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METHOD DEVELOPMENT FOR THE ANALYSIS OF FATTY ACIDS IN ADIPOSE TISSUE USING STIR BAR SORPTIVE EXTRACTION COUPLED WITH GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Dean of the Graduate School

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2020

# DEDICATION

In order to pursue higher education, my parents moved from rural Mexico to the city of Juarez which borders with the United States. For them, education was the way out of poverty and into a life with more opportunities. Therefore, within our family, they always placed great value in higher education; in such a way that my siblings and I are all pursuing Masters's degrees in our respective fields of study. I want to dedicate this thesis to their many efforts that are now bringing me here.

To my mom and dad, I love you, now and forever.

Thank you for making me the person I am today.

Para lograr tener una mejor educación, mis padres se mudaron del México rural, a ciudad Juárez, que es frontera con Estados Unidos. Para ellos, la educación fue un modo de alejarse de la pobreza y acercarse a una vida con más oportunidades. Por ello, dentro de nuestro núcleo familiar, siempre dieron un lugar importante a la educación. Tanto fue así que sus tres hijos hoy buscan conseguir maestrías en sus diferentes especialidades. Por eso, quiero dedicar esta tesis a sus esfuerzos que me han traído hasta aquí.

Para mi mamá y mi papá, los amo, ahora y siempre.

Gracias por haberme hecho la persona que soy hoy en día.

# METHOD DEVELOPMENT FOR THE ANALYSIS OF FATTY ACIDS IN ADIPOSE TISSUE USING STIR BAR SORPTIVE EXTRACTION COUPLED WITH GAS CHROMATOGRAPHY-MASS SPECTROMETRY

by

ANGELA MARISOL ENCERRADO MANRÍQUEZ, B.S.

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unconditional love you have given me, all the kindness you have shown me, and all the opportunities you provided me.

**ABSTRACT**

The project was aimed to develop an easy and sensitive analytical tool to study the role of fatty acids (FAs) profile in periprostatic adipose tissue (PPAT) and prostate cancer (PCa). PCa is the second leading cause of cancer-related death among American men. Although obesity has been mostly ruled out by many researchers as a risk factor for developing PCa, it has shown to be associated with PCa metastasis and progression. Periprostatic adipose tissue, which was present on 48% of prostatic surfaces, has been reported to act as energy sources for facilitating a positive microenvironment for PCa tumor progression. To understand the role of PPAT fatty acid profile in relationship to PCa aggressiveness, this project was set out to develop a green method to analyze the fatty acids (FAs) in adipose tissue using a solvent-less sample preparation technique, known as stir-bar sorptive extraction, coupled with thermal desorption-Gas Chromatography/Mass Spectrometry.

To detect FAs by Gas Chromatography-Mass Spectrometry, we used pork fat as the model adipose tissue. FAs in pork fat were first subjected to transesterification. Several conditions to optimize the transesterification process such as time, temperature, and acid amount in the solvent mixture were studied. The best transesterification condition was found to be at 60-70 $^{o}$C (regardless of the heating methods, either in an oven or sonicator), with the reaction time of 1hr and using a solvent mixture of $CH_3OH$:HCl:$CHCl_3$ (10:3:1, v/v/v). After transesterification, fatty acid methyl esters (FAMEs) were extracted by stir bar sorptive extraction (SBSE). We studied factors that could promote the extraction efficiency of FAMEs by SBSE. These factors included stirring time, solvent addition, and stirring speed. We found that 1hr at 1500 RPM and no solvent addition would give the best extraction efficiency of FAMEs. By estimate, the concentrations of FAMEs detected in the fat samples ranged from 0.18 ppb (µg/L) to 114.82 ppb (µg/L). Therefore, the limit

of detection for our method could be below 0.18 ppb (μg/L) for various FAMEs in fat tissue. The sample preparation developed in this report has provided a green and sensitive alternative for the study of FAs in adipose tissue, which will provide a valuable tool for future studies in the FAs profile in PPAT and its impacts on PCa progression.

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# CHAPTER 1 INTRODUCTION

## 1.1 Prostate Cancer Overview

Prostate cancer (PCa), according to the American cancer society, is the most commonly diagnosed cancer among American men, with more than 191,930 new cases diagnosed and about 33,330 deaths reported in 2020.[1] The numbers mean that 1 in 9 men will be diagnosed with PCa and about 1 in 41 will die from the disease, making PCa the second leading cause of cancer death among American men.[2] The rising incidence in PCa through the past 30 years, could be attributed to the improvement in testing awareness.[3] Other factors, such as adipose tissue (AT), could also play an important role in supporting metabolic changes happening in the tumor microenvironment to support tumor growth.[4] In a sense, PCa cells have become metabolically addicted to lipid metabolism, for achieving successful carcinogenesis and survival.[5–7] In a study done by Calle *et al.* (2003) overweight and obesity in the United States were said to decrease survival rate among all cancers and was estimated to account for 14% of all death from cancer in men.[8]

## 1.2 Prostate Cancer and Adipose Tissue

For a long time, AT was presumed to have no real relevance to biological processes in the human body. Scientists thought of AT as another form of energy storage and temperature regulation.[9] Nevertheless, AT in the body has been lifted off of the shadows and recognized as an active endocrine organ that secretes growth factors, chemokines, and proinflammatory molecules (adipokines).[9] AT in our body is divided into two categories: subcutaneous fat (underneath the skin) and visceral fat (surrounding the organs). Only about 10% of AT is visceral fat. Nonetheless, this type of body fat is one of the most metabolically active, which makes it of most interest for many cancer studies.[10] In the human body, the excessive accumulation of fat has been linked to a

variety of health problems that have detrimentally affected organs sensitive to metabolic changes, encouraging the development and progression of a range of metabolic disorders.[11,12]

For prostate cancer, the narration between its correlation to adiposity has long been debated. Some researchers have questioned whether high body mass index (BMI) is a risk factor for developing PCa. MacInnis *et al.* (2006) reported that obesity was weakly related to the risk of developing PCa.[13] On the other hand, De Pergola *et al.* (2013) reported that obesity was related to higher cancer risk through BMI, weight increase, visceral fat, and lifestyle factors.[14] More recently, Markozannes *et al.* (2016) reported that aspects of diet, adiposity, and physical activity may affect PCa risk, yet, evidence was not concise enough to identify these aspects as risk factors for PCa.[15] Other researchers have found stronger evidence that BMI is not related to PCa risk and rather is related to PCa grade. Cao *et al.* (2011) reported that cancer-free people with high BMI had a higher risk of dying from PCa if they developed the disease.[16] Discacciati *et al.* (2012) reported that as BMI increases, the risk of a localized PCa decreases, while a direct association with advanced PCa was observed.[17] Vidal *et al.* (2014) reported that BMI, independent of PSA levels and other clinical covariates, is associated with a low risk of developing PCa, but a high risk of having a high-grade non-localized more aggressive PCa.[18] Lastly, Zhang *et al.* (2015) reported that obesity was significantly associated with PCa aggressiveness and progression, thus increasing overall mortality.[19]

### 1.3 Effect of Fatty Acid Profile in Cancer - It Is Not All About Quantity, it is Also About the Quality

For a long time, BMI has been the main representation of adiposity and obesity in the human body. However, BMI does not show a direct correlation between obesity in men and PCa

aggressiveness.[3,10] Research has reported that the volume of AT surrounding the prostate, known as periprostatic adipose tissue (PPAT), has been significantly correlated to a high Gleason score, which is indicative of an advanced stage of PCa.[10] This is attributed to the extracapsular extension of PCa into the PPAT and the vascularization of the prostate, which promotes paracrine mechanisms for PCa progression.[3,20] As people get older, the amount of bone marrow adiposity increases, which tied to a large PPAT volume provides a pathway that fuels PCa cells to metastasize in the bone.[21] In an estimate, about 90% of the patients with an aggressive PCa develop metastasis in the bone.[9] Moreover, when looking at the composition of PPAT, it provides a better understanding of the possible correlation between PCa and AT, linking to metabolic alterations required for cancer cell survival.[7]

PPAT is composed of fatty acids (FAs) with different lengths of carbon chains and saturation. The FAs in PPAT are stored within complex lipids such as ceramides, phospholipids, diacylglycerol, and triacylglycerol.[22] In visceral adipose tissue, the majority of FAs (75%) present are oleate ($C_{18:1}$), palmitate ($C_{16:0}$), palmitoleate ($C_{16:1}$), stearate ($C_{18:0}$), and vacceneate ($C_{19:1}$).[23] Through paracrine mechanisms, PPAT provides PCa cells with FAs to fulfill metabolic necessities.[20] Also, as cancer progress and the demand for FA increases, *de novo* FA synthesis is used by PCa cells to allow tumor proliferation and metastasis.[5,9,22,24,25]

In PCa, FAs are used for membrane synthesis (allowing cell growth and proliferation), membrane saturation (providing oxidative stress resistance), lipid droplet formation (for survival under energy stress), beta-oxidation (energy production), NADH oxidation (for redox balance), and cholesterol lipid hormones (promoting cell proliferation and invasion).[5,24–26] In the human body, FA synthesis gives as main products palmitate ($C_{16:0}$), myristate ($C_{14:0}$), and stearate ($C_{18:0}$).[5] Further modifications, such as the addition of double bonds, or chain length increments take place

in these FA and that can further benefit the tumor microenvironment for PCa cell proliferation. For example, the elongation of FA chains is done by a family of elongates which add two carbons at the end of a chain in each cycle of reactions.[26] Tamura *et al.* reported that the overexpression of ELOVL7 in PCa was involved in FA elongation of saturated very long chains ($C_{20:0\sim}$) for the formation of phospholipids, which are essential for membrane stabilization and raft formation (signaling).[27] Longer and more unsaturated fatty acids in human visceral fat are associated with negative metabolic outcomes.[23]

Another modification is the degree of saturation or unsaturation of the FA chains. Saturation (SFA) and mono-unsaturation (MUFA) of FAs by *de novo* lipogenesis are needed for PCa cell membranes formation. Zadra *et al.* has reported that, when compared to normal tissue, PCa tumors showed an increase in the content of SFA and MUFA and a decrease in polyunsaturated chains (PUFA).[26] When lipid chains are saturated, they are more densely packed which alters the membrane cell dynamics affecting, for instance, the efficacy of chemotherapeutics.[24,25] The saturation protects the cancerous cells from lipid peroxidation and oxidative stress-induced cell death.[5] However, the FA profile in PPAT of PCa is yet to be closely investigated.

### 1.4 Current Analytical Methodologies for Fatty Acid Analysis

Lipids are often thought to be difficult to work with because of the complexity of the nomenclature surrounding them, the poor understanding of their role in cell functions, and the lack of methodologies for their study.[28] To study FAs in lipid tissue, these compounds first must be detached from their natural structure (Figure 1) through hydrolysis. After hydrolysis, free FAs undergo transesterification, also known as methylation, to form Fatty Acid Methyl Esters

(FAMEs). Contrary to FA, the apolar and semi-volatile structure of FAMEs allows for their identification and quantification using Gas Chromatography (GC).[29,30]

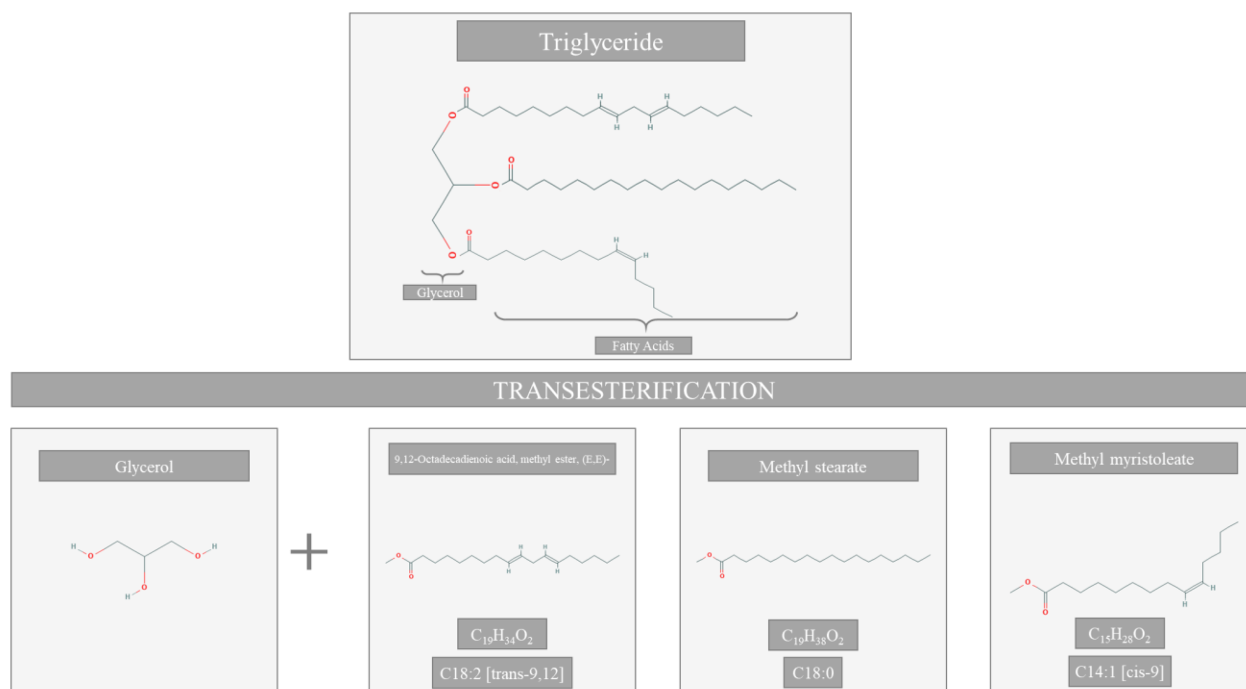



**Figure 1** An illustration showing the transesterification of FAs from lipids, triglyceride molecules. A triglyceride molecule is formed of glycerol and three molecules of FAs. The molecules of FAs forming the triglyceride can be of different length and have a different amount of saturations. The FAMEs structures were obtained from PubChem.[31]

