DETERMINATION OF FREE GLYCERIN AND MONO-, DI-, TRIGLYCERIDES IN PALM-BASED BIODIESEL USING FAST GAS CHROMATOGRAPHY

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ORIGINAL LITERARY WORK DECLARATION

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Name of Degree: Master of Science in Analytical Chemistry & Instrumental Analysis

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Abstract

As the tremendous growth of biodiesel industry, demand of fast analysis turnaround time becomes essential to sustain the drastic increase of samples quantity and to deliver the result on time. A fast gas chromatography (GC) method was developed using a shorter metal column to determine free glycerin and total glycerin, as well as mono-, di-, and triglyceride content in biodiesel. More than two times reduction of analysis time (16 minutes) was achieved by using this fast GC method without sacrificing the peaks resolution. Method validation results show good linearity ($r^2 > 0.95$) and repeatability (RSD < 8%) with acceptable limit of quantification. In addition, the calibration modules proposed in this method covers the quantification of biodiesel derived from wide range of fatty acids carbon chain, including lauric based methyl esters. This research covers the various parameters used in developing the fast GC method to analyse palm kernel and palm stearin based biodiesel.

Abstrak

Memandangkan perkembangan pesat industri biodiesel, permintaan masa analisis singkat menjadi penting untuk mengekalkan peningkatan drastik kuantiti sampel dan untuk menyampaikan keputusan pada masa yang tepat. Satu kaedah cepat mengenai kromatografi gas (GC) dibangunkan menggunakan kolom logam yang lebih pendek untuk menentukan kandungan gliserin dan jumlah gliserin, serta mono-, di-, dan trigliserida dalam biodiesel. Pengurangan lebih daripada dua kali ganda masa analisis (16 minit) telah dicapai dengan menggunakan kaedah GC cepat ini tanpa mengorbankan resolusi. Keputusan pengesahan kaedah menunjukkan kelinearan yang baik (r^2> 0.95) dan kebolehulangan (RSD <8%) dengan had kuantifikasi yang boleh diterima. Di samping itu, modul penentukuran dicadangkan dalam kaedah ini meliputi kuantifikasi biodiesel diperolehi daripada pelbagai asid lemak rantaian karbon, termasuk laurik ester. Kajian ini meliputi pelbagai parameter yang digunakan dalam membangunkan kaedah GC cepat untuk menganalisis biodiesel yang berasaskan minyak kernel sawit dan minyak stearin sawit.

Acknowledgements

First of all, I would like to express my deepest gratitude to my supervisor, Dr. Tay Kheng Soo, for his guidance and supervision as well as patience throughout the course of my research. Without his valuable suggestions and helpful comments, this project would not have been possible. Secondly, I would also like to acknowledge R&D Centre of KL-Kepong Oleomas for supporting and providing the study material and also facilities to succeed this work. I would also like to thank all my colleagues and friends who have offered their faithful help and technical support during my laboratory work. Next, I also like to thank my course mates for being supportive and making the postgraduate studies a great and memorable one. Last but not least, I wish to express my deep appreciation to my family members for their moral supports all the while in my life.

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List of Symbols & Abbreviations

ASTM	American Society for Testing and Materials
BSTFA	Bis(trimethylsilyl)-trifluoroacetamide
CCO	coconut oil
EPA	Environmental Protection Agency, US
FAME	Fatty acid methyl ester
FID	Flame-ionization detector
FT	Film thickness
GC	Gas Chromatography
DIN	European Deutsches Institut fur Normung
DG	Diglyceride
DMF	Dimethylformamide
ID	Internal diameter
LOD	Limit of detection
LOQ	Limit of quantification
ME	Methyl ester
MG	Monoglyceride
m/m	Mass over sample mass
MPOB	Malaysian Palm Oil Board
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
PKO	Palm kernel oil
PKME	Palm kernel methyl ester
PSME	Palm stearin methyl ester
PORIM	Palm Oil Research Institute of Malaysia

r^2	Correlation coefficient
RSD	Relative standard deviation
SD	Standard deviation
TG	Triglyceride

