Sample*Solvent	1	0.97	0.97	0.24	0.63

Table 3-A: The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.99	2.93	0.73	25.07

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Sample	2	661.29	330.65	612.31	<.0001
Solvent	1	1.73	1.73	3.20	0.097
Sample*Solvent	2	1.64	0.82	1.51	0.26

Table 3-B: The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.73	3.86	0.81	20.92

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Sample	1	14.19	14.19	21.78	0.0012
Moisture	1	0.54	0.54	0.83	0.386
Sample*Moisture	1	0.05	0.05	0.07	0.794

Table 4-A: Percentage of total lipid extraction, among three different methods

No	Samples	Method Name	Oil% Soxhlet	Moisture %	Oil %	SE
1	Chia_G8	Folch protocol	32.7	9.3	30.39	3.5
2	Chia_G8	Bligh & Dyer	32.7	9.3	24.84	1.47
3	Chia_G8	Hexane:isopropanol	32.7	9.3	23.41	0.76
4	Soybean_Jack	Folch	21.4	9.35	20.66	0.30
5	Soybean_Jack	Bligh & Dyer	21.4	9.35	19.01	0.66
6	Soybean_Jack	Hexane:isopropanol	21.4	9.35	14.08	0.43
7	Chia, G8	Folch protocol	32.7	9.27	29.52	0.64
8	Chia, G8	Bligh & Dyer	32.7	9.27	22.40	1.14
9	Chia, G8	Hexane:isopropanol	32.7	9.27	19.79	0.50
	Chia_G8	Folch protocol	32.7	10.6	32.17	0.42

Table 4-A (continued).

No	Samples	Method Name	Oil% Soxhlet	Moisture %	Oil %	SE
	JACK_R3	Folch protocol	21.4	10.8	16.60	0.28
	VGD1_2 R3	Folch protocol	25.3	10.9	19.31	0.54

Table 4-B: The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.97	5.32	1.26	23.67

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	3	437.6	145.87	92.02	<.0001
Sample	1	319.11	319.11	201.32	<.0001
Method*Sample	3	48.25	16.08	10.15	0.0003

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs Bli_Dyer	1	258.30	258.30	13.96	0.001
Soxhlet vs Folch	1	60.19	60.19	3.25	0.084
Soxhlet vs HIP	1	539.54	539.54	29.15	<.0001

Table 4-C: The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.98	3.86	0.99	25.73

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	57.00	57.00	57.82	<.0001
Sample	2	568.64	284.32	288.38	<.0001
Method*Sample	2	23.76	11.88	12.05	0.0009

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs Folch	1	79.02	79.02	2.20	0.1552

Table 5: HCH The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.94	6.018	1.51	25.05

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	104.84	104.84	46.13	<.0001
Sample	2	359.82	179.98	79.16	<.0001
Method*Sample	2	15.28	7.64	3.36	0.059

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Method
A	27.03	14	Soxhlet
B	21.97	9	HCH

Table 6-A: Direct Transesterification with four varities (soybean, JACK_R3, VGD1_2 R3 and Chia_G8)

Samples	Heat 100°C time min.	Moisture %	Soxhlet Oil%	DM Oil%	SE	Differ Soxhlet
Jack B2	10 min	2.29	21.1	15.93	0.52	5.17
Chia_G8	10 min	9.27	32.7	24.64	2.23	8.06
JACK_R3	10 min	10.1	21.4	17.44	0.99	3.96
VGD1_2 R3	10 min	9.4	25.3	15.85	0.91	9.45
Jack B2	30 min	2.29	21.1	20.74	1.23	0.36
Chia_G8	30 min	9.27	32.7	25.77	0.76	6.93
JACK_R3	30 min	10.1	21.4	19.24	0.56	2.16
VGD1_2 R3	30 min	9.4	25.3	19.91	1.65	5.39
Chia_G8	30 min	1.1	32.7	29.5	0.8	3.2
JACK_R3	30 min	2.4	21.4	20.2	0.55	1.2
VGD1_2 R3	30 min	3.2	25.3	23.2	0.62	2.1

Table 6-B: The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.91	7.98	1.95	24.43

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	229.86	114.93	30.21	<.0001
Sample	2	584.07	292.03	76.76	<.0001
Method*Sample	4	34.74	8.68	2.28	0.0887

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxh_Ben vs DT_LMC	1	22.04	22.04	4.92	0.0345
Soxh_Ben vs DT_MMC	1	236.22	236.22	52.76	<.0001
DT_LMC vs DT_MMC	1	236.22	236.22	52.76	<.0001

Means with the same letter are not significantly different.			
Scheffe Grouping	**Mean**	**N**	**Method**
A	27.03	14	Soxh_Ben
B	24.51	12	DT_LMC
C	19.76	8	DT_MMC

Table 7-A: DDTG protocol with two different techniques and four different amount of seeds mass.

Methods	Seed mass	Soxhlet Oil%	MC%	DDTG	S.E
Chia G8, Sonication	10 mg	32.7	9.3	21.5	0.8
	20 mg	32.7	9.3	23.1	0.8
	50 mg	32.7	9.3	23.8	1.1
	100 mg	32.7	9.3	22.3	0.8
Chia G8, Stir bar	10 mg	32.7	9.3	18.0	0.7
	20 mg	32.7	9.3	22.6	2.0
	50 mg	32.7	9.3	21.4	0.5
	100 mg	32.7	9.3	19.6	0.3

Table 7.A (continued).

Methods	Seed mass	Soxhlet Oil%	MC%	DDTG	S.E
Soybean Jack Sonication	10 mg	22.3	7.1	19.5	1.8
	20 mg	22.3	7.1	15.8	0.6
	50 mg	22.3	7.1	13.9	0.5
	100 mg	22.3	7.1	14.9	0.1
Soybean Jack stir bar	10 mg	22.3	7.1	17.4	0.7
	20 mg	22.3	7.1	16.4	1.0
	50 mg	22.3	7.1	14.3	0.1
	100 mg	22.3	7.1	13.9	0.5

Table 7-B: The SAS output by The GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.83	10.04	1.87	18.65

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	23.66	23.66	6.75	0.0140
Sample	3	22.88	7.63	2.18	0.1101
Amount of seeds	1	400.79	400.79	114.35	<.0001
Method*Sample	3	13.04	4.35	1.24	0.3113
Method*amount	1	9.28	9.28	2.65	0.1136
Sample*amount	3	86.27	28.76	8.21	0.0003
Method*Sample*amount	3	1.194	0.40	0.11	0.9515

Means with the same letter are not significantly different.			
Scheffe Grouping	**Mean**	**N**	**Sample**
A	19.48	12	20
A	19.09	12	10
A	18.37	12	50
A	17.68	12	100

Table 8: DDTG protocol with two varieties chia, G8 and soybean, Jack.

Sample	Soxhlet Oil%	MC%	DDTG Oil%	S.E
Chia, G8,Stir bar	32.7	9.3	10.8	0.8
Soybean, Jack Stir bar	22.3	7.1	8.1	0.3
Chia, G8, Sonication	32.7	9.3	11.4	0.8
Soybean, Jack Sonication	22.3	7.1	9.0	0.1

Table 9-A: DDTG protocol with two varieties soybean, Jack and VgD1

Sample	Soxhlet Oil%	MC%	DDTG	SE
Soybean, VgD1 Stir bar	25.3	6.8	22.5	0.3
Soybean, Jack, Stir bar	21.4	7.1	18.5	0.8
Soybean, VgD1 Sonication	25.3	6.8	21.5	0.5
Soybean, Jack Sonication	21.4	7.1	19.7	1.1

Table 9-B: The SAS output by the GLM procedure.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	7.43	3.72	2.39	0.1283
Sample	1	52.73	52.73	33.86	<.0001
Method*Sample	2	5.38	2.69	1.73	0.2135

Table 10-A: DDTG protocol with different incubate time for soybean, VgD1 and Jack1.

Sample/ Incubate time	Soxhlet Oil%	MC%	DDTG Oil%	SE
Stir bar 10 min. Soybean, VgD1	25.3	6.80	24.5	0.8
Stir bar 10 min. Soybean, Jack	21.4	7.10	23.2	0.4
Stir bar 20 min. Soybean, VgD1	25.7	6.8	23.2	1.2
Stir bar 20 min. Soybean, Jack	21.3	7.1	21.1	0.7
Stir bar 40 min. Soybean, VgD1	25.7	6.8	21.4	0.4
Stir bar 40 min. Soybean, Jack	21.3	7.1	20.4	0.7
Sonication 10 min. Soybean, VgD1	25.3	6.80	23.0	0.5
Sonication 10 min. Soybean, Jack	21.4	7.10	22.8	0.3

Table 10-A (continued).