Figure 2 summarizes some published methods for transesterification of FAs.[29,32–35] Generally, the transesterification reaction of FAs can be accelerated with the use of a catalyst and manipulation of other factors, such as temperature.[36] The transesterification time and temperature for most of the methodologies, ranges from 60-90 min and 90-100 °C, respectively.[29,32–34] A strong acid or a strong base is the common catalyst used in the reaction.[37] In the method described by Bligh and Dyer (1959), an excess of HCl is added to the sample before transesterification, and in Lewis *et al.* (2000) HCl is part of the solvent mixture added to the sample before

transesterification.[29,33] Also, excessive alcohol is added as a way to push the reaction towards the formation of FAMEs and glycerol, aiming for a 1:6 molar ratio of oil/alcohol.[37]

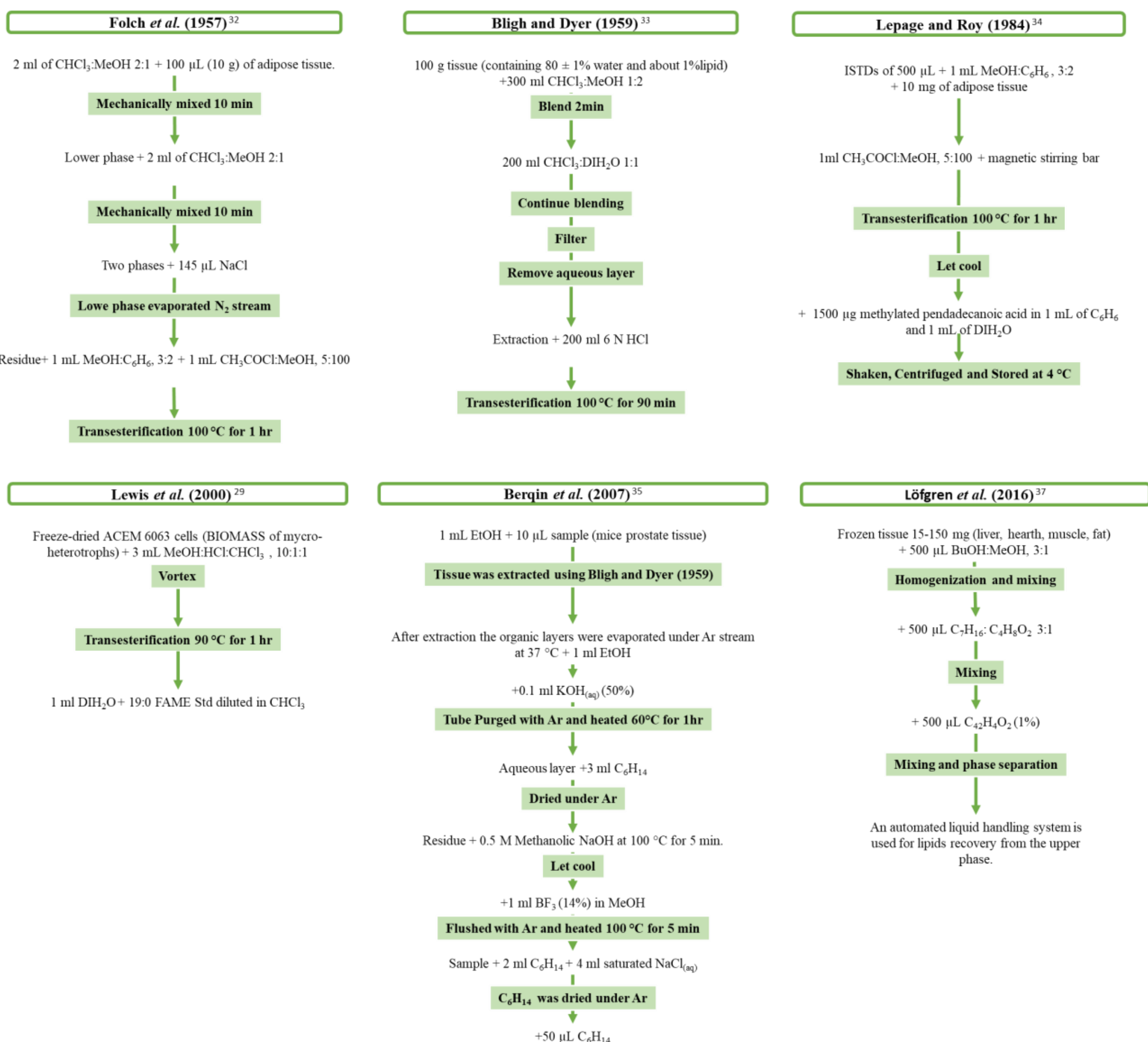



**Figure 2** Diverse methodologies developed for the extraction, transesterification, and analysis of FAMEs from biological samples.

Two of the main methodologies for the extraction and transesterification of FAs from tissue samples were developed by Folch *et al.* (1957) and Bligh and Dyer (1959).[32,33] (Figure 2) Other methods, such as Lepage and Roy (1984) made use of a sample size in the milligram range instead of the gram.[34] Also, in Löfgren *et al.* (2016) the methodology was mostly automated, with the help of a liquid handling system that facilitated the analysis of FAMEs using LC/MS.[38] As shown in

Figure 2, these methods either require a large volume of organic solvents or are labor-intensive. It is important to promote the use of green chemistry principles, such as the solvent-less sample preparation technique, known as stir-bar sorptive extraction (SBSE), in advancing new methodologies for the extraction and transesterification of FAs, as well as for the subsequent extraction of FAMEs from the sample matrix.

### 1.5 Significance and Objectives

Diagnosing cancer staging is one of the main areas of interest in cancer research.[39] The correct staging of PCa patients is important to provide a proper evaluation, develop a personalized treatment, and improve overall PCa prognosis for patients.[40,41] PCa cells have adapted mechanisms that ensure an optimal tumor microenvironment for cell survival and proliferation. Dietary and *de novo* FAs are important for PCa cells since they are part of the energy, structural, and signaling pathways. Many of these FAs are provided to PCa cells with the help of various molecules secreted from PPAT. For example, FABP4 promotes the binding of long chains of FAs, which in turn can be used in PCa cell membranes.[12] It was hypothesized that the type of FA chains present in PPAT is unique to the PCa tumor necessities, and in turn, the FA profile in PPAT could be a potential tool for understanding the cancer metabolic microenvironment for PCa. The overarching goal of our research is to use the FAs profile of the PPAT to develop an accurate staging tool for PCa. To accomplish the goal, a fast and sensitive analytical tool is essential. This project was aimed to develop a simple and solvent-less (i.e. green) method for the analysis of FAs profile in fat using stir bar sorptive extraction (SBSE) and gas chromatography-mass spectrometry (GC-MS). We hypothesized that by optimizing stirring time, speed, and solvent content of the sample matrix during SBSE we can effectively extract the highly hydrophobic fatty acid methyl esters from the

matrices and provide an overall more effective sample preparation process for the analysis of FAs in fat. The optimized method will allow us to study the FAs profile and PCa stage, helping in the understanding of the tumor microenvironment, and staging of the disease.

# CHAPTER 2 METHODOLOGY

## 2.1 Chemicals and Reagents

Commercially available pork fat was used as the model adipose tissue for all experiments. Pork fat was stored at 4°C until the preparation of the fat stock solution. Food Industry Fatty Acid Methyl Ester (FAME) Mix (2%wt/wt) standard was purchased from RESTEK Corporation (Bellefonte, PA). A list of the 37 FAMEs in the standard is included in Appendix 1 and 2. The concentration of FAME primary stock solution was $2.6512 \times 10^3$ ppm (mg/L) in methanol ($CH_3OH$, LC-MS Grade, Omni Solve Millipore Sigma, Billerica, MA).

Mirex, as the internal standard, was purchased from Crescent Chemical (Islandia, NY). Hydrochloric acid (HCl, 37% ACS grade) was purchased from Sigma Aldrich, Inc. (St. Louis, MO). Chloroform was purchased from Fisher Scientific (Fair Lawn, NY). Acetonitrile LC/MS grade was purchased from Fisher Chemical (Fair Lawn, NJ). Sodium Chloride (NaCl), ACS reagent (>=99.0%) was purchased from Sigma Aldrich, Inc. (St. Louis, MO). Deionized (DI) water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA).

## 2.2 Method Development

This methodology was developed following the diagram showed in Figure 3. The process was divided into two main sections: sample preparation and sample analysis. The first part was focused on fat sample handling, storage, homogenization, and transesterification parameters. Sample analysis was centered around the extraction conditions and instrumentation parameters to get an optimal separation, recovery, and identification of the analytes.

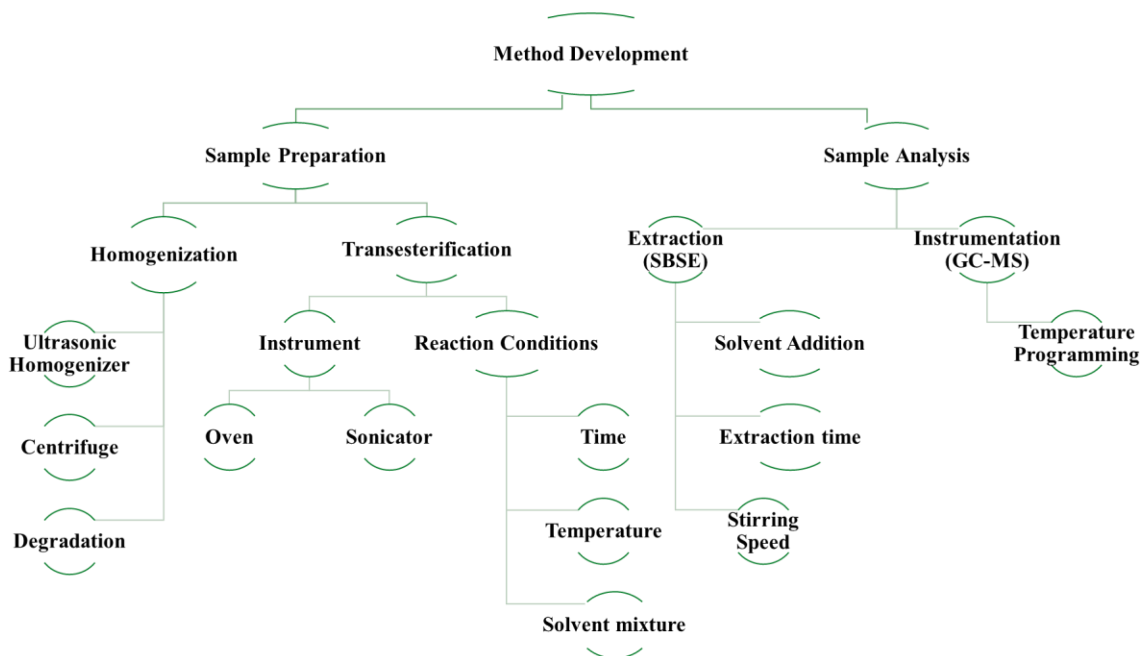



**Figure 3** A diagram describing the method development of this project. The two main areas of the study are sample preparation and sample analysis. Each area subdivided into more specific sections including all variables in this study.