List of Appendices

1.0 INTRODUCTION

1.1 Biodiesel, a renewable fuel

Biodiesel consist of a variety of ester-based oxygenated fuels derived from natural biological resources, such as vegetable oils and animal fats. It is one of the most promising alternative, non-toxic, biodegradable and renewable diesel fuel. The promising characteristics of biodiesel have gained worldwide attention since the energy crisis in 1970s (Choo *et al.,* 2005). In addition, high demand of energy consumption that leads to the fast diminishing energy reserves and also the increase of environmental awareness also upsurge the popularity of biodiesel. Biodiesel is produced from a great variety of sustainable natural feedstocks, commonly from vegetable oils, such as rapeseed oil, soybean oil, sunflower oil, palm oil, coconut, and so on, or animal fats (such as tallow), or even waste oils like used frying oils (Knothe *et al.*, 2005). Different oil sources may produce biodiesel with different chemical and physical properties in terms of viscosity, cloud point and pour point. However, the overall properties and performance of biodiesel are comparable as the petroleum-based diesel fuels that enable it to be used partially or totally replace the petrodiesel without modification of engine. Furthermore, it greatly reduces many environmental and transportation risks inherent to petroleum-based fuels (Ruppel *et al.*, 2012)

The emerge of biodiesel alternative energy source resolves the environmental issue with regard to petrochemical emissions and their contributions to the issues such as global warming and acid rain. Additionally, biodiesel can also be used in blends with conventional diesel while still achieving substantial reductions in tailpipe emissions, visible smoke and obnoxious odors. The tremendous growth of biodiesel industries also come across with the phenomenon of the steady increases of energy consumption

worldwide especially the developing and threshold countries, including Malaysia, which have a great demand to compete with the developed countries. Roland (1995) reported that the primary energy demand of Malaysia has doubly increased during the last 10 years.

1.2 The chemistry of biodiesel production

Commercially, biodiesel can be manufactured either by esterification of fatty acids or by transesterification of vegetable oils or animal fats with an alcohol to produce alkyl esters as shown in Figure 1.1 (Choo *et al.*, 2005). Thus, biodiesel is chemically known as fatty acids methyl esters (FAME).

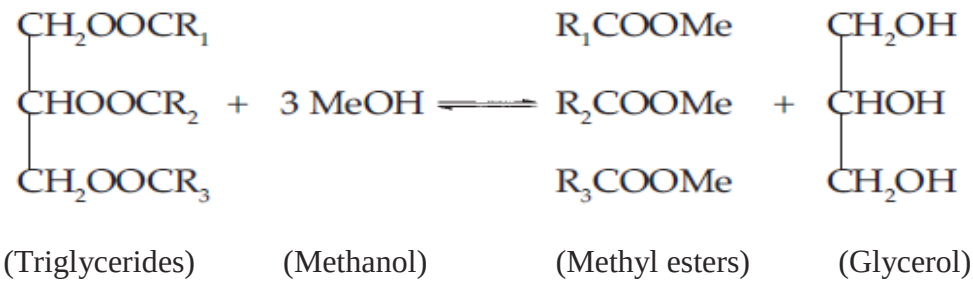


Figure 1.1: Reaction equation of transesterification of triglycerides.

Vegetable oils is the more commonly used feedstock because of its high heating value and the ease of handling. The oils are mainly consist of triglycerides that are made of glycerine backbone, with each attached with different type of fatty acid, which composition varies according to type of vegetable oils as shown in Table 1.1. Nevertheless, direct usage of the viscous vegetable oils without converting to alkyl esters in diesel engine is not workable due to poor fuel atomization, poor cold engine start-up, oil ring stickening, gum and other deposit formation (Jitputti *et al.*, 2006; Roland, 1995).

Table 1.1: Fatty acid composition of some vegetable oil feedstocks that can be used in the production of biodiesel (James & Chun, 2007).