Sample/ Incubate time	Soxhlet Oil%	MC%	DDTG Oil%	SE
Sonication 20 min. Soybean, VgD1	25.7	6.8	23.8	0.6
Sonication 20 min. Soybean, Jack	21.3	7.1	23.0	0.2
Sonication 40 min. Soybean, VgD1	25.7	6.8	21.4	0.9
Sonication 40 min. Soybean, Jack	21.3	7.1	20.4	0.5

Table 10-B: The SAS output by the GLM procedure.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	0.12	0.12	0.07	0.7961
Sample	1	9.1	9.10	5.07	0.0337
Incubate Time	2	40.24	20.12	11.22	0.0004
Method*Sample	1	1.82	1.82	1.02	0.3235
Method*Incubate	2	7.53	3.76	2.10	0.1447
Sample*Incubate	2	1.22	0.62	0.34	0.7160
Method*Sample*Incubate	2	1.13	0.56	0.31	0.7335

Means with the same letter are not significantly different.			
Scheffe Grouping	Mean	N	Incubate
A	23.36	12	10
A	22.81	12	20
B	20.90	12	40

Table 11-A: DDTG protocol with soybean, JACK_R3 and VGD1_2 R3 and Chia_G8 and 10 minutes incubate times.

Sample	Soxhlet Oil%	MC%	DDTG Oil%	SE
Stir bar chia, G 8	32.7	9.27	27.0	0.4
Stir bar soybean, Jack	21.4	7.10	23.2	0.4
Stir bar Soybean, VgD1	25.3	6.80	24.5	0.8
Sonication chia, G 8	32.7	9.27	23.6	0.8
Sonication soybean, Jack	21.4	7.10	22.8	0.3
Sonication soybean, VgD1	25.3	6.80	23.0	0.5

Table 11-B: The SAS output by the GLM procedure.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	57.84	28.92	22.87	<.0001
Sample	2	153.89	76.95	60.86	<.0001
Method*Sample	4	122.83	30.71	24.29	<.0001

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs Stir Bar	1	13.85	13.85	10.96	0.0031
Soxhlet vs Sonication	1	12.63	12.63	9.99	0.0044
Stir Bar vs Sonication	1	57.62	57.62	45.57	<.0001

Means with the same letter are not significantly different.			
Scheffe Grouping	Mean	N	Method
A	27.03	14	Soxhlet
B	24.88	9	Stir Bar
C	23.13	9	Sonication

Table 12-A: DDTG protocol for chia, G8 variety with different incubate

Sample	Soxhlet Oil%	MC%	DDTG Oil%	S.E.
Chia, G8, 20 minute	32.7	9.3	39.0	1.3
Chia, G8, 10 minute	32.7	9.3	40.1	0.6

Table 12-B: The SAS output by the GLM procedure.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	146.99	73.50	24.05	0.0002

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs DDTG_20	1	112.15	112.15	36.69	0.0002
Soxhlet vs DDTG_10	1	82.73	82.73	27.07	0.0006
DDTG_20 vs DDTG_10	1	1.67	1.67	0.55	0.48

Table 13-A: DDTG protocol for 11 bias samples of soybean and chia.

Sample	Soxhlet Oil%	MC%	DDTG Oil%	S.E
JACK-R3	21.4	10.1	29.0	0.1
VGD1-1 R2	24.3	9.1	28.8	0.4
VGD1-2 R3	25.3	9.4	30.8	1.3
VC - R3	16.5	10.1	24.8	1.1
VC -R1	16.4	10.3	24.1	0.5
VGD1-2 R2	20.3	9.6	31.5	0.1
JACK- R1	21.8	10.0	27.2	0.2
VGD1 -1R3	23.8	9.3	29.8	0.6
VC- R2	17.9	10.1	24.9	0.2
JACK -R2	21.5	9.7	28.4	0.5
Chia, G8	32.7	9.3	40.1	0.6

Table 13-B: The SAS output by the GLM procedure.

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.96	5.34	1.33	24.96

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	913.36	913.36	514.36	<.0001
Sample	10	1543.22	154.32	86.91	<.0001
Method*Sample	10	71.20	7.12	4.01	0.0004

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs DDTG	1	969.07	969.07	374.82	<.0001

Table 14: DDTQ protocol with soybean, Jack and VgD1 and chia, G8

Sample	Soxhlet Oil%	MC%	DDTQ Oil%	S.E.
Chia, G8	32.7	9.27	32.9	2.3
Soybean Jack 1	21.3	10.1	25.3	1.4
Soybean VgD1	25.3	9.4	28.4	0.3

Table 15: DDTQ protocol with soybean, Jack and VgD1 and chia, G8.

Sample	Soxhlet Oil%	MC%	DDTQ Oil%	S.E.
Chia, G8	32.7	9.27	35.7	0.8
Soybean Jack1	21.3	10.10	24.9	0.6
Soybean VgD1	25.3	9.40	27.8	1.5

Table 16-A: DDTQ protocol for 11 bias samples of soybean and chia.

Sample	Soxhlet Oil%	MC%	DDTQ Oil%	S.E
Soybean, JACK-R3	21.4	10.1	28.5	0.5
Soybean, VGD1-1 R2	24.3	9.1	28.9	1.6
Soybean, VGD1-2 R3	25.3	9.4	29.2	1.5
Soybean, VC - R3	16.5	10.1	25.3	1.0

Table 16-A (continued).

Sample	Soxhlet Oil%	MC%	DDTQ Oil%	S.E
Soybean, VC -R1	16.4	10.3	25.5	0.4
Soybean, VGD1-2 R2	20.3	9.6	30.7	1.1
Soybean, JACK- R1	21.8	10.0	29.4	1.1
Soybean, VGD1 -1R3	23.8	9.3	29.8	0.7
Soybean, VC- R2	17.9	10.1	24.5	1.2
Soybean, JACK -R2	21.5	9.7	29.6	0.2
Chia, G8	32.7	9.3	40.0	0.7

Table 16-B: The SAS output by the GLM procedure.

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.95	6.62	1.66	25.01

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	944.56	944.56	344.32	<.0001
Sample	10	1444.12	144.41	52.64	<.0001
Method*Sample	10	80.95	8.095	2.95	0.0048

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs DDTQ	1	1000.03	1000.03	281.37	<.0001

Table 16-C: The SAS output by the GLM procedure.

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.88	7.03	2.05	29.18

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	200.39	100.19	23.80	<.0001
Sample	2	810.19	405.09	96.24	<.0001
Method*Sample	4	25.49	6.371	1.51	0.2216

Table 16-C (continued).

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs DDTQ burr	1	55.71	55.71	13.23	0.0010
Soxhlet vs DDTQ coffee	1	200.01	200.01	47.52	<.0001
DDTQ burr vs DDTQ coffee	1	70.22	70.22	16.68	0.0003

Means with the same letter are not significantly different.			
Scheffe Grouping	Mean	N	Method
A	32.57	9	DDTQ coffee
B	29.15	18	DDTQ burr
C	27.03	14	Soxhlet

Table 17-A Bead Beating Extraction Method by following Folch method

Bead Beating Extraction Methods	Samples	Soxhlet Oil%	MC%	BBE Oil %	SE
First Tech- Follow Folch	Chia, G8	32.7	9.27	32.09	1.64
First Tech- Follow Folch	Soybean Jack1	21.4	10.1	17.34	3.44
First Tech- Follow Folch	Soybean VgD2	25.3	9.4	27.67	1.51

Tabel 17-B: The SAS output by the GLM procedure.

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.90	8.15	2.19	26.88

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	2.40	2.40	0.50	0.4908
Sample	2	561.64	280.82	58.49	<.0001
Method*Sample	2	27.07	13.53	2.82	0.0913

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs BBE Folch	1	3.46	3.46	0.59	0.4514

Table 17-A (continued).

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Method
A	27.03	14	Soxhlet
A	26.60	7	BBE_Folc

Table 18-A BBE method by following DDT & transesterification method

Sample	Soxhlet Oil%	MC%	BBE Oil%	S.E
Chia, G8	32.7	9.27	196.9	16.8
Soybean Jack1	21.4	10.1	124.3	2.9
Soybean VgD2	25.3	9.4	134.2	13.7

Table 18-B Bead Beating Extraction Method second protocol

Sample	Soxhlet Oil%	MC%	BBE Oil%	S.E
Chia, G8	32.7	9.27	94.5	28.5
Soybean Jack1	21.4	10.1	35.1	1.9
Soybean VgD2	25.3	9.4	35.4	1.1

Table 18-C: The SAS output by the GLM procedure.