### 2.2.1 Sample Preparation

***Homogenization***

This step aimed to achieve a homogeneous liquid fat stock sample. Pork fat was cut into small portions and placed in a 15ml centrifuge tube. From there the tube was left for one day in a Freeze Dry System (FreeZone 1 Liter Benchtop by LABCONCO, Kansas City, MO) to remove any water from the fat. About 3 g of freeze-dried pork fat were weight and added into a small beaker containing 90 ml of HCl:$CHCl_3$ (9:1 v:v). Then the mixture was homogenized for 1hr using Ultrasonic Homogenizer (Model 300V/T by BioLogics, Inc., Manassas, VA) at strength 80 and a pulse of 30%. The sample was then decanted into a 50ml centrifuge tube and centrifuged for 15 min at 3000 rpm. After these, the sample was carefully decanted on to a new 50 ml centrifuge tube and centrifuged for 10 min at 3500 rpm. These steps were to ensure that large fat particles were removed in the final stock fat solution. Finally, the sample was carefully decanted into a clean 50 ml centrifuge tube and stored in the 10 °C fridge until the analysis.

***Transesterification***

To promote the detection of FAs by GC-MS, transesterification of the FA contained in the stock pork fat was necessary prior to sample analysis. The transesterification procedure developed in this project was inspired by Lewis *et al.* (2000).[29] Optimization of transesterification to form fatty acid methyl esters (FAMEs) is described as follows.

*Heating Method by Oven (VWR International, 1326 Gravity Oven)*

300 µL pork fat stock solution (10 mg) were added into a 15 ml centrifuge tube containing 3ml of $CH_3OH$:HCl:$CHCl_3$ ,10:3:1 ratio. The sample was left in the oven at a temperature-controlled between 60°C -70 °C for 2 hr. After transesterification was completed, the sample was stored at 10 °C fridge until analysis.

*Heating Method by Sonicator (Bransonic®, ultrasonic cleaner)*

300 µL pork fat stock solution (10 mg) were added into a 15 ml centrifuge tube containing 3ml of $CH_3OH$:HCl:$CHCl_3$ ,10:3:1 ratio. Then the sample was left in the water bath on the sonicator at a temperature fluctuating between 60°C -70°C for 1hr. After transesterification was completed, the sample was stored at 10 °C fridge until analysis.

**2.2.2 Sample Analysis**

***Stir Bar Sorptive Extraction (SBSE)***

The basic steps of SBSE are illustrated in Figure 4. Pre-conditioned GERSTEL Twisters™ coated with polydimethylsiloxane (PDMS, 1 mm thickness, 10 mm length) were used

to extract FAMEs from the sample matrix. Briefly, 0.5 mL of fat sample was transferred into corresponding vials with 19.5 mL of DI water or desired solvent mix. Samples were then spiked with 400 µl of 1 ppm (mg/L) of mirex as the internal standard. Finally, preconditioned GERSTEL Twister® was added to each sample and all samples were set to stir for a pre-determined time at pre-determined speed on a GERSTEL Twister stir plate. After stirring, each twister was removed from solution with sterilized forceps and thoroughly rinsed with DI water. The twister was dried with lint-free wipes and individually placed into Thermal Desorber 3.5+ (TD 3.5+) desorption tube with glass frit. Sample was then analyzed by Thermal Desorber 3.5+ - Gas Chromatography /Mass Spectrometry (TD 3.5+ - GC-MS).

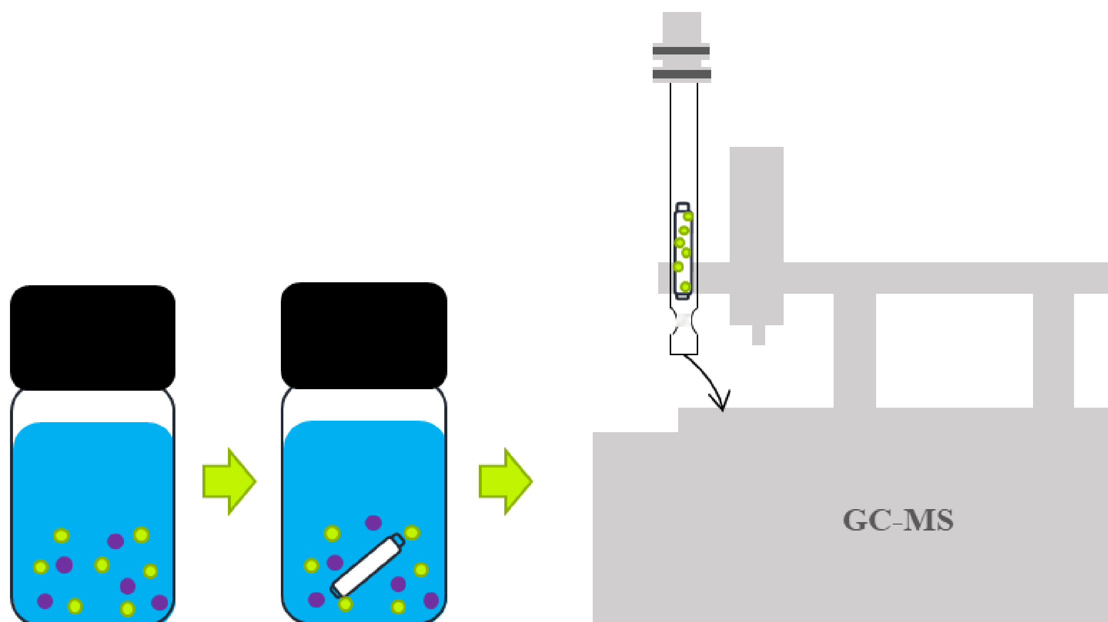



**Figure 4** Illustration of the SBSE extraction process for the analytes followed by GC-MS. A sample containing the analytes (i.e. FAMEs) under investigation, as well as other compounds that are part of the sample matrix, is placed in a vial. A GERSTEL Twister® is then added to the solution to extract the desired analytes. After the extraction is completed, the GERSTEL Twister® is placed on a desorption tube followed by chemical analysis on a TD/GC-MS system.

***Chemical Analysis (TD 3.5+ - GC-MS)***

After SBSE, the GERSTEL Twisters were removed and placed in a TD 3.5+ desorption tube with glass frit. The desorption tube was then placed in a GERSTEL TD 3.5+ for the analysis of FA methyl esters by GC-MS. Instrumental settings were as follows. TD 3.5+ initial temperature was programmed to have an initial temperature of 50 °C and then ramp to a final temperature of 260 °C (held for 3 min) at a rate of 100 °C/min. The transfer line temperature was set at 300 °C. During the thermal desorption, compounds were cryo-focused in a baffled glass liner CIS4 using the GERSTEL Cooled Injection System (CIS) under liquid nitrogen at -40 °C (equilibration time 0.5min). Once desorption was completed, the CIS was heated from -40 to 300 °C (held for 1 min) at a rate of 12 °C/s. The separation of all FAMEs analytes was completed on an Agilent 8890/5977B GC-MSD system (Agilent, CA, USA) fitted with a J&W HP-5MS ultra inert capillary column (0.25 mm × 30 m × 0.25 um, Agilent, CA, USA). The GC oven was programmed to have an initial temperature of 50°C which was first increased to 200 °C (held for 1 min) at a rate of 10°C/min, then the temperature was increased to 260 °C at a rate of 3 °C/min, and finally, the temperature was increased to 300 °C (held for 5 min) at a rate of 10 °C/min. Ultra-high purity helium was used as the carrier gas with a constant flow of 0.9 mL/min.

**2.3 Statistical Analysis**

T-test was performed using Microsoft Excel for Office 365 MSO to compare the means of the number of FAMEs found under various conditions while studying transesterification. Tukey test and ANOVA were performed using R to compare the recovery means of the 36 FAMEs across different times and percentages of $CH_3OH$.

# CHAPTER 3. RESULTS AND DISCUSSION

## 3.1 Sample Preparation

This section shows the results for the optimization of the transesterification process, for which several factors were studied. For transesterification, we studied the temperature effect on the formation of FAMEs (60-70 °C and 80-90 °C), as well as the effect of having different times of duration for the overall reaction (1 hr vs 2 hrs), and finally, we studied different quantities of the acid in the solvent mixture (10:1:1, $CH_3OH$:HCl:$CHCl_3$, v/v/v and 10:3:1, $CH_3OH$:HCl:$CHCl_3$, v/v/v). These conditions were studied using two available heating sources which were a sonicator and an oven. The performance of each condition was evaluated by the quantities of FAMEs which were determined by the instrument response on a GC-MS.

### 3.1.1 Transesterification

Two heating devices were used to determine the best transesterification temperatures for the fat samples: an oven and a sonicator. The studied parameters were temperature, transesterification time, and HCl ratio in the overall solvent mixture used for the transesterification. A total of six different conditions were investigated, t1:T60-70:A1, t2:T60-70:A1, t1:T60-70:A3, t2:T60-70:A3, t1:T80-90:A3, t2:T80-90:A3, where t stands for time, T stands for temperature, and A for the HCl used in the solvent mixture (A1: 10:1:1, $CH_3OH$:HCl:$CHCl_3$, v/v/v, and A3: 10:3:1, $CH_3OH$:HCl:$CHCl_3$, v/v/v)). Table 1 shows the number of FAMEs that were produced and detected by the conditions under investigation. Data collection and analysis protocols were detailed in Appendix 3. Only FAMEs that were identified by the library with a matching quality greater than 50% were considered in the data analysis. Samples were run in triplicates for each variable combination and the statistical analysis results are summarized in Appendix 4.

In table 1a., which shows the number of FAMEs obtained after transesterification using a sonicator for the reaction, the RSD (shown in parenthesis) of the different treatments was below 20%. Only one temperature setting was investigated using the sonicator (60-70 °C), due to the limitation of the instrument. Two solvent mixtures, A1 (10:1:1, $CH_3OH$:HCl:$CHCl_3$, v/v/v) and A3 (10:3:1, $CH_3OH$:HCl:$CHCl_3$, v/v/v), and two different transesterification times of 1hr and 2hrs were investigated for the experiments. Overall, t2:T60-70:A1 and t1:T60-70:A3 demonstrated the best performance to transesterify FAs into FAMEs. Using the t-test, we compared the resulting number of FAMEs detected under each condition against each other, and there was no significant difference between t1:T67:A1 and t2:T67:A1 (p=0.299), t1:T67:A1 and t1:T67:A3 (p=0.239), t1:T67:A1 and t2:T67:A3(p=0.184), t2:T60-70:A1 and t1:T60-70:A3 (p=0.420), t2:T67:A1 and t2:T67:A3 (p=0.059), as shown in Table 6 (Appendix 4). Nonetheless there was a significant difference between t1:T60-70:A3 and t2:T60-70:A3 (p=0.003). We also noticed that that higher HCl content, i.e. A3, produced a more consistent esterification, i.e. the RSD was decreased among the sampled variables. As there is no significant difference between t2:T60-70:A1 and t1:T60-70:A3, the transesterification reaction in a sonicator water bath at temperature of 60-70°C for one hour (for a shorter reaction time) and the solvent mix A3 (i.e. t1:T60-70:A3) was chosen as the optimized condition for transesterification when using the sonicator as the heating source.

**Table 1** Number of FAMEs from the transesterification of pork fat stock solution under various conditions. The two heat sources used for the formation of FAMEs were a) sonicator, and b) oven. Tables show the average number of FAMEs ± standard deviation that was detected after transesterification (n=3). The RSD is shown in parenthesis. The numbers in red indicate that there is a significant difference between the two values.

| **a) Sonicator** | | |
|---|---|---|
| **Time (hr)** | **Temperature (°C)** | |
| | **60-70** | |
| **1** | 18 ± 2.5 (14%) | 20 ± 1.5 (8%) |
| **2** | 21 ± 4.0 (20%) | 16 ± 1.0 (6%) |
| **A** | **1** | **3** |

| **b) Oven** | | | |
|---|---|---|---|
| **Time (hr)** | **Temperature (°C)** | | |
| | **60-70** | | **80-90** |
| **1** | 27 ± 5.7 (21%) | 21 ± 2.3 (11%) | 24 ± 2.3 (9%) |
| **2** | 24 ± 2.3 (10%) | 28 ± 4.9 (17%) | 20 ± 1.0 (5 %) |
| **A** | **1** | **3** | |

Table 1b. shows the data obtained for transesterification using an oven for the reaction. Two temperature variables were studied at 60-70 °C and 80-90 °C under the same solvent mixture systems and times as those tested in the sonication experiment. The highest number of FAMEs was produced under t2:T60-70:A3 followed by the results under t1:T60-70:A1. The higher temperature did not favor the degree of transesterification. Based on the t-test, there was a significant difference when comparing t1:T60-70:A3 to t2:T60-70:A3 (p=0.04); and no significant difference among any of the other conditions. The results showed that the conditions of temperature range 60-70 °C for two hours using the solvent mix A3 resulted in the greatest number of FAMEs (28 ± 5).