Oil Source	C8:0	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3 C20:0	C20:1 C22:0	C22:1
Rapeseed					2-5	0.2	1-2	10-15	10-20	5-10	0.9	50-60
Soybean				0.3	7-11	0-1	3-6	22-34	50-60	2-10	5-10	
Palm				1-6	32-47		1-6	40-52	2-11			
Coconut	5-9	4-10	45-52	13-18	7-10		1-4	5-8	1-3			
Palm kernel	2-4	3-7	44-51	14-19	6-9	0-1	1-3	10-18	1-2		1-2	

Commercially, biodiesel production via transesterification route with the presence of catalyst is more widely used. Indeed, transesterification of a vegetable oil was conducted as early as 1853, by scientists E. Duffy and J. Patrick, many years before the first diesel engine became functional (Kalyani *et al.,* 2012). Transesterificaton is a chemical reaction that replaces the glycerol portion of the triglyceride molecule with an alcohol (usually methanol) molecule to form the corresponding alkyl esters (known as methyl esters if methanol as reacting alcohol) and glycerol as a by-product. As a consequence of the reaction, biodiesel will be formed on the top layer, the next layer may contain soap, and the bottom layer will be glycerol. The glycerol and soap layers are drained off or further process to get pure glycerol. Whereas, the biodiesel is then washed with distilled water to remove any additional soap, alcohol, or other impurities in the biodiesel (Ekeoma, 2010).

In general, transesterification process can be accompanied by alkaline catalysis, acid catalysis, and lipase and alcohol catalysis in supercritical conditions. The mostly used methods are alkaline, such as metal hydroxide) and acid catalysis, such as mineral acids. However, Jitputti *et al.* (2006) had studied on the use of heterogenous catalysts for palm kernel oil (PKO) and coconut oil (CCO) transesterification with methanol. It was reported that the replacement of homogeneous catalysts by heterogeneous catalysts would have advantages of easy catalyst separation and reduction of environmental pollution. On the other hand, Saka and Kusdiana (2001) experimented on the transesterification reaction of rapeseed oil in super critical methanol, without using any catalyst at high temperature and high pressure (Kalyani *et al.,* 2012).

1.3 Characterization of biodiesel

The U.S. Environmental Protection Agency (EPA) requires the manufacturers or distributers to meet certain standards of biodiesel for legal registration as fuel and fuel additive. Various quality specifications including physical and chemical tests has been outlined by American Society for Testing and Materials (ASTM) and the European Deutsches Institut fur Normung (DIN) in Europe are associated with the composition and structure of the fatty esters comprising in the biodiesel. For instance, ASTM D6751 and EN 14214 standards specified a range of quality criteria, which must be met for good performance of the fuel in a diesel engine. Some essential properties requirements are cetane number, kinematic viscosity, oxidative stability, elemental analysis and cold-flow properties in form of the cloud and pour points (Sokoto *et al.*, 2011). One of the important analytical methods is gas chromatography (GC) determination of free and total glycerine in biodiesel, which was cited in method ASTM D6584 and EN 14105. The analysis of glycerine is critical to ensure the free glycerine content in the final product meet the acceptable level. Or else, the excessive amounts of glycerine can make long term storage problematic, or cause the formation of unwanted deposits, leading to injector fouling and accelerated engine wear. Whereas, determination of bonded glycerine, such as mono-, di-, triglycerides indicates the completeness of transesterification reaction.

1.4 Objectives of study

Recent discoveries and tremendous growth in the field of biodiesel has created a necessity to use fast, accurate, and easy methods for the analysis of the in-process and also final product. This research study focused on GC analysis of free and total

glycerine in biodiesel samples by using fast GC method. It is a technique to reduce the GC run time without sacrificing the quality of analysis, i.e. peaks resolution, precision and elution order, compare to the conventional GC standard method. This study demonstrates the parameters and optimization settings of GC used to test some palm based biodiesel, was known as methyl esters in the following reports, with shorter GC run time using metal capillary column. In addition, different calibration modules were also introduced for better quantification of lauric based methyl esters, which was not covered in the standard method ASTM D6584 and EN 14105. Method validation was also carried out to meet the standard method requirements.