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.935216	5.49	1.44	26.30

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	132.06	66.03	31.71	<.0001
Sample	2	429.34	214.67	103.10	<.0001
Method*Sample	4	29.92	7.48	3.59	0.0204

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs BBE First	1	64.25	64.25	22.29	<.0001
Soxhlet vs BBE Second	1	19.46	19.48	6.75	0.015
BBE First vs BBE Second	1	128.53	128.53	44.60	<.0001

Table 18-C (continued).

Means with the same letter are not significantly different.			
Scheffe Grouping	Mean	N	Method
A	28.41	9	BBE Second
A	27.03	14	Soxhlet
B	23.07	9	BBE First

Table 19-A: Second-Technique via Bead Beating Extraction with/out Sonication

Sample	Soxhlet Oil%	MC%	BBE Oil%	S.E
Chia, G8 &Sonication	32.7	9.27	38.0	0.4
Soybean Jack1&Sonication	21.4	10.1	28.2	0.3
Soybean VgD2 &Sonication	25.3	9.4	31.0	0.7
Chia, G8	32.7	9.27	39.4	1.4
Soybean Jack1	21.4	10.1	28.6	1.1
Soybean VgD2	25.3	9.4	28.9	1.6

Table 19-B: The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.93	5.69	1.70	30.02

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	262.83	131.42	45.39	<.0001
Sample	2	653.60	326.80	112.86	<.0001
Method*Sample	4	16.07	4.02	1.39	0.2693

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Method
A	32.40	9	BBE Sonication
A	32.30	9	BBE No Sonication
B	27.03	14	Soxhlet

Table 20-A: BBE second protocol with sonication 15 minutes and homogenized tubes twice times in an MP Bio Fastprep®-24 homogenizers with soybean seed program.

Sample	Soxhlet Oil%	MC%	BBE Oil%	S.E
Chia, G8	32.7	9.27	40.5	2.1
Soybean Jack1	21.4	10.1	26.1	1.8
Soybean VgD2	25.3	9.4	30.1	0.3

Table 20-B: The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.93	6.25	1.87	30.00

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	2	259.99	129.99	37.01	<.0001
Sample	2	777.48	388.74	110.68	<.0001
Method*Sample	4	21.24	5.31	1.51	0.2317

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs BBE_MT1	1	197.84	197.84	52.36	<.0001
Soxhlet vs BBE_MT2	1	187.80	187.80	49.70	<.0001
BBE_MT1 vs BBE_MT2	1	0.11	0.11	0.03	0.8665

Table 21-A: BBE protocol for 11 bias samples of soybean and chia.

	Sample	Soxhlet Oil%	MC%	BBE Oil%	S.E
1	JACK-R3	21.4	10.1	28.4	0.3
2	VGD1-1 R2	24.3	9.1	30.5	0.5
3	VGD1-2 R3	25.3	9.4	31.6	0.3
4	VC - R3	16.5	10.1	26.1	0.3
5	VC -R1	16.4	10.3	25.9	0.3
6	VGD1-2 R2	20.3	9.6	26.9	0.7
7	JACK- R1	21.8	10.0	29.6	0.3
8	VGD1 -1R3	23.8	9.3	29.4	0.4
9	VC- R2	17.9	10.1	25.3	0.1
10	JACK -R2	21.5	9.7	29.0	0.1
11	Chia, G8	32.7	9.3	36.6	0.5

Table 21-B : The SAS output by the GLM procedure

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.97	4.60	1.15	24.96

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	911.55	911.55	692.06	<.0001
Sample	10	1191.10	119.11	90.43	<.0001
Method*Sample	10	61.58	6.16	4.68	<.0001

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs BBE	1	932.88	932.88	454.94	<.0001

Table 22: The SAS output by the GLM procedure

Square	Coeff Var	Root MSE	Oil Percent Mean
0.96	4.81	1.08	22.39

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	1	17.05	17.05	14.70	0.0003
Sample	10	1184.91	118.49	102.15	<.0001
Method*Sample	10	88.29	8.83	7.61	<.0001

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs NIRS	1	22.05	22.05	9.50	0.0030

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Method
A	22.96	33	NIRS
B	21.97	45	Soxhlet

Table 23: Different amount of soybean oil with different concentration of Nile Red

Well Row	Soybean oil µg	Nile Red µg/mL	Average
A	0	10	10998
B	1	10	5887
C	10	10	8645
D	25	10	17721
E	100	10	13892
A	0	25	28827
B	1	25	50162
C	10	25	47945
D	25	25	91933
E	100	25	83972
A	0	100	71980
B	1	100	69284
C	10	100	69664
D	25	100	71038
E	100	100	57589

Table 24: Comparisons significant at the 0.05 level are indicated by four methods compared with the Soxhlet method

R-Square	Coeff Var	Root MSE	Oil Percent Mean
0.96	4.90	1.28	26.13

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Method	4	1901.71	475.43	289.81	<.0001
Sample	10	2437.84	243.78	148.61	<.0001
Method*Sample	40	213.48	5.34	3.25	<.0001

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Method
A	29.15	33	DDTQ
A	29.03	33	DDTG
A	29.03	33	BBE
B	22.96	33	NIRS
C	21.97	45	Soxhlet

Table 24 (continued).

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Soxhlet vs DDTG	1	988.94	988.94	387.34	<.0001
Soxhlet vs DDTQ	1	1021.76	1021.76	400.19	<.0001
Soxhlet vs BBE	1	987.03	987.03	386.59	<.0001
Soxhlet vs NIRS	1	25.58	25.58	10.02	0.0019

APPENDIX CHAPTER 3

Table 1:Forty genotypes planted in two different locations.

Entry	Identify	Entry	Identify
1	15.G8 Remutation Single Plants	21	15.G8 Remutation Single Plants
2	15.G8 Remutation Single Plants	22	15.G8 Remutation Single Plants
3	15.G8 Remutation Single Plants	23	15.G8 Remutation Single Plants
4	15.G8 Remutation Single Plants	24	15.G8 Remutation Single Plants
5	15.G8 Remutation Single Plants	25	15.G8 Remutation Single Plants White seed
6	15.G8 Remutation Single Plants	26	15.G8 Remutation Single Plants
7	15.G8 Remutation Single Plants	27	15.G8 Remutation Single Plants
8	15.G8 Remutation Single Plants	28	15.G8 Remutation Single Plants
9	15.G8 Remutation Single Plants	29	15.G8 Remutation Single Plants
10	15.G8 Remutation Single Plants	30	15. G8 Remutation late harvest
11	15.G8 Remutation Single Plants	31	15.R2N Row 15.1 White seed sBc (Salba $\times$ G8)
12	15.G8 Remutation Single Plants	32	15. KBSP - 5 White seed (KW13.1) (Salba X G8)
13	15.G8 Remutation Single Plants	33	15. KBSP - 3 G8
14	15.G8 Remutation Single Plants	34	15. KBSP - 7 G8
15	15.G8 Remutation Single Plants	35	15. KBSP - 11 G8
16	15.G8 Remutation Single Plants	36	15. KBSP - 1 Kummers 15 G8
17	15.G8 Remutation Single Plants	37	15. KBSP - 10 White seed from G8
18	15.G8 Remutation Single Plants	38	15. KBSP - 12 G8
19	15.G8 Remutation Single Plants	39	15. KW13 - 1 - 3 White seed (Salaba$\times$G8)
20	15.G8 Remutation Single Plants	40	15. KBSP - 8 G8

Table 2: Flowering date at Spindletop Farm on 8/6/2016 and Quicksamd Farm on 8/16/2016.

Treatments	Spindletop Flowering% on 8/6/2016	Quicksand Flowering% on 8/16/2016
3	60%	20%
3	15%	20%
4	25%	30%
4	60%	25%
8	60%	45%
8	60%	40%
14	60%	45%
14	60%	45%
19	60%	35%
19	60%	75%
21	75%	20%
21	70%	90%
28	60%	25%
28	60%	60%
29	60%	25%
29	60%	3%
33	60%	35%
33	60%	60%
36	60%	25%
36	60%	20%
39	60%	50%
39	60%	55%
49	60%	30%
49	60%	80%

Table 2 (continued).

Treatments	Spindletop Flowering% on 8/6/2016	Quicksand Flowering% on 8/16/2016
51	60%	55%
51	60%	70%
53	60%	75%
53	60%	20%
56	50% on 7/21/2016	90%
56	70%	35%
57	60%	50%
57	60%	20%
59	60%	75%
59	60%	60%
61	60%	40%
61	60%	30%
63	60%	25%
63	35%	45%
73	15%	20%
73	60%	15%
75	60%	20%
75	60%	50%
81	60%	20%
81	15%	30%
82	15%	20%
82	10%	20%
84	60%	0%
84	60%	45%
88	60%	20%

Table 2 (continued).