We noticed that the heating in the oven may produce a more consistent heat transfer to the reaction which, in turn, resulted in better transesterification of FAs into FAMEs. Table 1a had less number of recovered FAMEs when compared to the conditions on table 1b. Nevertheless, the RSD was lower for most of the conditions in Table 1a (t1:T67:A1, t1:T67:A3, t2:T67:A3) in contrast to Table 1b, except for one condition ( t2:T67:A1). In regard to reaction time, the production of FAMEs under 1 hour reaction time was generally equal to or better than the performance in 2 hours using sonicator as shown in table 1a. As for the oven heating experiment, two hours of the reaction was better for achieving a high number of FAMEs formed. This difference of time between the oven and the sonicator could be due to the active mixing of the sample happening in the sonicator, while in the oven the sample remains static through the heating process.

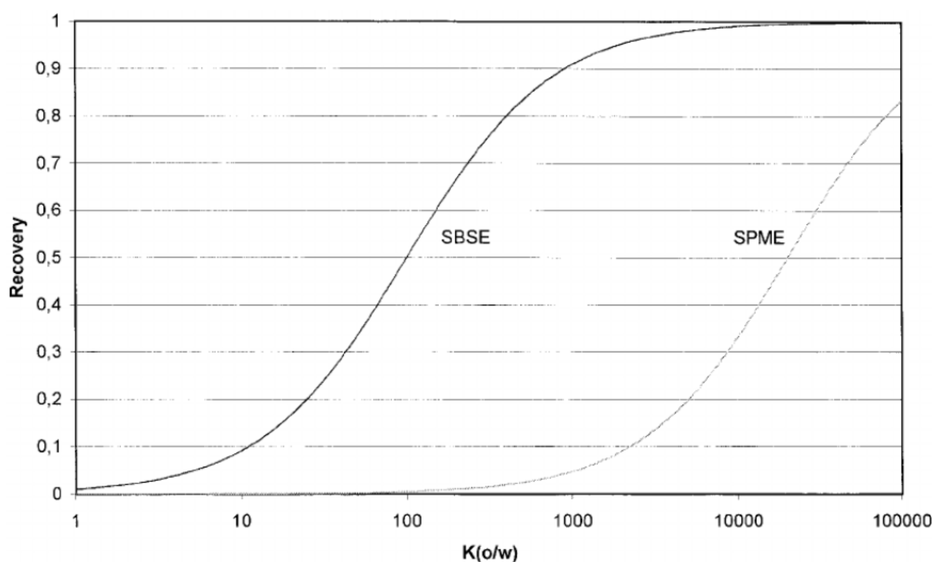
Overall a transesterification protocol was optimized for both the oven and the sonicator. When comparing the number of FAMEs in obtained in the best condition of the sonicator (t1:T60-70:A3) versus the best condition in the oven (t2:T60-70:A3) there was a significant difference between both results (p=0.028). But for safety concerns, the sonicator was selected as the heating

source of choice considering the volatility of the compounds used in the solvent mixture and the acids used in the reaction. A sonicator can be placed inside of a fume hood, making it a safer instrument to be used during the transesterification of FAs. Overall based on the findings it was decided that the condition for the optimal transesterification of FAs moving forward would be maintaining a temperature between 60-70 °C for 1 hr using a solvent mixture of 10:3:1, $CH_3OH$:HCl:$CHCl_3$, v/v/v using the sonicator.

### 3.2 FAMEs Extraction

After the esterification, FAMEs were extracted from the matrices prior to the chemical analysis. As showed in the schematic of Figure 1, the extraction of the FAMEs from the sample was achieved using SBSE. SBSE was first introduced to the analytical world of extractions by *Baltussen et al.* (1999). [42] The technique is solventless and acts to preconcentrate the analyte before instrumental analysis. It is similar to solid-phase micro-extraction (SPME), which is also a solvent-free sample preparation technique that includes the use of a solid sorbent in the form of a syringe for the extraction of the analyte of interest from the sample.[43] Nevertheless, the surface area occupied by the polymer is larger in SBSE than that in SPME, allowing a higher amount of analyte to be extracted. The theory behind the technique is based on the sorption of the analyte on to the specific polymer coating the stir bar (twister). The extraction then depends on the octanol-water partition coefficient ($K_{o/w}$, also expressed in Log scale, i.e. log P) of each specific analyte. SBSE is used for the extraction of medium-polar and non-polar compounds. In general, the theoretical extraction efficiency reaches 100% for solutes with log P greater than 2.7 (Figure 5).

**Figure 5** Recovery for solutes in the function of the octanol-water partitioning coefficient Ko/w for SPME (10 mL sample, 0.5 μL PDMS) and for SBSE (10 mL sample, 100 μL PDMS-fiber coated stir bar).[42]




To achieve the optimal extraction of a specific analyte, parameters such as the extraction phase (twister coating), the sampling mode (direct immersion, or headspace), and other extraction parameters (organic modifier or salt addition, pH adjustment, temperature, extraction time, stirring speed during the extraction) have to be considered. For this experiment, the extraction phase was set to be Polydimethylsiloxane (PDMS) which is the type of stir bar available in the laboratory, and the chosen sampling mode was direct immersion. A FAMEs standard containing 37 saturated and unsaturated FAMEs was used for these experiments. Table 2 shows the list of the 37 compounds tested. The pH adjustment and temperature as extraction parameters were not studied. In this section we studied the extraction parameters of solvent addition, stirring time, and stirring speed.

**Table 2** List of the chemical and physical characteristics of both saturated and unsaturated from the 37 FAMEs found in the standard solution. An explanation of how to read the shorthand notation can be found in Figure 9 (Appendix 5).

| 37 FAMEs from standard | Molecular Formula | Shorthand notation | BP (760 mm Hg) | Log P ($K_{o/w}$) |
|---|---|---|---|---|
| Butanoic acid, methyl ester | $C_5H_{10}O_2$ | C4:0 | 102.8 | 1.295 |
| Hexanoic acid, methyl ester | $C_7H_{14}O_2$ | C6:0 | 149.5 | 2.314 |
| Octanoic acid, methyl ester | $C_9H_{18}O_2$ | C8:0 | 192.9 | 3.333 |
| Decanoic acid, methyl ester | $C_{11}H_{22}O_2$ | C10:0 | 224 | 4.352 |
| Undecanoic acid, methyl ester | $C_{12}H_{24}O_2$ | C11:0 | 246 | 4.861 |
| Dodecanoic acid, methyl ester | $C_{13}H_{26}O_2$ | C12:0 | 267 | 5.371 |
| Tridecanoic acid, methyl ester | $C_{14}H_{28}O_2$ | C13:0 | 289.6 | 5.88 |
| Methyl myristoleate | $C_{15}H_{28}O_2$ | C14:1 [cis-9] | 306.6 | 5.98 |
| Methyl tetradecanoate | $C_{15}H_{30}O_2$ | C14:0 | 295 | 6.39 |
| Methyl (Z)-10-pentadecenoate | $C_{16}H_{30}O_2$ | C15:1 [cis-10] | 320.9 | 6.49 |
| Pentadecanoic acid, methyl ester | $C_{16}H_{32}O_2$ | C15:0 | 309.3 | 6.899 |
| 9-Hexadecenoic acid, methyl ester, (Z)- | $C_{17}H_{32}O_2$ | C16:1 [cis-9] | 394.2 | 6.999 |
| Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | C16:0 | 332.1 | 7.409 |
| cis-10-Heptadecenoic acid, methyl ester | $C_{18}H_{34}O_2$ | C17:1 [cis-10] | 353.1 | 7.509 |
| Heptadecanoic acid, methyl ester | $C_{18}H_{36}O_2$ | C17:0 | 337.1 | 7.918 |
| Methyl .gamma.-linolenate | $C_{19}H_{32}O_2$ | C18:3 [cis-6,9,12] | 385.4 | 7.111 |
| 9,12-Octadecadienoic acid, methyl ester, (E,E)- | $C_{19}H_{34}O_2$ | C18:2 [trans-9,12] | 373.3 | 7.615 |
| 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | $C_{19}H_{34}O_2$ | C18:2 [cis-9,12] | 373.3 | 7.615 |
| 9-Octadecenoic acid, methyl ester, (E)- | $C_{19}H_{36}O_2$ | C18:1 [trans-9] | 351.4 | 8.018 |
| 9-Octadecenoic acid (Z)-, methyl ester | $C_{19}H_{36}O_2$ | C18:1 [cis-9] | 351.4 | 8.018 |
| Methyl stearate | $C_{19}H_{38}O_2$ | C18:0 | 355.5 | 8.428 |
| 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)- | $C_{21}H_{34}O_2$ | C20:4 [cis-5,8,11,14] | 403.9 | 7.628 |
| 5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)- | $C_{21}H_{32}O_2$ | C20:5 [cis-5,8,11,14,17] | 402.8 | 7.132 |
| 8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)- | $C_{21}H_{36}O_2$ | C20:3 [cis-8,11,14] | 405 | 8.138 |
| cis-11,14-Eicosadienoic acid, methyl ester | $C_{21}H_{38}O_2$ | C20:2 [cis-11,14] | 396.6 | 8.634 |
| 11,14,17-Eicosatrienoic acid, methyl ester | $C_{21}H_{36}O_2$ | C20:3 [cis-11,14,17] | 398.9 | 8.138 |
| Eicosanoic acid, methyl ester | $C_{21}H_{42}O_2$ | C20:0 | 375 | 9.447 |
| cis-Methyl 11-eicosenoate | $C_{21}H_{40}O_2$ | C20:1 [cis-11] | 394.3 | 9.037 |
| 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- | $C_{19}H_{32}O_2$ | C18:3 [cis-9,12,15] | 364.4 | 7.119 |
| Heneicosanoic acid, methyl ester | $C_{22}H_{44}O_2$ | C21:0 | 386.7 | 9.956 |
| 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)- | $C_{23}H_{34}O_2$ | C22:6 [cis-4,7,10,13,16,19] | 429.9 | 7.645 |
| cis-13,16-Docasadienoic acid, methyl ester | $C_{23}H_{42}O_2$ | C22:2 [cis-13,16] | 425.1 | 9.653 |
| 13-Docosenoic acid, methyl ester, (Z)- | $C_{23}H_{44}O_2$ | C22:1 [cis-13] | 422.9 | 10.056 |
| Docosanoic acid, methyl ester | $C_{23}H_{46}O_2$ | C22:0 FAME | 398 | 10.466 |
| Tricosanoic acid, methyl ester | $C_{24}H_{48}O_2$ | C23:0 | 408.9 | 10.975 |
| 15-Tetracosenoic acid, methyl ester, (Z)- | $C_{25}H_{48}O_2$ | C24:1 [cis-15] | 450.3 | 11.075 |
| Tetracosanoic acid, methyl ester | $C_{25}H_{50}O_2$ | C24:0 | 419.5 | 11.485 |

### 3.2.1 Solvent Addition and Stirring Time

Solvent addition and stirring time were analyzed together as possible variables that had the capability of improving the extraction efficiency of FAMEs (Figure 6). The use of an organic solvent in the sample matrix can increase the solubility of non-polar compounds (log p >3) by minimizing the interaction of the analyte to the container (e.g. glass vial).[42] Tables 4 & 5 (Appendix 1 & 2) show a detailed list of the physical and chemical characteristics of the FAMEs analyzed in this project. The Log P ($K_{o/w}$) is a measurement that allows us to understand the hydrophilicity of a compound. Since most of the FAMEs have a Log P>3, it was considered that the use of an organic solvent would help to maximize the recovery of the analytes. $CH_3OH$ was the solvent of choice for these experiments. The addition of $CH_3OH$ to the sample matrix was studied at 0%, 5%, 10%, and 30% of the total solvent composition. The statistical analysis of the experiment is shown in Table 6 (Appendix 4). Results indicate that all $CH_3OH$ conditions were significantly different as compared to 0% $CH_3OH$. After looking at the peak area (i.e. the instrument response) of each compound under every condition, it was concluded that the extraction of most of the FAMEs from the matrix solution was achieved with better efficiency without adding $CH_3OH$ to the extraction matrix. It could be due to the fact that $CH_3OH$ also increases the solubility of FAMEs in the solvent, hence reduce the extraction of those compounds onto the stir bar.