2.0 LITERATURE REVIEW

2.1 Background of palm-based biodiesel

In year 1895, the first invention of diesel engine using vegetable oil as a fuel by Dr Rudolf Diesel has enlighten the fast development and researches of biodiesel in worldwide (Choo *et al.*, 2005). Nowadays, biodiesel has evolved to become an alternative replacement for diesel fuel in conjunction with the dwindling of non-renewable energy sources and the rising of environmental concerns. FAME derived from palm oil is one of the viable biofuel with promising potential that getting attention worldwide, especially Asia including Malaysia. Research and development of palm biodiesel in Malaysia has been carried out since 1980s (Puah *et al.*, 2010). Thereafter, numerous researches were reported on various processing as well as analytical methods in order to cater with the technology growth of biodiesel production.

Oil palm (*Elaeis guineensis Jacquin*) is among the top oil-producing plant, as it gives highest oil yield of about 21.6-24.5% by weight of a fresh fruit bunch (Vanichseni *et al*., 2002). The two sources of oil obtained from the oil palm fruit are palm oil from the mesocarp and palm kernel oil from the kernel inside the nut. Oil quality can be affected by various factors, such as harvesting of fruits at the optimum stage of ripeness, handling of fruits during transportation, and processing conditions during oil extraction (Siew, 2011). USDA reported that an elevating proportion of palm oil is being used for non-food purposes as shown in Figure 2.1 (Gunstone, 2011). This includes biodiesel production and also reflects the growth of the oleochemical industry in Malaysia.

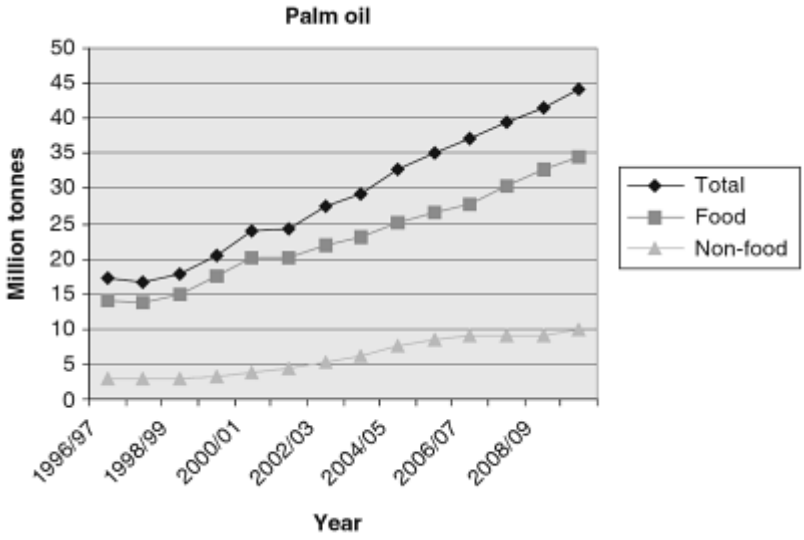


Figure 2.1: Consumption of palm oil from 1996/97 to 2008/09. *Note: Non-food uses include animal feed, oleochemicals and biodiesel.* (Gunstone, 2011)

Various intensive studies had been reported on the production and performance of palm-based biodiesel. Palm Oil Research Institute of Malaysia (PORIM), which later was known as Malaysian Palm Oil Board (MPOB), shows promising results when trial production of their pilot plant and then utilize the produced biodiesel as a fuel in diesel engines (Körbitz, 2005). The development of palm-based utilization may become an additional venue for Malaysia's palm oil industry as the feedstock limitations in Europe. Additionally, Choo *et al.* (2005) also reported that crude palm diesel were systematically and exhaustively evaluated as diesel substitute since 1983 to 1994. These included laboratory evaluation, stationary engine testing and field trials on a large number of vehicles including taxis, trucks, passenger cars and buses. Generally, palm diesel has very similar fuel properties to petroleum diesel. It exhibits shorter ignition time delay compared to petroleum diesel due to a higher cetane number. However, further improvement by adding pour point depressants or other processes was investigated for possible use in temperate climate countries (Choo *et al*., 2005).