Treatments	Spindletop Flowering% on 8/6/2016	Quicksand Flowering% on 8/16/2016
88	60%	70%
89	25%	35%
89	60%	40%
92	60%	10%
92	5%	15%
94	30%	15%
94	10%	35%
99	60%	70%
99	60%	75%
109	10%	10%
109	10%	20%
138	3%	0%
138	2%	0%
142	60%	50%
142	60%	80%
145	60%	50%
145	60%	50%
147	60%	60%
147	60%	40%
148	60%	30%
148	60%	20%
152	60%	60%
152	10%	35%
153	60%	25%
153	10%	30%

Table 2 (continued).

Treatments	Spindletop Flowering% on 8/6/2016	Quicksand Flowering% on 8/16/2016
157	60%	20%
157	60%	45%
161	60%	40%
161	60%	35%
222	60%	80%
222	60%	10%
Average	51%	38%

Table 3:Percentage of oil content in progeny seeds among treatments (varieties) from high to low.

Treatment	Oil%	A	B	C	D	E	F	G	H	I	H	K	L	M	N	O	P	Q	R	S
30	35.6	A																		
24	32.3		B																	
7	31.8		B	C																
28	31.8		B	C																
19	31.7		B	C																
8	31.6		B	C	D															
27	31.4		B	C	D	E														
11	31.4		B	C	D	E														
14	31.4		B	C	D	E														
36	31.2		B	C	D	E	F													
37	31.1		B	C	D	E	F	G												
35	31.0			C	D	E	F	G	H											
12	31.0			C	D	E	F	G	H											
3	30.8			C	D	E	F	G	H	I										

Table 3 (continued).

Treatment	Oil%	A	B	C	D	E	F	G	H	I	H	K	L	M	N	O	P	Q	R	S
13	30.7			C	D	E	F	G	H	I	J									
20	30.7			C	D	E	F	G	H	I	J	K								
17	30.7			C	D	E	F	G	H	I	J	K								
22	30.6			C	D	E	F	G	H	I	J	K	L							
10	30.4				D	E	F	G	H	I	J	K	L							
15	30.2					E	F	G	H	I	J	K	L	M						
40	30.1						F	G	H	I	J	K	L	M	N					
16	30.1						F	G	H	I	J	K	L	M	N					
5	30.0						F	G	H	I	J	K	L	M	N	O				
29	30.0						F	G	H	I	J	K	L	M	N	O				
26	30.0						F	G	H	I	J	K	L	M	N	O				
9	29.8							G	H	I	J	K	L	M	N	O	P			
38	29.8								H	I	J	K	L	M	N	O	P			
18	29.6									I	J	K	L	M	N	O	P			
33	29.6									I	J	K	L	M	N	O	P			
23	29.5										J	K	L	M	N	O	P			
1	29.5											K	L	M	N	O	P			
34	29.4												L	M	N	O	P			
21	29.3												L	M	N	O	P	Q		
2	29.1													M	N	O	P	Q		
4	29.0														N	O	P	Q		
32	28.8															O	P	Q	R	
6	28.7																P	Q	R	
25	28.1																	Q	R	S
31	27.6																		R	S
39	27.0																			S

Table 3 shows categorizes treatments intro groups and shows the average of progeny oil% seeds content among treatments within each group.Treatments within each group are not significantly different between each other in terms of oil content. Group A (32), group B (24, 7, 28, 19, 8, 27, 11, 14, 36, and 37), group C (7, 28, 19, 8, 27, 11, 14, 36, 37,, 35, 12, 3, 13, 20, 17, and 22), group D (8, 27, 11, 14, 36, 37,, 35, 12, 3, 13, 20, 17, 22, and 10), group E (27, 11, 14, 36, 37,, 35, 12, 3, 13, 20, 17, 22, 10, and 15) group F (36, 37,, 35, 12, 3, 13, 20, 17, 22, 10, 15,, 40, 16, 5, 29, and 26) group G (37,, 35, 12, 3, 13, 20, 17, 22, 10, 15,, 40, 16, 5, 29, 26, and 9), group H (35, 12, 3, 13, 20, 17, 22, 10, 15,, 40, 16, 5, 29, 26, 9, and 38), group I(3, 13, 20, 17, 22, 10, 15,, 40, 16, 5, 29, 26, 9, 38, 18, and 33), group J (13, 20, 17, 22, 10, 15, 40, 16, 5, 29, 26, 9, 38, 18, 33, and 23), group K (20, 17, 22, 10, 15,, 40, 16, 5, 29, 26, 9, 38, 18, 33, 23, and 1), group L (22, 10, 15,, 40, 16, 5, 29, 26, 9, 38, 18, 33,23, 1, 34, 21), group M (15, 40, 16, 5, 29, 26, 9, 38, 18, 33,23, 1, 34, 21, 2), group N (40, 16, 5, 29, 26, 9, 38, 18, 33,23, 1, 34, 21, 2, and 4), group O (5, 29, 26, 9, 38, 18, 33,23, 1, 34, 21, 2, 4, and 32), group P (9, 38, 18, 33,23, 1, 34, 21, 2, 4, 32, and 6), group Q (21, 2, 4, 32, 6, and 25), group R (32, 6, 25, 21, 2, 4, 32, 6, 25, and 31), and group S (25, 31and 39). More details below on each treatment

1. **Treatment 30** (group A) was the highest oil% with an average 35.6%, and it was not overlapping with other treatments.
2. **Treatment 24** (group B) showed an average 32.3% oil, and there was not significantly different between treatment 24 with other treatments in the same group. **The treatment 24 was the highest oil treatment selected from parents.**
3. **Treatment 7** provided an average 31.8% oil and it carried characteristic for two groups, including group B and group C.
4. **Treatment 28** obtained an average 31.8% oil and it carried characteristic for two groups, including group B and group C.
5. **Treatment 19** obtained an average 31.7% oil content and it carried characteristic for two groups, including group B and group C. **Treatment 19 was the fifth highest oil% treatment selected from parents.**
6. **Treatment 8** provided an average 31.6% oil and it carried characteristic for three groups, including group B, group C and group D.

7. **Treatment 27** showed an average 31.4% oil content and it carried characteristic for four groups, including group B, group C, group D and group E.

8. **Treatment 11** provided an average 31.4 oil% content and it carried characteristic for four groups, including group B, group C, group D, and group E.

9. **Treatment 14** obtained an average 31.4 oil% content and it carried characteristic for four groups, including group B, group C, group D, and group E. **Treatment 14 was the third highest oil% treatment selected from parents.**

10. **Treatment 36** showed an average 31.2 oil% content and it carried characteristic for five groups, including group B, group C, group D, group E (27, 11, 14, 36, 37, 35, 12, 3, 13, 20, 17, 22, 10, and 15) ,and group F.

11. **Treatment 37** obtained an average 31.1% oil content and it carried characteristic for six groups, including group B, group C, group D, group E, group F and group G.

12. **Treatment 35** provided an average 31.0% oil content and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H.

13. **Treatment 12** provided an average 31.0% oil content and it carried characteristic for six group, including group C, group D, group E, group F, group G, and group H.

14. **Treatment 3** showed an average 30.8% oil content and it carried characteristic for seven groups, including group C, group D, group E, group F, group G, group H, and group I. **Treatment 3 was the second highest oil% treatment selected from parents.**

15. **Treatment 13** obtained an average 30.7% oil content and it carried characteristic for eight groups, including group C, group D, group E, group F, group G, group H, group I, and group J.

16. **Treatment 20** provided an average 30.7% oil content and it carried characteristic for nine groups, including group C, group D, group E, group F, group G, group H, group I, group J, and group K.

17. **Treatment 17** showed an average 30.7% oil and it carried characteristic for nine groups, including group C, group D, group E, group F, group G, group H, group I, group J, and group K.

18. **Treatment 22** provided an average 30.6% oil content and it carried characteristic for ten groups, including group C, group D, group E, group F, group G, group H, group I, group J, group K, and group L. **Treatment 22 was the forth highest oil% treatment selected from parents.**

19. **Treatment 10** obtained an average 30.4% oil content and it carried characteristic for nine groups, including group D, group E, group F, group G, group H, group I, group J, group K, and group L.

20. **Treatment 15** provided an average 30.2% oil content and it carried characteristic for nine groups, including group E, group F, group G, group H, group I, group J, group K, group L, and group M.

21. **Treatment 40** obtained an average 30.1% oil content and it carried characteristic for nine groups, including group F, group G, group H, group I, group J, group K, and group L, group M, and group N.

22. **Treatment 16** showed an average 30.1% oil content and it carried characteristic for nine groups, including group F, group G, group H, group I, group J, group K, group L, group M, and group N.

23. **Treatment 5** provided an average 30.0% oil content and it carried characteristic for ten groups, including group F, group G, group H, group I, group J, group K, group L, group M, group N, and group O.