Stirring time was also evaluated since it is known that extraction time plays an important role in extraction effectiveness.[42] In Figure 6 every $CH_3OH$ condition was evaluated at the stirring times of 1 hr, 2 hr, 3 hr, 6 hr, and 12 hr. The effect of the studied variables was analyzed in each of the compounds both individually and as a group. Figure 6a shows the response of each FAMEs found in the sample while stirring for 1hr and having different percentages of $CH_3OH$ in the extraction matrix. Figure 6a had the highest response for the majority of the compounds at 0%

$CH_3OH$. Figure 6b shows the response of each FAMEs found in the sample while stirring for 2 hrs and having different percentages of $CH_3OH$ in the extraction matrix. Figure 6b had the highest response at 0% $CH_3OH$ for the majority of the compounds. Figure 6c shows the response of each FAMEs found in the sample while stirring for 3hr and having different percentages of $CH_3OH$ in the extraction matrix. Figure 6c had a good response for small FAMEs chains through the samples with different percentages of $CH_3OH$, but the overall best response for the majority of the compounds was at 0% $CH_3OH$. Figure 6d shows the response of each FAMEs found in the sample while stirring for 6hr and having different percentages of $CH_3OH$ in the extraction matrix. Figure 6d had the highest response for the majority of the compounds at 0% $CH_3OH$. Figure 6e shows the response of each FAMEs found in the sample while stirring for 12hr and having different percentages of $CH_3OH$ in the extraction matrix. Figure 6e had the highest response for small chain FAMEs at 30% $CH_3OH$.

The statistical analysis summary can be found in Table 7 (Appendix 6). There was no significant difference found for the majority of the compounds at different times, therefore out of effectiveness the shortest time was selected as the condition for the extraction of FAMEs using SBES. ANOVA results showed a significant difference between $CH_3OH$ and Stirring time, meaning that the $CH_3OH$ contents had a significant difference in the extraction efficiency while stirring times did not have much impact on the recovery.

After the statistical analysis and looking over the responses of the FAMEs through the different variables, it was concluded that the best condition would be to have no $CH_3OH$ as part of the sample matrix during SBSE. Also, it was concluded that 1hr would be the ideal time to achieve a good response from all FAMEs being extracted from the sample while using SBSE.

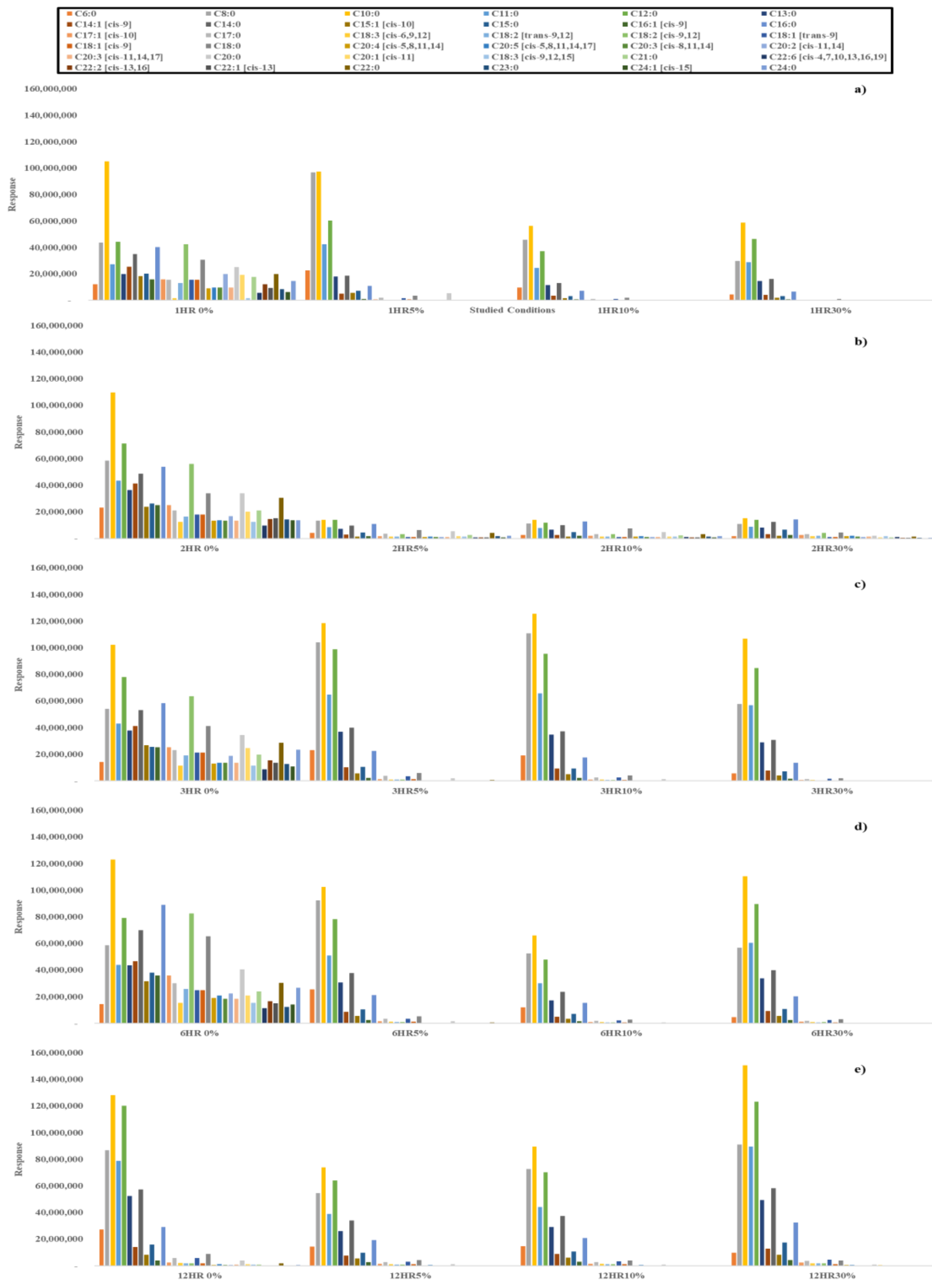



**Figure 6** Comparison of FAMEs recoveries using SBSE across different stirring times and $CH_3OH$ percentages. Each graph shows a stirring time for the extraction of each of the 36 FAMEs found in the standard stock solution, at times of **a)**1 hr, **b)**2 hrs, **c)**3 hrs, **d)**6 hrs, and **e)**12 hrs. The presence of different percentages of $CH_3OH$ (0%, 5%, 10%, and 30%) in the extraction matrix was evaluated as a possible factor to increase the recovery of FAMEs with Log P >3 (n=6).

### 3.2.2 Stirring Speed

The stirring speed during extraction was analyzed as a possible variable to improve the extraction efficiency of FAMEs. Based on previous results, 1 hour of stirring time was used in the study of the effect of stirring speed on the recovery. To avoid excessive or too little agitation, the studied speeds were 700, 1000, and 1500 RPM, which are normally the recommended speeds.[42] As shown in Figure 7, the recovery of each FAME compound was impacted by the different stirring speeds. Also, it was observed that the recovery of smaller chains of FAMEs was higher compared to the recovery of larger chains of FAMEs through all stirring speeds.

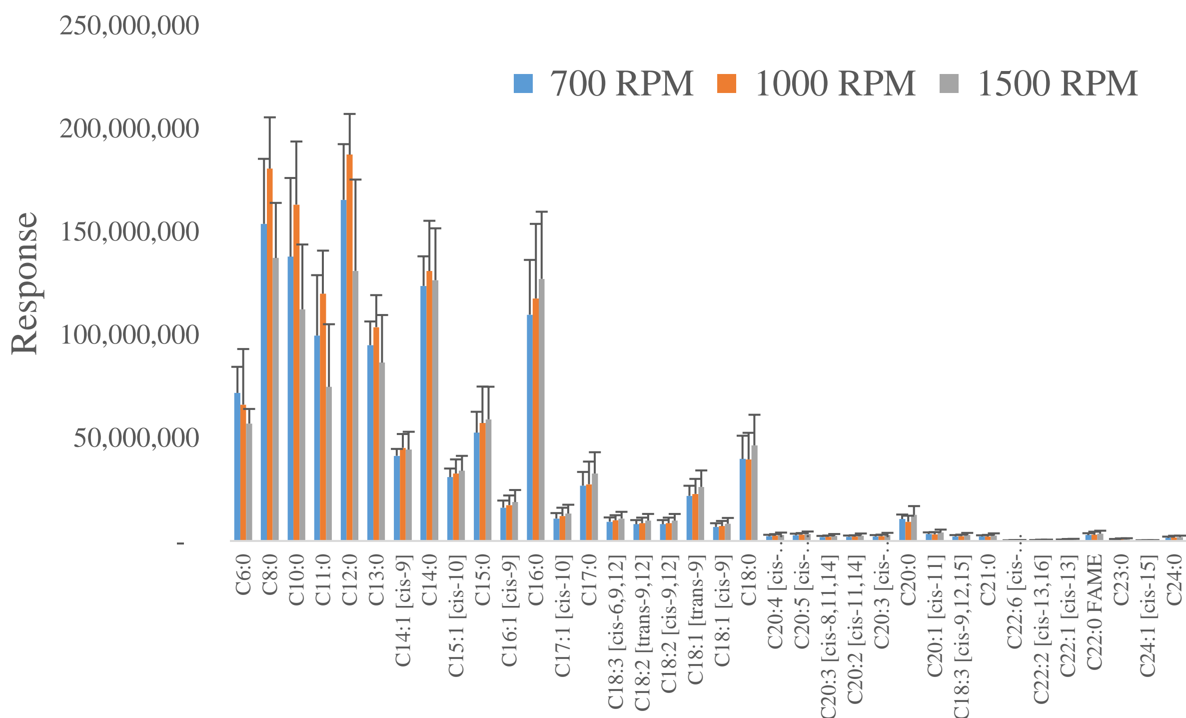



**Figure 7** Recovery of FAMEs using SBSE. The extraction was studied at 700 RPM, 1000 RPM, 1500 RPM for the duration of 1hour. (n=4)

Taking the average mean of the total response for the compounds at different stirring speeds, a t-test looked at the statistical difference between these means. The statistical analysis results in Table 3 showed that there was a large significant difference (p-value= 0.004) between

using 700 RPM and 1500 RPM; also there was a slightly significant difference (p-value= 0.035) between using 1000 RPM and 1500 RPM as stirring speed, but no significant difference (p-value=0.182) between using 1000 RPM and 700 RPM as stirring speed. When looking at individual compounds and comparing them through different stirring speeds Table 8 (Appendix 7) it was observed that at higher speed (1500 RPM) promotes the recovery of long-chain FAMEs from the sample. Therefore, 1500 RPM was the stirring speed chosen for the method.

**Table 3** Statistical analysis summary for stirring speed experiments. T-test showed that there was a significant difference (p-value<0.05) between using 700 RPM or 1500 RPM, and between 1000 RPM and 1500 RPM.

| T-test (p-value) | | |
|---|---|---|
| **RPM** | **1000** | **1500** |
| **700** | 0.182 | 0.004 |
| **1000** | | 0.035 |

Finally, we attempted to use the optimized methodology to estimate the concentration of the FAMEs extracted from pork fat. During SBSE procedures, 1 μL of the stock standard (at $2.6512\times10^3$ ppm, mg/L) was diluted in 20 mL of water giving the final concentration of the solution at 0.132 ppm (mg/L). This concentration and the percent contribution of each of FAMEs in the standard[44] were then used to calculate the concentration of each FAMEs in the SBSE solution. The response obtained from each extracted FAMEs was assumed to account for 100% extraction efficacy for that compound. Using the relationship between the concentration of each FAME in the standard solution and their instrument response, we were then able to estimate the concentration of the FAMEs extracted from pork fat by doing a linear extrapolation.

Table 9 shows the calculated concentrations of FAMEs in the standard solution and the instrument response, which is the area below the peak integrated by the instrument data analysis software. When analyzing the extract from port fat, we obtained the instrument responses of each FAME, compared the response to the FAMEs in the standard, and roughly calculated the concentration in the fat extract. Our estimation showed that the FAMEs in the fat tissue have concentrations from 0.18 ppb (µg/L) to 114.82 ppb (µg/L). The response of the FAME with the lowest concentration was still very high (i.e. almost $3\times10^6$ counts). This allowed us to predict that the limit of detection could be lower than 0.18 ppb (µg/L). This also indicated that we could use much less amount of sample during transesterification from the current 10 mg fat tissue. This is a substantial attribute, especially when dealing with human tissue samples which often come with a small amount available for the analysis. Thus, this estimate gives us the confidence that the optimized method developed in this project could be a sensitive analytical tool for our future study in PPAT in prostate cancer research.