2.2 Free and total glycerin in biodiesel analysis

There are a variety of ways to determine biodiesel composition and quality. Both ASTM D6751 and EN 14214 standard specifications defined a set of requirements for biodiesel commercialization as pure biofuel or blending stock for heating and diesel fuels. These standards list out the maximum allowable concentrations of contaminants in pure (B100) finished product, along with other chemical physical properties necessary for a safe and satisfactory engine operation (Munari *et al*., 2007).

The current study only focus on the determination of free glycerine and mono-, di-, triglycerides in FAME, which is outlined in ASTM D 6584 and EN 14105 standard methods. Table 2.1 indicates the specification of free glycerine and glycerides content that must be fulfilled by using ASTM D 6584 and EN 14105 test methods.

Table 2.1 Free and total glycerine specifications for biodiesel (McCurry *et al.*, 2007)

	EN 14214		ASTM D6571	
	Limit (% m/m)	Test method	Limit (% m/m)	Test method
Free glycerin	0.02 max	EN 14105	0.020 max	D 6584
Monoglycerides	0.80 max	EN 14105	NA	D 6584
Diglycerides	0.20 max	EN 14105	NA	D 6584
Triglycerides	0.20 max	EN 14105	NA	D 6584
Total glycerin	0.25 max	EN 14105	0.240 max	D 6584

The above mentioned method is based on high temperature GC analysis of glycerol, mono-, di-, and triglycerides after converted into more volatile silylated derivatives with *N*-(trimethylsilyl)trifluoroacetamide (MSTFA). The total glycerol content is obtained by summing the free glycerol content with the bound glycerol, i.e., the glycerol content of the mono-, di-, and triglycerides (Pauls, 2011). According to

EN14105: 2003 version, two internal standards, i.e. 1,2,4-butanetriol for the glycerol and 1,2,3-tridecanolylglycerol for the glycerides are employed for quantification. However, in the updated version (EN14105:2011), the method was modified to obtain better precision by introducing separate internal standards for glyceryl monononadecanoate (C19 MG), 1,3-glyceryl dinonadecanoate (C19 DG), and glyceryl trinonadecanoate (C19 TG) instead of using calibration solutions for glycerides compounds quantifications. Several inherent challenges of implementing these methods are the tests run at very high temperatures (above 380 ℃) that causes the polyimide coating of most fused silica columns starts to degrade, eventually becoming brittle and inflexible. Besides, the standard methods also do not cover the lauric based FAME, such as FAME derived from coconut and palm kernel oils because of overlapping of different glyceride peaks.

Nevertheless, there are several alternative methods that have been reported for the determination of free and bound glycerol. Ebenezer (2010) developed alternative analytical methods to determine total and bound glycerol to make modifications to both ASTM and EN standards. The study showed poor repeatability of the use of a programmable temperature volatilization as a substitute injector for the recommended on-column injector to determine free and bound glycerol. In contrary, better performance was shown by using normal phase high performance liquid chromatography with binary gradient elution for the qualitative and quantitative determination of bound glycerols.

In addition, other related studies including Mittelbach (1993) reported a GC method to determine glycerol directly in FAME after reaction with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) in dimethylformamide (DMF) solvent using a 60m DB-5 column. Mariani *et al.* (1991) reported a similar silylation GC method to determine

mono-, di-, and triglycerides on a 10m x 32mm DB-5 column with a 0.1 μm film same as those adopted in ASTM and EN methods (Pauls, 2011).

2.3 Fast GC analysis

As the rapid growth of biodiesel demands that lead to drastic expansion of production capacities, the fast analysis turnaround time becomes essential to cater the increase of sample quantities. Janssen (2014) has reported about the principle of selecting the best route to speed up a chromatography run time starts with an understanding of why a chromatographic separation takes time. The total run time of a chromatogram is actually the sum of all empty baseline segments plus the sum of the width of all baseline peaks. The important steps to obtain faster GC analysis are minimize resolution to a value which is just sufficient to achieve desired separation, maximize the selectivity of the chromatographic system, and implement a method that can reduce analysis time while holding the resolution in constant (Janssen, 2014).