24. **Treatment 29** obtained an average 30.0% oil content and it carried characteristic for ten groups, including group F, group G, group H, group I, group J, group K, group L, group M, group N, and group O.

25. **Treatment 26** showed an average 30.0% oil content and it carried characteristic for ten groups, including group F, group G, group H, group I, group J, group K, group L, group M, group N, and group O.

26. **Treatment 9** obtained an average 29.8% oil content and it carried characteristic for ten groups, including group G, group H, group I, group J, group K, and group L, group M, group, group O, and group P.

27. **Treatment 38** provided an average 29.8% oil content and it carried characteristic for nine groups, including group H, group I, group J, group K, and group L, group M, group N, group O, and group P.

28. **Treatment 18** showed an average 29.6% oil content and it carried characteristic for eight groups, include group I, group J, group K, and group L, group M, group N, group O, and group P.

29. **Treatment 33** provided an average 29.6% oil content and it carried characteristic for eight groups, including group I, group J, group K, and group L, group M, group N, group O, and group P.

30. **Treatment 23** obtained an average 29.55% oil content and it carried characteristic for seven groups, including group J, group K, group L, group M, group N, group O, and group P.

31. **Treatment 1** obtained an average 29.55% oil content and it carried characteristic for six groups, include group K, and group L, group M, group N, group O, and group P.

32. **Treatment 34** obtained an average 29.4 % oil content and it carried characteristic for five groups, including group L, group M, group N, group O, and group P.

33. **Treatment 21** showed an average 29.3% oil content and it carried characteristic for six groups, including group L, group M, group N, group O, group P, and group Q.

34. **Treatment 2** provided an average 29.1% oil content and it carried characteristic for five groups, including group M, group N, group O, group P, and group Q. **Treatment 2 was the second lowest oil% treatment selected from parents.**

35. **Treatment 4** obtained an average 29.0% oil content and it carried characteristic for four groups, including group N, group O, group P, and group Q. **Treatment 4 was the lowest oil% treatment selected from parents.**

36. **Treatment 32** provided an average 28.8% oil content and it carried characteristic for four groups, including group O, group P, group Q, group R, and group S.

37. **Treatment 6** showed an average 28.7% oil content and it carried characteristic for four groups, including group P, group Q, group R, and group S.

38. **Treatment 25** obtained an average 28.1% oil content and it carried characteristic for three groups, including group Q, group R, and group S. **Treatment 25 was the fourth lowest oil% treatment selected from parents.**

39. **Treatment 31** provided an average 27.6% oil content, and it carried characteristic for two groups, including group R, and group S.

40. **Treatment 39** was the lowest oil% content among treatments with an average 27.0% content, and there was not significantly different between treatment 39 with other treatments (25, 31, and 39) in the group S.

Table 4:percentage of protein content (P%) in progeny seeds among treatments (varieties) from high to low.

T	P %	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
31	28.49	A																			
23	26.31		B																		
20	25.86		B	C																	
39	25.22		B	C	D																
38	25.05		B	C	D	E															
13	24.90			C	D	E	F														
2	24.75			C	D	E	F	G													
37	24.63			C	D	E	F	G	H												
25	24.42				D	E	F	G	H												
1	24.39				D	E	F	G	H	I											
28	24.28				D	E	F	G	H	I											
27	24.28				D	E	F	G	H	I											
32	24.22				D	E	F	G	H	I	J										
10	24.01				D	E	F	G	H	I	J	K									
22	23.87					E	F	G	H	I	J	K	L								
18	23.86					E	F	G	H	I	J	K	L								
21	23.76					E	F	G	H	I	J	K	L	M							
24	23.60						F	G	H	I	J	K	L	M	N						

Table 4 (continued).

T	P %	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
34	23.48							G	H	I	J	K	L	M	N	O					
35	23.42								H	I	J	K	L	M	N	O					
4	23.40								H	I	J	K	L	M	N	O					
17	23.09									I	J	K	L	M	N	O	P				
26	22.94										J	K	L	M	N	O	P	Q			
11	22.85											K	L	M	N	O	P	Q	R		
30	22.73											K	L	M	N	O	P	Q	R		
7	22.72											K	L	M	N	O	P	Q	R		
19	22.68												L	M	N	O	P	Q	R		
3	22.52													M	N	O	P	Q	R		
5	22.40														N	O	P	Q	R		
14	22.23															O	P	Q	R	S	
29	21.99																P	Q	R	S	T
36	21.94																P	Q	R	S	T
8	21.87																P	Q	R	S	T
33	21.77																	Q	R	S	T
12	21.68																	Q	R	S	T
15	21.63																	Q	R	S	T
40	21.60																		R	S	T
6	21.02																			S	T
9	20.89																				T
16	20.76																				T

Table 4 shows categorizes treatments intro groups and shows the average of progeny protein% seeds content among treatments within each group. Treatments within each group are not significantly different between each other in terms of oil content. Group A (31), group B (23, 20, and 39), group C (20, 39, 38, 13, 2, and 37), group D (39, 38, 13, 2, 37, 25, 1, 28, 27, 32, and 10), group E (38, 13, 2, 37, 25, 1, 28, 27, 32, 10, 22, 18, and 21),

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group F (13, 2, 37, 25, 1, 28, 27, 32, 10, 22, 18, 21, and 24), group G (2, 37, 25, 1, 28, 27, 32, 10, 22, 18, 21, 24, and 34), group H (37, 25, 1, 28, 27, 32, 10, 22, 18, 21 ,24, 34, 35, and 4), group I (1, 28, 27, 32, 10, 22, 18, 21, 24, 34, 35, 4, and 17), group J (32, 10, 22, 18, 21, 24, 34, 35, 4, 17, and 26), group K (10, 22, 18, 21, 24, 34, 35, 4, 17, 26, 11, 30, and 7), group L (22, 18, 21, 24, 34, 35, 4, 17, 26, 11, 30, 7, and 19), group M (21, 24, 34, 35, 4, 17, 26, 11, 30, 7, 19, 3), group N (24, 34, 35, 4, 17, 26, 11, 30, 7, 19, 3, and 5), group O (34, 35, 4, 17, 26, 11, 30, 7, 19, 3, 5, and 14), group P (17, 26, 11, 30, 7, 19 , 3, 5, 14, 29, 36, and 8), group Q (26, 11, 30, 7, 19, 3, 5, 14, 29, 36, 8, 33, 12, and 15), group R (11, 30, 7, 19, 3, 5, 14, 29, 36, 8, 33, 12, 15, and 40), group S (14, 29, 36, 8, 33, 12, 15, 40, and 6), and group T (29, 36, 8, 33, 12, 15, 40, 6, 9, and 16). More details for each treatment:

1. **Treatment 31** (group A) was the highest percentage of protein with an average 28.49% and it was not overlapping with other treatments.

2. **Treatment 23** (group B) showed an average 26.31% protein, and there was not significantly different between treatment 23 with other treatments (23, 20, and 39) in the group B.

3. **Treatment 20** provided an average 25.86% protein, and it carried characteristic for two groups, including group B and group C. **Treatment 20 was the fourth highest protein% treatment selected from parents.**

4. **Treatment 39** obtained an average 25.22% protein and it carried characteristic for three groups, including group B, group C, and group D.

5. **Treatment 38** obtained an average 25.05% protein content and it carried characteristic for four groups, including group B, group C, group D, and group E.

6. **Treatment 13** provided an average 24.90% protein and it carried characteristic for four groups, including group C, group D, group E, and group F.

7. **Treatment 2** showed an average 24.75 % protein and it carried characteristic for five groups, including group C, group D, group E, group F, and group G. **Treatment 2 was the fifth highest protein% treatment selected from parents.**

8. **Treatment 37** provided an average 24.63% protein and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H.

9. **Treatment 25** obtained an average 24.42% protein content and it carried characteristic for five groups, including group D, group E, group F, group G, and group H.

10. **Treatment 1** showed an average 24.39% protein and it carried characteristic for six groups, including group D, group E, group F, group G, group H, and group I.

11. **Treatment 28** provided an average 24.28% protein and it carried characteristic for six groups, including group D, group E, group F, group G, group H, and group I.

12. **Treatment 27** obtained an average 24.28% protein content and it carried characteristic for six groups, including group D, group F, group G, group H, and group I.

13. **Treatment 32** showed an average 24.22% protein and it carried characteristic for seven groups, including group D, group E, group F, group G, group H, group I, and group J.

14. **Treatment 10** provided an average 24.01% protein and it carried characteristic for eight groups, including group D, group F, group G, group H, group I, group J, and group K.

15. **Treatment 22** obtained an average 23.87% protein content and it carried characteristic for eight groups, including group E, group F, group G, group H, group I, group J, group K, and group L.