**Table 4** Estimated concentrations of the extracted FAMEs from pork fat. First, the concentration for the standard solution was calculated for all FAMEs. These calculated concentrations were then used to estimate (based on the response of the FAMEs obtained from the sonicated pork fat) the concentration of the recovered FAMEs. The concentrations detected ranged from 0.18 ppb (µg/L) to 114.82 ppb (µg/L).

| | Extract from standard solution | | Extract from Pork fat | |
|---|---|---|---|---|
| FAMEs | Instrument Response (Area) | Calculated concentration (ppb) | Instrument Response (Area) | Estimated concentration (ppb) |
| Hexanoic acid, methyl ester | 56,942,599 | 5.28 | 1,238,327,699 | 114.82 |
| Octanoic acid, methyl ester | 137,232,460 | 5.28 | 1,047,539,122 | 40.30 |
| Decanoic acid, methyl ester | 112,324,947 | 5.28 | 277,513,385 | 13.04 |
| Dodecanoic acid, methyl ester | 130,969,624 | 5.28 | 58,185,748 | 2.35 |
| Methyl myristoleate | 44,306,252 | 2.64 | 2,939,322 | 0.18 |
| Methyl tetradecanoate | 126,446,694 | 5.28 | 30,615,993 | 1.28 |
| 9-Hexadecenoic acid, methyl ester, (Z)- | 18,854,006 | 2.64 | 5,183,963 | 0.73 |
| Hexadecanoic acid, methyl ester | 127,111,517 | 7.92 | 51,651,523 | 3.22 |
| 9-Octadecenoic acid, methyl ester, (E)- | 26,213,122 | 2.64 | 34,566,586 | 3.48 |
| Heptadecanoic acid, 16-methyl-, methyl ester | 46,358,035 | 5.28 | 12,005,919 | 1.37 |

# CHAPTER 4. CONCLUSIONS

This project developed a methodology for the transesterification and extraction of FAMEs from adipose tissue as shown in Figure 9. Pork fat was used as the model adipose tissue during the development of the methodology. For transesterification studies, the variables studied were solvent mixture composition, transesterification time, and heating method. Furthermore, for the improvement of the SBSE extraction process, the studied variables were stirring time, stirring speed, and solvent composition.

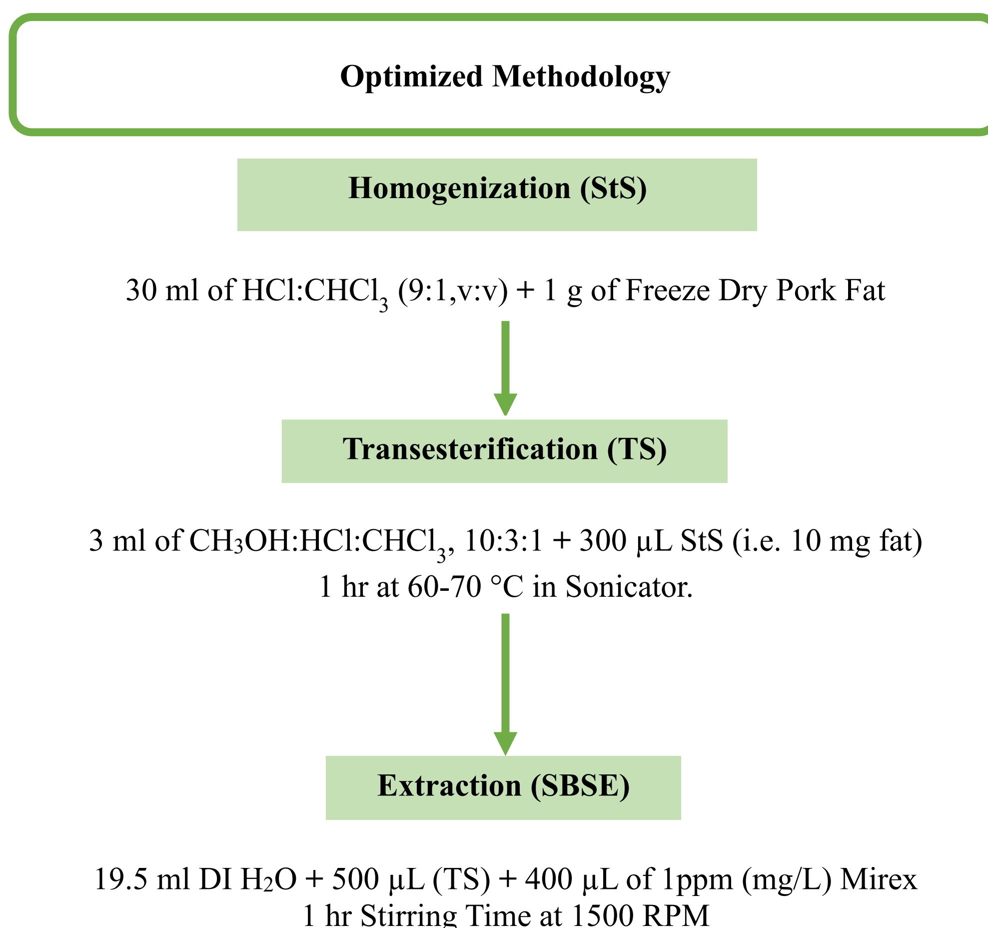



**Figure 8** Diagram showing the steps for the optimized method for the analysis of FAs in fat. StS: Stock sample after homogenization; TS: Transesterified sample.

Based on our results, we chose the solvent mixture $CH_3OH$:HCl:$CHCl_3$, 10:3:1 (v/v/v) as the solvent condition tested for the transesterification as it gave the most number of FAs extracted from the fat while using the sonicator as the heating source for1 hr. The transesterification could take place in either heating source, nevertheless, using the sonicator was recommended over the oven for safety concern, since a sonicator could be used inside the hood during transesterification of the samples to avoid the emission of harmful chemical fumes.

For SBSE there was no significant difference found across different extraction times, therefore it was decided that 1hr was the best stirring time to be used. There was a significant difference found among various $CH_3OH$ contents, with 0% $CH_3OH$ giving the highest recovery for the majority of the compounds.

These results from this study demonstrated a green chemistry approach for the analysis of FAs (in forms of FAMEs) in adipose tissue samples. In contrast to previous methodologies, this method used a solventless (i.e. green chemistry) extraction techniques by using SBSE. As compared to the existing methods, the optimized method used no organic solvent and minimal labor during the extraction process. Also, the sample size required for the developed methodology was less (10 mg) than that reported in previously developed methodologies (15 – 1000 mg shown in Figure 2).

Based on our estimation, the optimized method could be capable of detecting FAMEs at a concentration below 0.18 ppb (µg/L). The amount of fat tissue used in this study was 10 mg. This finding indicated that we could potentially handle the analysis of FAs in a PPAT sample as small as 1 mg or less. This will be an important benefit for this kind of study when the mass of samples is limited.

Overall, the project has laid a foundation for an easy, green, and sensitive method for the analysis of FAs in fat tissue. To our knowledge, it is the first methodology currently developed that uses SBSE as the extraction technique. For future improvement, we will continue working on determining the linearity and the method detection limit. In the future, this project could become a tool to study the profile of FAs in PPAT for a better understanding of the role of FAs in prostate cancer progression and aggressiveness.

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# APPENDICES

## APPENDIX 1

**Table 5** Saturated FAMEs chemical and physical characteristics. The retention time (RT) for each compound according to the developed methodology is also shown.

| Saturated FAMEs from standard | CAS # | Molecular Formula | #C | Shorthand notation | BP (760 mm Hg) | Log P ($K_{o/w}$) | RT |
|---|---|---|---|---|---|---|---|
| Butanoic acid, methyl ester | 623-42-7 | $C_5H_{10}O_2$ | 4 | C4:0 | 102.8 | 1.295 | |
| Hexanoic acid, methyl ester | 000106-70-7 | $C_7H_{14}O_2$ | 6 | C6:0 | 149.5 | 2.314 | 8.31 |
| Octanoic acid, methyl ester | 000111-11-5 | $C_9H_{18}O_2$ | 8 | C8:0 | 192.9 | 3.333 | 12.20 |
| Decanoic acid, methyl ester | 000110-42-9 | $C_{11}H_{22}O_2$ | 10 | C10:0 | 224 | 4.352 | 15.25 |
| Undecanoic acid, methyl ester | 001731-86-8 | $C_{12}H_{24}O_2$ | 11 | C11:0 | 246 | 4.861 | 16.59 |
| Dodecanoic acid, methyl ester | 000111-82-0 | $C_{13}H_{26}O_2$ | 12 | C12:0 | 267 | 5.371 | 17.86 |
| Tridecanoic acid, methyl ester | 001731-88-0 | $C_{14}H_{28}O_2$ | 13 | C13:0 | 289.6 | 5.88 | 19.04 |
| Methyl tetradecanoate | 000124-10-7 | $C_{15}H_{30}O_2$ | 14 | C14:0 | 295 | 6.39 | 20.18 |
| Pentadecanoic acid, methyl ester | 007132-64-1 | $C_{16}H_{32}O_2$ | 15 | C15:0 | 309.3 | 6.899 | 21.44 |
| Hexadecanoic acid, methyl ester | 000112-39-0 | $C_{17}H_{34}O_2$ | 16 | C16:0 | 332.1 | 7.409 | 22.90 |
| Heptadecanoic acid, methyl ester | 001731-92-6 | $C_{18}H_{36}O_2$ | 17 | C17:0 | 337.1 | 7.918 | 24.57 |
| Methyl stearate | 000112-61-8 | $C_{19}H_{38}O_2$ | 18 | C18:0 | 355.5 | 8.428 | 26.44 |
| Eicosanoic acid, methyl ester | 001120-28-1 | $C_{21}H_{42}O_2$ | 20 | C20:0 | 375 | 9.447 | 30.62 |
| Heneicosanoic acid, methyl ester | 6064-90-0 | $C_{22}H_{44}O_2$ | 21 | C21:0 | 386.7 | 9.956 | 32.85 |
| Docosanoic acid, methyl ester | 000929-77-1 | $C_{23}H_{46}O_2$ | 22 | C22:0 | 398 | 10.466 | 35.13 |
| Tricosanoic acid, methyl ester | 002433-97-8 | $C_{24}H_{48}O_2$ | 23 | C23:0 | 408.9 | 10.975 | 37.41 |
| Tetracosanoic acid, methyl ester | 002442-49-1 | $C_{25}H_{50}O_2$ | 24 | C24:0 | 419.5 | 11.485 | 39.67 |

## APPENDIX 2

**Table 6** Unsaturated FAMEs chemical and physical characteristics. The retention time (RT) for each compound according to the developed methodology is also shown.

| Unsaturared FAMEs from standard | CAS # | Molecular Formula | #C | Shorthand notation | Unsaturations | | BP (760 mm Hg) | Log P ($K_{o/w}$) | RT |
|---|---|---|---|---|---|---|---|---|---|
| Methyl myristoleate | 056219-06-8 | $C_{15}H_{28}O_2$ | 14 | C14:1 | [cis-9] | mono | 306.6 | 5.98 | 20.05 |
| Methyl (Z)-10-pentadecenoate | 1000426-92-2 | $C_{16}H_{30}O_2$ | 15 | C15:1 | [cis-10] | mono | 320.9 | 6.49 | 21.29 |
| 9-Hexadecenoic acid, methyl ester, (Z)- | 001120-25-8 | $C_{17}H_{32}O_2$ | 16 | C16:1 | [cis-9] | mono | 394.2 | 6.999 | 22.60 |
| cis-10-Heptadecenoic acid, methyl ester | 1000333-62-1 | $C_{18}H_{34}O_2$ | 17 | C17:1 | [cis-10] | mono | 353.1 | 7.509 | 24.23 |
| Methyl .gamma.-linolenate | 016326-32-2 | $C_{19}H_{32}O_2$ | 18 | C18:3 | [cis-6,9,12] | poly | 385.4 | 7.111 | 25.56 |
| 9,12-Octadecadienoic acid, methyl ester, (E,E)- | 002566-97-4 | $C_{19}H_{34}O_2$ | 18 | C18:2 | [trans-9,12] | poly | 373.3 | 7.615 | 25.85 |
| 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | 000112-63-0 | $C_{19}H_{34}O_2$ | 18 | C18:2 | [cis-9,12] | poly | 373.3 | 7.615 | 25.85 |
| 9-Octadecenoic acid, methyl ester, (E)- | 001937-62-8 | $C_{19}H_{36}O_2$ | 18 | C18:1 | [trans-9] | mono | 351.4 | 8.018 | 25.97 |
| 9-Octadecenoic acid (Z)-, methyl ester | 000112-62-9 | $C_{19}H_{36}O_2$ | 18 | C18:1 | [cis-9] | mono | 351.4 | 8.018 | 26.08 |
| 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)- | 002566-89-4 | $C_{21}H_{34}O_2$ | 20 | C20:4 | [cis-5,8,11,14] | poly | 403.9 | 7.628 | 29.18 |
| 5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)- | 002734-47-6 | $C_{21}H_{32}O_2$ | 20 | C20:5 | [cis-5,8,11,14,17] | poly | 402.8 | 7.132 | 29.33 |
| 8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)- | 021061-10-9 | $C_{21}H_{36}O_2$ | 20 | C20:3 | [cis-8,11,14] | poly | 405 | 8.138 | 29.56 |
| cis-11,14-Eicosadienoic acid, methyl ester | 1000333-61-8 | $C_{21}H_{38}O_2$ | 20 | C20:2 | [cis-11,14] | poly | 396.6 | 8.634 | 29.96 |
| 11,14,17-Eicosatrienoic acid, methyl ester | 055682-88-7 | $C_{21}H_{36}O_2$ | 20 | C20:3 | [cis-11,14,17] | poly | 398.9 | 8.138 | 30.11 |
| cis-Methyl 11-eicosenoate | 002390-09-2 | $C_{21}H_{40}O_2$ | 20 | C20:1 | [cis-11] | mono | 394.3 | 9.037 | 30.07 |
| 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- | 000301-00-8 | $C_{19}H_{32}O_2$ | 18 | C18:3 | [cis-9,12,15] | poly | 364.4 | 7.119 | 30.11 |
| 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)- | 2566-90-7 | $C_{23}H_{34}O_2$ | 22 | C22:6 | [cis-4,7,10,13,16,19] | poly | 429.9 | 7.645 | 33.41 |
| cis-13,16-Docasadienoic acid, methyl ester | 1000333-60-3 | $C_{23}H_{42}O_2$ | 22 | C22:2 | [cis-13,16] | poly | 425.1 | 9.653 | 34.46 |
| 13-Docosenoic acid, methyl ester, (Z)- | 001120-34-9 | $C_{23}H_{44}O_2$ | 22 | C22:1 | [cis-13] | mono | 422.9 | 10.056 | 34.54 |
| 15-Tetracosenoic acid, methyl ester, (Z)- | 002733-88-2 | $C_{25}H_{48}O_2$ | 24 | C24:1 | [cis-15] | mono | 450.3 | 11.075 | 39.12 |