Freedman *et al.* (1986) developed a fast capillary silylation GC method using a 1.8 m x 0.32 mm SE-30 column with oven temperature programmed to 350℃ to monitor transesterification reactions. Besides, Buchanan (2009) has reported on the use of metal column, which designed specifically for the determination of free and total glycerine in B100 biodiesel, in order to obtain the acceptable resolution in as fast of a run time as possible.

In this study, the first challenge in establish this fast GC method is to obtain a good resolution of the analyte peaks in conjunction shorter the GC run time. The second challenge is the method should able to analyse the lauric based FAME which derived from palm kernel oil. It is difficult due to the wide carbon chain distribution of palm kernel oil, whereof the medium chain glycerides will overlap with long chain glycerides.

3.0 EXPERIMENTAL

3.1 Instrumentation

A GC system connected to flame-ionization detector (FID) from Agilent was used for this work. A metal capillary column (named as MET-Biodiesel column), dimensions 14 m x 0.53 mm ID x 0.16μm FT with integrated 2 m x 0.53 mm ID guard, manufactured by Supelco was used. Table 3.1 shows the GC operating conditions used in this study.

Table 3.1: GC operating configuration

GC system	Agilent 6890N Series GC with FID
Injector	Cool-on-column inlet with electronic pneumatics control (EPC); oven track
Data system	Agilent Multitechnique ChemStation
Oven program	Initial temperature 80℃, programmed at 30℃/min up to 230℃, programmed at 10℃/min up to 250℃, programmed at 30℃/min up to 380℃, final temperature hold for 4.67min.
Total run time	16 min.
Carrier gas	Helium; 4mL/min (constant flow)
Detector temperature	380℃
Auxiliary gas	Hydrogen flow: 40.0 mL/min Air flow: 400.0 mL/min Makeup flow: 25.0 mL/min (Nitrogen)
Injection volume	1 μL

3.2 Materials and reagents

Three types of samples – palm stearin methyl ester (PSME), palm kernel methyl esters (PKME), and B100 biodiesel, were given by KLK Bioenergy Sdn Bhd. The glycerol and 1,2,4-butanetriol pure standards were purchased from Sigma-Aldrich. Individual standard of glyceryl monononadecanoate (C19 MG), glyceryl dinonadecanoate (C19 DG) and glyceryl trinonadecanoate (C19 TG), and standard mixture of mono-, di-, and trilaurin were purchased from Nu-Chek Prep, Inc. Other standards used for retention time identification, such as C8 to C22 methyl esters standards and C8 to C18 mono-, di-, triglycerides from Sigma-Aldrich and also Nu-Chek Prep were also used. The chemicals like pyridine, tetrahydrofurane (THF) and *n*-heptane were purchased from Merck Malaysia. Whereas, the N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) silylating agent was from Thermo Scientific.

3.3 Preparation of stock solutions

3.3.1 1,2,4-butanetriol stock solution, 1 mg/mL

Approximately 50 mg (accuracy ± 0.1 mg) of 1,2,4-butanetriol was weighed in a 50 mL volumetric flask and make up to the mark with pyridine.

3.3.2 Glycerol stock solution, 0.5 mg/mL

Approximately 50 mg (accuracy ± 0.1 mg) of glycerol was weighed in a 10 mL volumetric flask and make up to the mark with pyridine. 1 mL of this solution was transferred into a 10 mL volumetric flask using a pipette and make up to the mark with pyridine.

3.3.3 Standard glycerides stock solution

(i) 2.5 mg/mL of C19 MG, DG and TG

Approximately 62.50 mg (accuracy ± 0.1 mg) of individual C19 MG, C19 DG, and C19 TG was weighed in a 25 mL volumetric flask and make up to the mark with tetrahydrofurane. The stability of the solution can be maintained for almost 3 months if stored at 4 ℃. However, the solution might show precipitate when refrigerated at 4 ℃ that must re-dissolve spontaneously when restored at ambient temperature.