16. **Treatment 18** provided an average 23.86% protein and it carried characteristic for eight groups, including group E, group F, group G, group H, group I, group J, group K, and group L.

17. **Treatment 21** obtained an average 23.76% protein content and it carried characteristic for nine groups, including group E, group F, group G, group H, group I, group J, group K, group L, and group M.

18. **Treatment 24** showed an average 23.60% protein and it carried characteristic for nine groups, including group F, group G, group H, group I, group J, group K, group L, group M, and group N.

19. **Treatment 34** obtained an average 23.48% protein content and it carried characteristic for nine groups, including group G, group H, group I, group J, group K, group L, group M, group N, and group O.

20. **Treatment 35** provided an average 23.42% protein and it carried characteristic for eight groups, including group H, group I, group J, group K, group L, group M, group N, and group O.

21. **Treatment 4** obtained an average 23.40 % protein content and it carried characteristic for eight groups, including group H, group I, group J, group K, group L, group M, group N, and group O.

22. **Treatment 17** obtained an average 23.09% protein content and it carried characteristic for eight groups, including group I, group J, group K, group L, group M, group N, group O, and group P.

23. **Treatment 26** provided an average 22.94% protein and it carried characteristic for eight groups, including group J, group K, group L, group M, group N, group O, group P, and group Q.

24. **Treatment 11** obtained an average 22.85% protein content and it carried characteristic for eight groups, including group K, group L, group M, group N, group O, group P, group Q, and group R.

25. **Treatment 30** showed an average 22.73% protein and it carried characteristic for eight groups, including group K, group L, group M, group N, group O, group P, group Q, and group R.

26. **Treatment 7** obtained an average 22.72% protein content and it carried characteristic for eight groups, including group K, group L, group M, group N, group O, group P, group Q, and group R.

27. **Treatment 19** provided an average 22.68% protein and it carried characteristic for seven groups, including group L, group M, group N, group O, group P, group Q, and group R.

28. **Treatment 3** obtained an average 22.52% protein content and it carried characteristic for six groups, including group M, group N, group O, group P, group Q, and group R.

29. **Treatment 5** showed an average 22.40% protein and it carried characteristic for five groups, including group N, group O, group P, group Q, and group R.

30. **Treatment 14** provided an average 22.23% protein and it carried characteristic for five groups, including group O, group P, group Q, group R, and group S. **Treatment 14 was the sixth lowest protein% treatment selected from parents.**

31. **Treatment 29** obtained an average 21.99% protein content and it carried characteristic for five groups, including group P, group R, group S, and group T.

32. **Treatment 36** showed an average 21.94% protein and it carried characteristic for five groups, including group P, group Q, group R, group S, and group T.

33. **Treatment 8** provided an average 21.87% protein and it carried characteristic for five groups, including group P, group Q, group R, group S, and group T.

34. **Treatment 33** obtained an average 21.77% protein content and it carried characteristic for four groups, including group Q, group R, group S, and group T.

35. **Treatment 12** provided an average 21.77% protein and it carried characteristic for four groups, including group Q, group R, group S, and group T. **Treatment 12 was the fifth lowest protein% treatment selected from parents.**

36. **Treatment 15** obtained an average 21.68% protein content and it carried characteristic for four groups, including group Q, group R, group S, and group T.

37. **Treatment 40** provided an average 21.60% protein and it carried characteristic for three groups, including group R, group S, and group T.

38. **Treatment 6** showed an average 21.02% protein and it carried characteristic for two groups, including group S and group T.

39. **Treatment 9** obtained an average 20.89% protein content, and there was not significantly difference between treatment 39 with other treatments (29, 36, 8, 33, 12, 15, 40, 6, 9, and 16) in the group T.

40. **Treatment 16** was the lowest protein with an average 20.76% content among treatments with 20.76% content, and there was not significantly different between treatment 39 with other treatments (29, 36, 8, 33, 12, 15, 40, 6, 9, and 16) in the group T.

Table 5:The average of progeny yield produced kg/ha among treatments (varieties) from high to low.

Treatment	Yield kg/ha	A	B	C	D	E	F	G	H	I
30	512	A								
7	437	A	B							
28	356		B	C						
10	352		B	C	D					
13	340		B	C	D	E				
1	333		B	C	D	E	F			
24	332		B	C	D	E	F			
11	292		B	C	D	E	F	G		
37	290		B	C	D	E	F	G		
2	262			C	D	E	F	G	H	
22	259			C	D	E	F	G	H	
36	256			C	D	E	F	G	H	
27	254			C	D	E	F	G	H	
38	246			C	D	E	F	G	H	
19	236			C	D	E	F	G	H	
3	217			C	D	E	F	G	H	I
20	212			C	D	E	F	G	H	I
26	211			C	D	E	F	G	H	I
35	208			C	D	E	F	G	H	I
23	202			C	D	E	F	G	H	I
12	201			C	D	E	F	G	H	I
34	200				D	E	F	G	H	I
29	200				D	E	F	G	H	I
8	199				D	E	F	G	H	I
17	197				D	E	F	G	H	I

Table 5 (continued)

Treatment	Yield kg/ha	A	B	C	D	E	F	G	H	I
18	196					E	F	G	H	I
40	188					E	F	G	H	I
31	179						F	G	H	I
21	174							G	H	I
14	168							G	H	I
33	166							G	H	I
5	165							G	H	I
15	165							G	H	I
4	162							G	H	I
16	148							G	H	I
25	143							G	H	I
39	134								H	I
32	131								H	I
9	108								H	I
6	79									I

Table 5 shows categorizes treatments intro groups and shows the average of progeny yield seeds production among treatments within each group. Treatments within each group are not significantly different between each other in terms of oil content. Group A (30 and 7), group B (7, 28, 10, 13, 1, 24, 11, 37), group C (28, 10, 13, 1, 24, 11, 37, 2, 22, 36, 27, 38, 19, 3, 20, 26, 35, 23, 12), group D (10, 13, 1, 24, 11, 37, 2, 22, 36, 27, 38, 19, 3, 20, 26, 35, 23, 12 34, 29, 8, 17), group E (13, 1, 24, 11, 37, 2, 22, 36, 27, 38, 19, 3, 20, 26, 35, 23, 12, 34, 29, 8, 17, 18, 40), group F (1, 24, 11, 37, 2, 22, 36, 27, 38, 19, 3, 20, 26, 35, 23, 12, 34, 29, 8, 17, 18, 40, 31), group G (11, 37, 2, 22, 36, 27, 38, 19, 3, 20, 26, 35, 23, 12 34, 29, 8, 17, 18, 40, 31, 21, 14, 33, 5, 15, 4, 16, 25), group H (2, 22, 36, 27, 38, 19, 3, 20, 26, 35, 23, 12 34, 29, 8, 17, 18, 40, 31, 21, 14, 33, 5, 15, 4, 16, 25, 39, 32, and 9), and group I (3, 20, 26, 35, 23, 12, 34, 29, 8, 17, 18, 40, 31, 21, 14, 33, 5,15 , 4, 16, 25, 39, 32, 9, and 6). More details below on each treatment

1. **Treatment 30** (group A) was the highest yield amount with an average 512 kg/ha and it was not overlapping with other treatments.

2. **Treatment 7** provided an average 437 kg/ha yield, and it carried characteristic for two groups, including group A and group B. **Treatment 7 was the fifth highest yield treatment selected from parents.**

3. **Treatment 28** obtained an average 356 kg/ha yield and it carried characteristic for two groups, including group B, and group C.

4. **Treatment 10** showed an average 352 kg/ha yield and it carried characteristic for three groups, including group B, group C, and group D.

5. **Treatment 13** provided an average 340 kg/ha yield and it carried characteristic for four groups, including group B, group C, group D, and group E.

6. **Treatment 1** obtained an average 333 kg/ha yield and it carried characteristic for five groups, including group B, group C, group D, group E, and group F.

7. **Treatment 24** provided an average 332 kg/ha yield and it carried characteristic for five groups, including group B, group C, group D, group E, and group F.

8. **Treatment 11** showed an average 292 kg/ha yield and it carried characteristic for six groups, including group B, group C, group D, group E, group F, and group G.

9. **Treatment 37** obtained an average 290 kg/ha yield and it carried characteristic for six groups, including group B, group C, group D, group E, group F, and group G.

10. **Treatment 2** showed an average 262 kg/ha yield and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H.

11. **Treatment 22** provided an average 259 kg/ha yield and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H. **Treatment 22 was the third highest yield treatment selected from parents.**

12. **Treatment 36** obtained an average 256 kg/ha yield and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H.

13. **Treatment 27 showed** an average 254 kg/ha yield and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H.

14. **Treatment 38** provided an average 246 kg/ha yield and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H.