**APPENDIX 3**

Protocol for Data Analysis of Transesterification Experiments

1. Data Extraction from the Chromatogram.
   i. Instrument Analysis
   ii. File<Load the File
   iii. Integrate
   iv. Spectrum<Percent Report
   v. Chromatography<Library Search Report
2. Filter the Extracted Data.
   i. Copy the data from the GC-MS computer to the Excel.
   ii. Organize the data by peak number, retention time, area, library, CAS, and quality.
   iii. Filter Mirex and methyl esters from data.
   iv. Deal with the replicated compounds
      - Find out the compound with the highest quality.
      - Delete the compound(s) with quality <10% of that of the highest quality peak.
      - Consider the retention time. If the difference between the retention times of peaks identified with the same CAS# is more than 0.5s, delete the one with lower quality and keep the one with higher quality (when the qualities are the same, keep the one with higher amount of area).
      - Mark in the compound list of the sample, the compound(s) which can be found in empty TDT and control samples. Then delete the compound in the sample list, if it has the same area, as the compound from empty TDT or control.
   v. Look over the quality and the retention time of every methyl ester, then delete those which have the quality <50%.
   vi. Count the number of methyl esters of every sample.

## APPENDIX 4

**Table 7** Statistical analysis summary for transesterification experiments. Temperature (T), time (t), and HCl (A) amount in the solvent mix were the studied variables. The studied variables were a) t1:T60-70:A1, b) t2:T60-70:A1, c) t1:T60-70:A3, d) t2:T60-70:A3, e) t1:T80-90:A3, f)t2:T80-90:A3. T-test showed a significant difference (p-value<0.05) when comparing **c vs d** in both oven and sonicator.

| Studied variables | T-test (p-value) | |
|---|---|---|
| | Sonication | Oven |
| a vs b | 0.299 | 0.225 |
| a vs c | 0.239 | 0.160 |
| a vs d | 0.184 | 0.386 |
| a vs e | - | 0.322 |
| a vs f | - | 0.067 |
| b vs c | 0.420 | 0.162 |
| b vs d | 0.059 | 0.190 |
| b vs e | - | 0.333 |
| b vs f | - | 0.064 |
| c vs d | 0.003 | 0.041 |
| c vs e | - | 0.055 |
| c vs f | - | 0.377 |
| d vs e | - | 0.191 |
| d vs f | - | 0.053 |
| e vs f | - | 0.072 |

**APPENDIX 5**

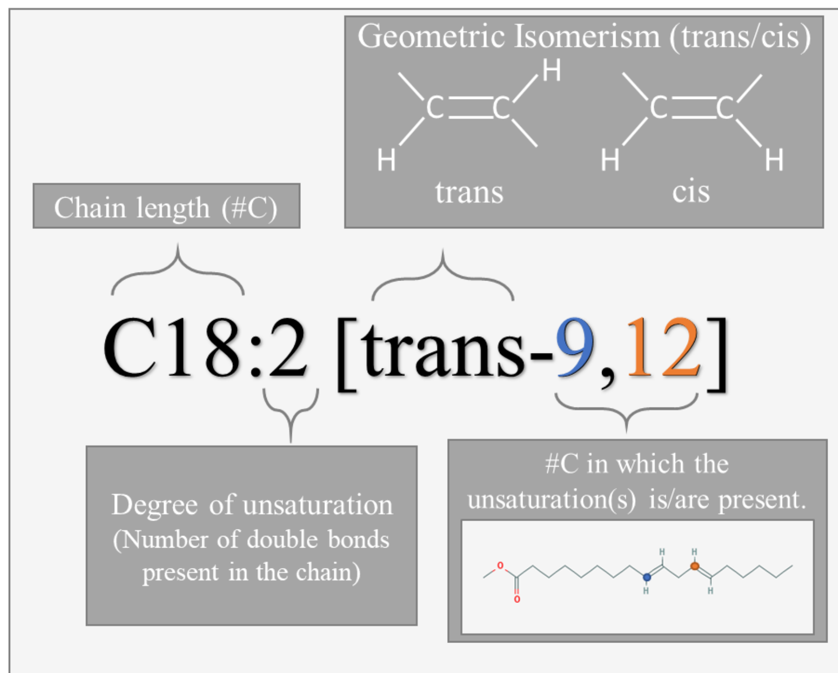


**Figure 9** This figure gives a short explanation of the shorthand notation used for naming FAMEs. The number after C represents the number of carbons present on the chain (this including the methyl group present on the ester side). The next number represents the degree of unsaturation of specific FAME. The next portion on the brackets gives further specifications about the unsaturations present in the carbon chain. If the unsaturations are on a cis or trans mode, and where are the unsaturations located throughout the chain. The FAME structure showed on the white box was obtained from PubChem.[31]

## APPENDIX 6

**Table 8** Statistical analysis summary for SBSE stirring time and solvent addition experiments. ANOVA test showed a significant difference (f-value<0.05) between CH3OH and time. Tukey test showed that there was no significant difference between different times, nevertheless, there was a significant difference (p-value<0.05) among different percentages of CH3OH in the sample matrix.