15. **Treatment 19** obtained an average 236 kg/ha yield and it carried characteristic for six groups, including group C, group D, group E, group F, group G, and group H.

16. **Treatment 3** provided an average 217 kg/ha yield and it carried characteristic for seven groups, including group C, group D, group E, group F, group G, group H, and group I.

17. **Treatment 20** showed an average 212 kg/ha yield and it carried characteristic for seven groups, including group C, group D, group E, group F, group G, group H, and group I.

18. **Treatment 26** obtained an average 211 kg/ha yield and it carried characteristic for seven groups, including group C, group D, group E, group F, group G, group H, and group I.

19. **Treatment 35** provided an average 208 kg/ha yield and it carried characteristic for seven groups, including group C, group D, group E, group F, group G, group H, and group I.

20. **Treatment 23** showed an average 202 kg/ha yield and it carried characteristic for seven groups, including group C, group D, group E, group F, group G, group H, and group.

21. **Treatment 12** obtained an average 201 kg/ha yield and it carried characteristic for seven groups, including group C, group D, group E, group F, group G, group H, and group I.

22. **Treatment 34** showed an average 200 kg/ha yield and it carried characteristic for six groups, including group D, group E, group F, group G, group H, and group I.

23. **Treatment 29** provided an average 200 kg/ha yield and it carried characteristic for six groups, including group D, group E, group F, group G, group H, and group I.

24. **Treatment 8** showed an average 199 kg/ha yield and it carried characteristic for six groups, including group D, group E, group F, group G, group H, and group I.

25. **Treatment 17** obtained an average 197 kg/ha yield and it carried characteristic for six groups, including group D, group E, group F, group G, group H, and group I.

26. **Treatment 18** showed an average 196 kg/ha yield and it carried characteristic for five groups, including group E, group F, group G, group H, and group I.

27. **Treatment 40** provided an average 188 kg/ha yield and it carried characteristic for five groups, including group E, group F, group G, group H, and group I.

28. **Treatment 31** showed an average 179 kg/ha yield and it carried characteristic for four groups, including group F, group G, group H, and group I.

29. **Treatment 21** provided an average 174 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

30. **Treatment 14** showed an average 168 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

31. **Treatment 33** obtained an average 166 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

32. **Treatment 5** showed an average 165 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

33. **Treatment 15** provided an average 165 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

34. **Treatment 4** obtained an average 162 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

35. **Treatment 16** showed an average 148 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

36. **Treatment 25** provided an average 143 kg/ha yield and it carried characteristic for three groups, including group G, group H, and group I.

37. **Treatment 39** obtained an average 134 kg/ha yield and it carried characteristic for two groups, including group H and group I.

38. **Treatment 32** provided an average 131 kg/ha yield and it carried characteristic for two groups, including group H and group I.

39. **Treatment 9** showed an average 108 kg/ha yield, and it carried characteristic for two groups, including group H and group I.

40. **Treatment 6** was the lowest yield an average 79 kg/ha among treatments and there was not significantly different between treatment 39 with other treatments (3, 20, 26, 35, 23, 12, 34, 29, 8, 17, 18, 40, 31, 21, 14, 33, 5,15, 4, 16, 25, 39, 32, 9, and 6 in the group I.

APPENDIX CHAPTER 4

Table 1: First Project (Omega-3): 14 chia bulk plant planted as a single plant in a greenhouse on January 9, 2017, and harvested on June 19, 2017.

Entry	ID	source	values: oil/18.3
1	Salba	Commercial	35.94/66.49
2	G8	Single Plant 2014	33.29/61.78
3	sbc	15.R1N row 38-1	35.97/64.29
4	G8 normal	15.46 3-2	34.46/57.12
5	505	SC2 charcoal 13.R2N (white)	28.9/65.83
6	507	SC4 charcoal 13.R2N	28.6/66.46
7	530	SC1 white 13.R2N	28.4/66.60
8	906	sBc F4 F2-1.4 13.42	33.8/58.41
9	907	sBc F4 F2-1.7 13.42	31.5/56.32
10	909	sBc F4 F2-1.9 13.42	33.0/64.78
11	914	sBc F4 F2-1.9 13.42	33.2/64.49
12	936	sBc F4 F2-1.9 13.42	35.2/59.94
13	940	sBc F4 F2-1.9 13.42	33.4/54.09
14	948	sBc F4 F2-1.9 13.42	36.2/58.67

Table 2: The first project (Omega-3) 14 single plant shows LS-MEAN six replications progeny seeds oil%, protein%, oil+protein%, seeds yield kg/ha, Palmitic Acid%, Stearic Acid%, Oleic Acid%, Linoleic Acid% and α-Linolenic Acid%.

Entry	Progeny Oil% LSMEAN	Progeny Protein% LSMEAN	Progeny Oil+Protein% LSMEAN	Progeny Seeds yield LSMEAN	Progeny Palmitic Acid LSMEAN
1	34.74	28.01	62.77	11.99	7.70
2	39.25	19.27	58.52	10.66	7.26
3	36.75	23.32	60.08	16.21	7.10
4	38.74	18.66	57.40	11.26	7.15
5	36.83	22.86	59.68	12.62	6.96
6	35.22	22.85	58.05	11.70	7.35
7	34.01	27.42	61.42	9.70	7.30
8	35.90	24.47	60.35	12.86	7.24
9	35.11	25.56	60.67	14.04	7.22
10	35.90	24.36	60.25	13.35	6.94
11	36.49	24.51	61.00	15.49	7.06
12	36.40	23.90	60.32	15.06	7.23
13	37.47	23.04	60.48	14.40	7.16
14	35.95	25.03	60.97	12.73	6.92

Table 2 (continued).

Entry	Progeny Stearic Acid LSMEAN	Progeny Oleic Acid LSMEAN	Progeny Linoleic Acid LSMEAN	Progeny α-Linolenic Acid LSMEAN
1	2.90	4.72	17.28	66.57
2	4.02	6.63	18.79	62.55
3	3.19	5.63	17.98	65.30
4	3.85	6.54	17.99	63.73
5	2.92	5.58	17.94	65.80
6	2.52	5.31	18.01	65.93
7	2.80	5.18	17.71	66.19
8	3.13	5.58	17.36	65.87
9	3.00	5.31	17.43	66.22
10	3.22	5.89	18.10	65.04
11	3.07	5.08	17.77	66.20
12	2.95	5.90	18.99	64.11
13	2.88	5.71	18.34	65.10
14	3.07	5.63	19.47	64.08

Table 3 shows R-squares and levels of significance for parental values used as a covariate for progeny values for oil%, protein%, linoleic acid, α-linolenic acid…etc.

Table 3: Diffrences between parents and progeny seeds

Parents/ Progeny	R-Square	Coeff Var	P- Value
Oil%	0.52	4.72	0.686
Protein%	0.65	9.45	0.001
Oil&Protein%	0.64	1.99	<.0001
Palmitic Acid	0.50	3.28	0.051
Stearic Acid	0.70	9.71	<.0001
Oleic Acid	0.59	9.79	0.003
Linoleic Acid	0.36	5.79	0.297
α-Linolenic Acid	0.47	2.47	0.830

Table 4: Second project (Omega-3) 52 chia F2 single plants planted at Spindletop Farm on May 25, 2016, transplant into Spindletop Farm on June 27, 2016 and harvested on October 24, 2016.

Entry	Oil%	Protein %	Oil + Protein %	Weight , g	18;3n3 me	Selected Line -Cont Mean % 18:3
Contro 1	28.91	21.90	50.8	34.97	54.76	0.00
1	29.60	24.81	54.4	19.34	58.65	3.89
2	30.07	26.48	56.6	35.74	60.69	5.93
3	30.87	23.56	54.4	25.85	55.47	0.71
4	30.74	24.91	55.7	14.51	54.60	-0.16
5	27.70	25.08	52.8	7.61	52.75	-2.01
6	29.28	23.45	52.7	17.09	57.00	2.24
7	29.14	26.05	55.2	14.03	58.60	3.84
8	29.20	26.14	55.3	18.57	57.12	2.36
9	28.43	26.29	54.7	25.39	54.79	0.03
10	28.79	25.93	54.7	14.83	52.73	-2.03
11	28.52	26.11	54.6	28.17	57.08	2.32
12	28.94	26.37	55.3	28.33	55.37	0.61
13	28.83	26.73	55.6	9.85	53.86	-0.90
14	26.82	28.11	54.9	8.93	55.34	0.58
15	31.07	25.14	56.2	33.99	54.10	-0.66
16	26.29	27.26	53.5	21.96	53.79	-0.97
17	29.27	26.38	55.7	18.09	55.32	0.56
18	27.10	27.04	54.1	7.53	55.94	1.18
19	28.80	25.14	53.9	15.59	54.02	-0.74
20	29.78	25.23	55.0	15.74	54.13	-0.63
21	31.09	26.84	57.9	24.50	63.04	8.28

Table 4 (continued).

Entry	Oil%	Protein %	Oil + Protein %	Weight , g	18;3n3 me	Selected Line -Cont Mean % 18:3
22	29.06	24.95	54.0	17.68	55.25	0.49
23	31.39	23.03	54.4	17.20	56.08	1.32
24	28.75	25.99	54.7	18.82	52.00	-2.76
25	28.33	26.14	54.5	26.70	52.43	-2.33
26	28.45	24.39	52.8	17.34	51.64	-3.12
27	29.04	24.79	53.8	31.55	53.18	-1.58
28	27.50	25.78	53.3	11.67	51.97	-2.79
29	26.83	27.26	54.1	18.66	55.68	0.92
30	30.66	24.21	54.9	17.75	54.65	-0.11
31	28.07	26.88	54.9	22.14	55.79	1.03
32	31.01	25.04	56.1	17.64	54.45	-0.31
33	28.49	25.75	54.2	18.03	53.95	-0.81
34	27.02	26.06	53.1	6.18	53.04	-1.72
35	28.07	25.51	53.6	13.95	54.63	-0.13
36	28.24	27.65	55.9	18.92	57.48	2.72
37	28.11	24.88	53.0	7.67	51.63	-3.13
38	27.39	27.19	54.6	12.23	53.58	-1.18
39	28.96	25.14	54.1	11.63	50.46	-4.30
40	28.17	25.69	53.9	15.56	54.51	-0.25
41	26.96	25.68	52.6	13.88	53.59	-1.17
42	29.57	25.84	55.4	11.32	53.92	-0.84
43	28.66	24.79	53.5	18.77	53.35	-1.41
44	29.27	26.50	55.8	14.36	60.36	5.60
45	30.20	26.62	56.8	12.24	57.28	2.52
46	29.72	26.82	56.5	16.40	56.89	2.13

Table 4 (continued).

Entry	Oil%	Protein %	Oil + Protein %	Weight , g	18;3n3 me	Selected Line -Cont Mean % 18:3
47	29.79	26.07	55.9	16.74	60.12	5.36
48	30.32	24.97	55.3	13.39	59.21	4.45
49	29.37	26.94	56.3	7.95	59.75	4.99
50	33.79	23.90	57.7	3.03	65.43	10.67
51	34.44	23.55	58.0	2.82	63.03	8.27
52	37.50	19.16	56.7	4.44	65.25	10.49

Table 5: 19 plants selected from 52 single plants with 4 parent plant and grandparent and planted into the greenhouse on January 20, 2017, and harvested June 30, 2017.

NO	Parent Entery	Identify	Parent Oil%	Omega- 3%
1	Parent	2016.34 sbC bulk , Parent 9 sbC X Omega-3] F2	28.91	54.76
2	Parent	Mi Costenita	37.83	61.74
3	Grand	Salba 2015	36.37	63.57
4	Grand	G8 2015	29.90	60.94
5	2	2016.34[sbC X Omega-3] F2	30.07	60.69
6	5	2016.34[sbC X Omega-3] F2	27.7	52.75
7	6	2016.34[sbC X Omega-3] F2	29.28	57
8	13	2016.34[sbC X Omega-3] F2	28.83	53.86
9	14	White Flower- 2016.34[sbC X Omega-3] F2	26.82	55.34
10	16	2016.34[sbC X Omega-3] F2	26.29	53.79
11	21	2016.34[sbC X Omega-3] F2	31.09	63.04
12	23	2016.34[sbC X Omega-3] F2	31.39	56.08
13	26	2016.34[sbC X Omega-3] F2	28.45	51.64
14	28	2016.34[sbC X Omega-3] F2	27.5	51.97
15	29	White Flower- 2016.34[sbC X Omega-3] F2	26.83	55.68
16	36	White Flower- 2016.34[sbC X Omega-3] F2	28.24	57.48

Table 5 (continued).

NO	Parent Entery	Identify	Parent Oil%	Omega-3%
17	37	White Flower- 2016.34[sbC X Omega-3] F2	28.11	51.63
18	39	2016.34[sbC X Omega-3] F2	28.96	50.46
19	41	2016.34[sbC X Omega-3] F2	26.96	53.59
20	45	White Flower- 2016.34[sbC X Omega-3] F2	30.2	57.28
21	50	White Flower- 2016.34[sbC X Omega-3] F2	33.79	65.43
22	51	White Flower- 2016.34[sbC X Omega-3] F2	34.44	63.03
23	52	White Flower- 2016.34[sbC X Omega-3] F2	37.5	65.25

Table 6: Second project (Omega-3) shows LS-MEAN six replications progeny seeds oil%, protein%, oil+protein%, seeds weight g, Palmitic Acid%, Stearic Acid%, Oleic Acid%, Linoleic Acid% and α-Linolenic Acid%.

Entry	Progeny Oil% LSMEAN	Progeny Protein% LSMEAN	Progeny Oil+Protein % LSMEAN	Progeny Seeds weight LSMEAN	Progeny Palmitic Acid LSMEAN
1	36.67	19.61	56.27	6.71	6.66
2	39.16	16.29	55.45	2.74	7.19
3	39.22	19.68	58.92	7.89	7.53
4	39.71	17.59	57.30	7.96	6.80
5	36.96	19.76	56.72	10.07	6.78
6	36.55	19.24	55.78	7.37	6.80
7	35.80	20.60	56.42	4.85	6.99
8	37.25	18.08	55.32	7.62	6.59
9	37.85	18.17	56.02	7.88	6.76
10	38.12	16.87	54.98	10.34	6.64
11	39.85	16.47	56.33	8.26	6.65
12	41.16	15.32	56.48	6.88	6.81
13	37.75	17.62	55.37	7.81	6.71

Table 6 (continued).

Entry	Progeny Oil% LSMEAN	Progeny Protein% LSMEAN	Progeny Oil+Protein % LSMEAN	Progeny Seeds weight LSMEAN	Progeny Palmitic Acid LSMEAN
14	37.95	16.27	54.22	8.56	6.37
15	35.39	20.56	55.95	7.52	6.77
16	35.83	20.58	56.40	7.81	6.76
17	36.98	19.19	56.15	9.38	6.74
18	37.36	17.85	55.22	10.42	6.88
19	38.09	17.77	55.85	9.35	6.55
20	37.65	18.86	56.52	8.34	6.67
21	37.60	17.55	55.17	7.50	6.85
22	35.49	20.32	55.80	6.67	6.96
23	37.91	17.55	55.45	9.74	6.53

Entry	Progeny Stearic Acid LSMEAN	Progeny Oleic Acid LSMEAN	Progeny Linoleic Acid LSMEAN	Progeny α-Linolenic Acid LSMEAN
1	2.89	4.65	19.06	65.91
2	3.10	4.85	16.38	67.67
3	4.30	5.61	18.51	63.25
4	3.91	6.28	18.40	63.88
5	2.90	5.60	19.36	64.58
6	2.94	5.51	21.47	62.49
7	2.46	4.79	18.66	66.23
8	3.06	5.81	21.93	61.88
9	2.92	5.49	20.16	63.89
10	2.85	5.32	19.47	64.91
11	3.00	5.64	18.96	64.97

Table 6 (continued).

Entry	Progeny Stearic Acid LSMEAN	Progeny Oleic Acid LSMEAN	Progeny Linoleic Acid LSMEAN	Progeny α-Linolenic Acid LSMEAN
12	3.86	5.87	17.56	65.20
13	3.15	5.40	20.52	63.48
14	3.04	5.33	20.59	63.92
15	2.75	5.32	22.55	61.78
16	2.77	5.16	19.62	64.86
17	3.01	5.34	20.51	63.62
18	2.79	5.68	21.18	62.66
19	3.02	5.45	20.12	64.12
20	2.89	5.28	21.41	62.93
21	2.89	5.25	22.49	61.73
22	2.86	5.50	21.16	62.71
23	2.90	5.49	19.77	64.52

Table 7 shows R-squares and levels of significance for parental values used as a covariate for progeny values for oil%,, protein% linoleic acid, α-linolenic acid…etc.

Table 7: Diffrences between parents and progeny seeds.

Parents/ Progeny	R-Square	Coeff Var	P- Value
Oil%	0.54	4.26	0.002
Protein%	0.49	11.63	0.184
Oil&Protein%	0.66	1.48	<.0001
Palmitic Acid	0.65	2.78	0.155
Stearic Acid	0.81	7.41	<.0001
Oleic Acid	0.68	5.31	0.579
Linoleic Acid	0.70	5.52	<.0001
α-Linolenic Acid	0.61	2.12	0.194

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