| Name | Tukey test (p-value) | | | | | | | | | | | | | | | | ANOVA f-value | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MeOH | | | | | | Time | | | | | | | | | | | |
| | 0.05-0% | 0.1-0% | 0.3-0% | 0.1-0.05% | 0.3-0.05% | 0.3-0.1% | 1 to 12 | 2 to 12 | 3 to 12 | 6 to 12 | 2 to 1 | 3 to 1 | 6 to 1 | 3 to 2 | 6 to 2 | 6 to 3 | MeOH | Time |
| Mirex | 0.0356 | 0.0592 | 0.7910 | 0.9910 | 0.1680 | 0.2620 | 0.9530 | 0.2900 | 1.0000 | 0.7100 | 0.0999 | 0.9460 | 0.3340 | 0.3030 | 0.9250 | 0.7270 | 0.0220 | 0.0990 |
| 000106-70-7 Hexanoic acid, methyl ester | 1.0000 | 0.3820 | 0.0293 | 0.4180 | 0.0331 | 0.4080 | 0.8690 | 0.3450 | 0.9990 | 0.9820 | 0.8540 | 0.9440 | 0.9920 | 0.4550 | 0.6310 | 0.9980 | 0.3670 | 0.0199 |
| 000111-11-5 Octanoic acid, methyl ester | 0.8710 | 0.9990 | 0.8940 | 0.8200 | 0.4870 | 0.9330 | 0.7150 | 0.0685 | 0.9980 | 0.9660 | 0.4510 | 0.5380 | 0.9670 | 0.0405 | 0.1900 | 0.8740 | 0.0428 | 0.5578 |
| 000110-42-9 Decanoic acid, methyl ester | 0.3100 | 0.1230 | 0.5100 | 0.9260 | 0.9770 | 0.7450 | 0.5450 | 0.0233 | 1.0000 | 0.9860 | 0.2920 | 0.4670 | 0.8230 | 0.0182 | 0.0550 | 0.9640 | 0.0144 | 0.1486 |
| 001731-86-8 Undecanoic acid, methyl ester | 0.9310 | 0.6170 | 0.9990 | 0.9170 | 0.8740 | 0.5270 | 0.0973 | 0.0130 | 0.9900 | 0.6180 | 0.7610 | 0.2000 | 0.6660 | 0.0286 | 0.1470 | 0.8590 | 0.0099 | 0.5157 |
| 000111-82-0 Dodecanoic acid, methyl ester | 0.6500 | 0.2470 | 0.9500 | 0.8510 | 0.9140 | 0.4930 | 0.0461 | 0.0049 | 0.9960 | 0.6270 | 0.6900 | 0.0841 | 0.4090 | 0.0090 | 0.0553 | 0.8220 | 0.0029 | 0.2738 |
| 001731-88-0 Tridecanoic acid, methyl ester | 0.0540 | 0.0131 | 0.1560 | 0.8490 | 0.9170 | 0.4950 | 0.0080 | 0.0050 | 0.9090 | 0.6030 | 0.9990 | 0.0342 | 0.0958 | 0.0214 | 0.0608 | 0.9710 | 0.0018 | 0.0162 |
| 056219-06-8 Methyl myristoleate | 0.0005 | 0.0003 | 0.0006 | 0.9970 | 0.9990 | 0.9850 | 0.9990 | 0.9970 | 0.7480 | 0.7250 | 0.6020 | 0.5780 | 0.8980 | 0.8820 | 1.0000 | 0.4653 | 0.0001 | 0.0000 |
| 000124-10-7 Methyl tetradecanoate | 0.0023 | 0.0007 | 0.0070 | 0.8860 | 0.9080 | 0.5280 | 0.0053 | 0.0047 | 0.7890 | 0.9560 | 1.0000 | 0.0366 | 0.0171 | 0.0323 | 0.0150 | 0.9910 | 0.0010 | 0.0006 |
| 1000426-92-2 Methyl (Z)-10-pentadecenoate | 0.0008 | 0.0004 | 0.0007 | 0.9860 | 1.0000 | 0.9940 | 1.0000 | 1.0000 | 0.8620 | 0.7010 | 1.0000 | 0.8500 | 0.6840 | 0.8900 | 0.7380 | 0.9970 | 0.5559 | 0.0002 |
| 007132-64-1 Pentadecanoic acid, methyl ester | 0.0011 | 0.0005 | 0.0014 | 0.9630 | 0.9990 | 0.9280 | 0.6400 | 0.9230 | 1.0000 | 0.8940 | 0.9740 | 0.6820 | 0.2130 | 0.9440 | 0.4670 | 0.8640 | 0.2593 | 0.0003 |
| 001120-25-8 9-Hexadecenoic acid, methyl ester, (Z)- | 0.0018 | 0.0017 | 0.0020 | 1.0000 | 1.0000 | 0.9990 | 0.9990 | 0.8510 | 0.8430 | 0.4880 | 0.9420 | 0.9380 | 0.6350 | 1.0000 | 0.9600 | 0.9640 | 0.5022 | 0.0006 |
| 000112-39-0 Hexadecanoic acid, methyl ester | 0.0021 | 0.0013 | 0.0023 | 0.9910 | 1.0000 | 0.9830 | 0.8260 | 0.9980 | 0.9980 | 0.7060 | 0.9360 | 0.6630 | 0.1990 | 0.9740 | 0.5410 | 0.8600 | 0.2749 | 0.0006 |
| 1000333-62-1 cis-10-Heptadecenoic acid, methyl ester | 0.0018 | 0.0017 | 0.0019 | 1.0000 | 1.0000 | 1.0000 | 0.9850 | 0.7000 | 0.7780 | 0.4300 | 0.9310 | 0.9660 | 0.7180 | 1.0000 | 0.9880 | 0.9690 | 0.4614 | 0.0006 |
| 001731-92-6 Heptadecanoic acid, methyl ester | 0.0007 | 0.0005 | 0.0004 | 0.9930 | 0.9850 | 1.0000 | 0.9980 | 0.7380 | 0.7550 | 0.4630 | 0.8740 | 0.8860 | 0.6210 | 1.0000 | 0.9870 | 0.9840 | 0.4351 | 0.0002 |
| 016326-32-2 Methyl .gamma.-linolenate | 0.0123 | 0.0115 | 0.0125 | 1.0000 | 1.0000 | 1.0000 | 0.9870 | 0.7980 | 0.9150 | 0.6970 | 0.5270 | 0.6870 | 0.4250 | 0.9990 | 1.0000 | 0.9890 | 0.3746 | 0.0052 |
| 002566-97-4 9,12-Octadecadienoic acid, methyl ester, (E,E)- | 0.0014 | 0.0013 | 0.0014 | 1.0000 | 1.0000 | 1.0000 | 0.9650 | 0.7210 | 0.7270 | 0.4290 | 0.9700 | 0.9720 | 0.7850 | 1.0000 | 0.9830 | 0.9820 | 0.4798 | 0.0005 |
| 000112-63-0 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | 0.0015 | 0.0015 | 0.0015 | 1.0000 | 1.0000 | 1.0000 | 0.8990 | 0.6260 | 0.6350 | 0.3890 | 0.9810 | 0.9830 | 0.8610 | 1.0000 | 0.9920 | 0.9910 | 0.4387 | 0.0005 |
| 001937-62-8 9-Octadecenoic acid, methyl ester, (E)- | 0.0004 | 0.0003 | 0.0003 | 0.9960 | 0.9960 | 1.0000 | 1.0000 | 0.9940 | 0.7970 | 0.6090 | 0.9990 | 0.8660 | 0.6950 | 0.9500 | 0.8280 | 0.9970 | 0.5510 | 0.0001 |
| 000112-62-9 9-Octadecenoic acid (Z)-, methyl ester | 0.0008 | 0.0007 | 0.0007 | 1.0000 | 1.0000 | 1.0000 | 0.8880 | 0.7140 | 0.5960 | 0.4340 | 0.9960 | 0.9780 | 0.9070 | 1.0000 | 0.9860 | 0.9980 | 0.4799 | 0.0002 |
| Methyl stearate | 0.0023 | 0.0017 | 0.0013 | 0.9980 | 0.9860 | 0.9980 | 0.9780 | 0.8180 | 0.7940 | 0.3550 | 0.9850 | 0.9790 | 0.6580 | 1.0000 | 0.9070 | 0.9230 | 0.4332 | 0.0006 |
| 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)- | 0.0016 | 0.0016 | 0.0018 | 1.0000 | 1.0000 | 1.0000 | 0.9210 | 0.5050 | 0.7220 | 0.3740 | 0.9200 | 0.9910 | 0.8170 | 0.9950 | 0.9990 | 0.9670 | 0.3971 | 0.0006 |
| 5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)- | 0.0019 | 0.0019 | 0.0020 | 1.0000 | 1.0000 | 1.0000 | 0.9520 | 0.5560 | 0.7780 | 0.3810 | 0.9130 | 0.9910 | 0.7690 | 0.9940 | 0.9970 | 0.9480 | 0.4112 | 0.0007 |
| 021061-10-9 8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)- | 0.0012 | 0.0012 | 0.0012 | 1.0000 | 1.0000 | 1.0000 | 0.8940 | 0.4980 | 0.6660 | 0.3840 | 0.9390 | 0.9900 | 0.8630 | 0.9980 | 0.9990 | 0.9840 | 0.4031 | 0.0004 |
| 1000333-61-8 cis-11,14-Eicosadienoic acid, methyl ester | 0.0006 | 0.0006 | 0.0006 | 1.0000 | 1.0000 | 1.0000 | 0.5600 | 0.5530 | 0.5810 | 0.4350 | 1.0000 | 1.0000 | 0.9990 | 1.0000 | 0.9990 | 0.9990 | 0.4313 | 0.0002 |
| 055682-88-7 11,14,17-Eicosatrienoic acid, methyl ester | 0.0011 | 0.0011 | 0.0011 | 1.0000 | 1.0000 | 1.0000 | 0.8980 | 0.5000 | 0.6610 | 0.3820 | 0.9370 | 0.9880 | 0.8570 | 0.9980 | 0.9990 | 0.9850 | 0.4008 | 0.0004 |
| 001120-28-1 Eicosanoic acid, methyl ester | 0.0008 | 0.0005 | 0.0004 | 0.9890 | 0.9550 | 0.9970 | 0.7580 | 0.3380 | 0.5600 | 0.4120 | 0.9330 | 0.9960 | 0.9690 | 0.9920 | 1.0000 | 0.9990 | 0.3497 | 0.0002 |
| 002390-09-2 cis-Methyl 11-eicosenoate | 0.0005 | 0.0004 | 0.0004 | 1.0000 | 1.0000 | 1.0000 | 0.6640 | 0.4840 | 0.4010 | 0.5800 | 0.9980 | 0.9880 | 1.0000 | 1.0000 | 1.0000 | 0.9970 | 0.4111 | 0.0001 |
| 000301-00-8 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- | 0.0115 | 0.0108 | 0.0111 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.5150 | 0.7880 | 0.5600 | 0.5150 | 0.7890 | 0.5610 | 0.9870 | 1.0000 | 0.9940 | 0.3276 | 0.0048 |
| Heneicosanoic acid, methyl ester | 0.0005 | 0.0005 | 0.0004 | 1.0000 | 0.9980 | 0.9990 | 0.6910 | 0.3250 | 0.5810 | 0.4080 | 0.9560 | 1.0000 | 0.9850 | 0.9860 | 1.0000 | 0.9980 | 0.3463 | 0.0002 |
| 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)- | 0.0012 | 0.0013 | 0.0014 | 1.0000 | 1.0000 | 1.0000 | 0.8910 | 0.3060 | 0.6500 | 0.4060 | 0.7840 | 0.9880 | 0.8840 | 0.9620 | 0.9990 | 0.9910 | 0.3168 | 0.0004 |
| 1000333-60-3 cis-13,16-Docasadienoic acid, methyl ester | 0.0007 | 0.0007 | 0.0006 | 1.0000 | 1.0000 | 1.0000 | 0.7090 | 0.4350 | 0.5040 | 0.4450 | 0.9870 | 0.9960 | 0.9890 | 1.0000 | 1.0000 | 1.0000 | 0.3970 | 0.0002 |
| 001120-34-9 13-Docosenoic acid, methyl ester, (Z)- | 0.0008 | 0.0008 | 0.0007 | 1.0000 | 1.0000 | 1.0000 | 0.8290 | 0.3560 | 0.5510 | 0.4640 | 0.9000 | 0.9850 | 0.9610 | 0.9950 | 0.9990 | 1.0000 | 0.3678 | 0.0003 |
| 000929-77-1 Docosanoic acid, methyl ester | 0.0006 | 0.0005 | 0.0004 | 1.0000 | 0.9960 | 0.9990 | 0.8250 | 0.2490 | 0.5190 | 0.4620 | 0.7890 | 0.9790 | 0.9620 | 0.9760 | 0.9880 | 1.0000 | 0.2925 | 0.0002 |
| 002433-97-8 Tricosanoic acid, methyl ester | 0.0008 | 0.0007 | 0.0006 | 1.0000 | 0.9980 | 0.9990 | 0.8210 | 0.2350 | 0.5150 | 0.5500 | 0.7730 | 0.9790 | 0.9870 | 0.9710 | 0.9590 | 1.0000 | 0.2979 | 0.0002 |
| 15-Tetracosenoic acid, methyl ester, (Z)- | 0.0020 | 0.0022 | 0.0019 | 1.0000 | 1.0000 | 1.0000 | 0.9420 | 0.3840 | 0.7000 | 0.4550 | 0.7910 | 0.9790 | 0.8570 | 0.9770 | 1.0000 | 0.9200 | 0.3696 | 0.0007 |
| 002442-49-1 Tetracosanoic acid, methyl ester | 0.0024 | 0.0023 | 0.0020 | 1.0000 | 1.0000 | 1.0000 | 0.8720 | 0.7800 | 0.5330 | 0.4180 | 1.0000 | 0.9660 | 0.9100 | 0.9910 | 0.9630 | 0.9990 | 0.4555 | 0.0008 |

## APPENDIX 7

**Table 9** Recovery value of FAMEs while using different stirring velocities during SBSE. (n=4)

| FAMEs | Stirring speed (RPM) | | |
|---|---|---|---|
| | 700 | 1,000 | 1,500 |
| C6:0 | 71,792,185 | 66,105,725 | 56,942,599 |
| C8:0 | 153,789,251 | 180,594,424 | 137,232,460 |
| C10:0 | 137,856,983 | 163,132,981 | 112,324,947 |
| C11:0 | 99,618,114 | 119,911,065 | 74,711,739 |
| C12:0 | 165,414,181 | 187,356,252 | 130,969,624 |
| C13:0 | 94,927,677 | 103,690,270 | 86,496,856 |
| C14:1 [cis-9] | 41,287,039 | 45,188,887 | 44,306,252 |
| C14:0 | 123,689,337 | 130,914,201 | 126,446,694 |
| C15:1 [cis-10] | 30,944,218 | 32,715,415 | 34,031,592 |
| C15:0 | 52,523,223 | 57,166,874 | 58,960,583 |
| C16:1 [cis-9] | 16,118,929 | 17,366,558 | 18,854,006 |
| C16:0 | 109,738,907 | 117,621,937 | 127,111,517 |
| C17:1 [cis-10] | 10,858,534 | 12,096,793 | 13,305,543 |
| C17:0 | 26,821,387 | 27,481,563 | 32,721,734 |
| C18:3 [cis-6,9,12] | 9,336,847 | 10,026,374 | 10,887,375 |
| C18:2 [trans-9,12] | 8,110,943 | 8,593,242 | 9,876,008 |
| C18:2 [cis-9,12] | 8,108,528 | 8,597,122 | 9,873,233 |
| C18:1 [trans-9] | 21,875,117 | 22,814,655 | 26,213,122 |
| C18:1 [cis-9] | 6,816,435 | 7,187,783 | 8,343,793 |
| C18:0 | 39,768,756 | 39,531,534 | 46,358,035 |
| C20:4 [cis-5,8,11,14] | 2,243,014 | 2,558,067 | 2,918,057 |
| C20:5 [cis-5,8,11,14,17] | 2,733,338 | 3,097,456 | 3,365,965 |
| C20:3 [cis-8,11,14] | 1,870,817 | 1,999,924 | 2,421,500 |
| C20:2 [cis-11,14] | 2,020,182 | 2,061,451 | 2,658,158 |
| C20:3 [cis-11,14,17] | 2,183,866 | 2,277,628 | 2,853,186 |
| C20:0 | 10,649,261 | 9,292,889 | 12,635,276 |
| C20:1 [cis-11] | 3,328,205 | 3,207,304 | 4,157,989 |
| C18:3 [cis-9,12,15] | 2,196,454 | 2,291,266 | 2,844,454 |
| C21:0 | 2,126,241 | 2,101,508 | 2,627,311 |
| C22:6 [cis-4,7,10,13,16,19] | 212,950 | 298,726 | 297,671 |
| C22:2 [cis-13,16] | 294,758 | 388,928 | 406,951 |
| C22:1 [cis-13] | 530,839 | 607,473 | 710,978 |
| C22:0 FAME | 2,823,635 | 2,982,855 | 3,488,113 |
| C23:0 | 676,044 | 800,420 | 884,778 |
| C24:1 [cis-15] | 192,013 | 222,667 | 229,946 |
| C24:0 | 1,463,715 | 1,661,083 | 1,781,791 |

# VITA

Angela Marisol Encerrado Manríquez was born in Hidalgo del Parral, Chihuahua, México. She grew up in Ciudad Juarez, Chihuahua and at age 18 she emigrated to the neighboring city of El Paso, Texas, to pursue her dream of becoming an environmental researcher. She started volunteering research laboratories in the Chemistry and Biochemistry department at UTEP during her second week of college. She attended the University of Texas at El Paso (UTEP) starting in Fall 2013 to pursue a bachelor's degree in environmental science and chemistry and graduated in Spring 2018. During most of this time, Angela worked under the mentoring of Dr. Wen Yee Lee in her environmental analytical chemistry laboratory, developing various project in the area of analytical chemistry and participating three years as an intern in the annual program MERITUS (Mentor Experiences in Research, Inquiry, and Creativity for Undergraduate Students), as well as in the Diana Natalicio Environmental internship. Her active role in research made her recipient of the Undergraduate Academic & Research Excellence in Environmental Science Award in Spring 2018.

In Fall 2018 Angela continued her studies at UTEP to pursue her Master of Science in Chemistry. Here she continued to explore the area of method development while collaborating on various projects across the Chemistry department. While pursuing her graduate degree she worked as a Teaching Assistant for General and Organic Chemistry laboratories in the Department of Chemistry. After receiving her Master's degree during the COVID pandemic, she will be attending the University of California at Davis to continue her graduate education in the Agricultural and Environmental Chemistry Doctoral program.

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