(ii) 2.0 mg/mL of C12 MG, DG and TG

Approximately 30 mg (accuracy ± 0.1 mg) of C12 MG, DG and TG standard mixture was weighed in a 5 mL volumetric flask and make up to the mark with tetrahydrofurane.

3.4 Calibration solutions preparation and analysis

3.4.1 Calibration of glycerol

It is based on the variation of weight ratio versus area ratio makes it possible to verify the linearity of the response and to work out a calibration function. Four calibration solutions were prepared by transferring into a series of vials the volumes of stock solutions of glycerol (3.3.2) and 1,2,4-butanetriol (3.3.1) as given in Table 3.2.

Table 3.2: Preparation of calibration solutions of glycerol

Stock solution	**1**	**2**	**3**	**4**
Glycerol solution	10 µL	40 µL	70 µL	100 µL
1,2,4-butanetriol solution	80 µL	80 µL	80 µL	80 µL

150 µL of MSTFA was added to each of the four calibration solutions, the vials were closed hermetically and shaken vigorously. The solutions were stored 15 min at 70 ℃, then 8 mL of *n*-heptane was added using a graduated cylinder. 1 µL of each reaction mixture was analysed by GC under the conditions defined in Table 3.1.

3.4.2 Calibration of mono-, di-, triglycerides

It is assumed that the detector response is regarded as linear within the considered concentration range.

For long chain (C16 and above) glycerides, a fixed amount (200 µL) of C19 glycerides standard stock solution (3.3.3i) was added into each sample and analyse together under the same condition. This is based on the calibration module of EN 14105:2011 standard method.

For medium chain (C12 and C14) glycerides, five calibration solutions were prepared by transferring into a series of vials the volumes of stock solutions of C12 mono-, di-, triglycerides mixture (3.3.3ii) to get concentration from 0.1 – 2.0 mg/mL.

200 µL of pyridine, and 200 µL of MSTFA were added. Avoid contact with humidity. The vial was closed hermetically and shaken vigorously. The solutions were stored 15 min at 70 ℃, then 8 mL of *n*-heptane was added using a graduated cylinder. 1 µL of each reaction mixture was analysed by GC under the conditions defined in Table 3.1.

3.5 Sample preparation and analysis

Approximately 100 mg (accuracy ± 0.1 mg) of homogenized sample was weighed in a 10 mL vial. 80 µL of 1,2,4-butanetriol stock solution (3.3.1), 200 µL of C19 standard glycerides stock solution (3.3.3i), 200 µL of pyridine, and 200 µL of MSTFA were added. Avoid contact with humidity. The vial was closed hermetically and shaken

vigorously. The solutions were stored 15 min at 70 ℃, then 8 mL of *n*-heptane was added using a graduated cylinder. 1 µL of each reaction mixture was analysed by GC under the conditions defined in Table 3.1.

3.6 Results calculation

3.6.1 Free glycerol determination

The calibration function is given by the following equation, obtained from the calibration curve plotted from the experimental data using the linear regression method:

$$M_g/M_{ei} = a_g(A_g/A_{ei}) + b_g \qquad \text{(Equation 1)}$$

Where

M_g is the weight of glycerol (mg);

M_{ei} is the weight of internal standard 1,2,4-butanetriol (mg);

A_g is the peak area of glycerol;

A_{ei} is the peak area of the internal standard 1,2,4-butanetriol;

a_g, b_g are the regression coefficients of the calibration function for glycerol.

The mass percentage of free glycerol (G) in % (m/m) in the sample is calculated using the following equation:

$$G = [a_g(A_g/ A_{ei}) + b_g] \text{ x } (M_{ei}/m) \text{ x } 100 \qquad \text{(Equation 2)}$$

Where

A_{ei} is the peak area of internal standard 1,2,4-butanetriol (mg);

M_{ei} is the weight of internal standard 1,2,4-butanetriol (mg);

A_g is the peak area of glycerol;

m is the weight of sample

3.6.2 Glycerides determination

For mono-, di- and triglycerides of carbon chain C12 and C14,

Response factor (RF) for respective C12 MG, DG and TG was determined from the external standard calibration (3.4.2):

$$RF_{MonoC12} = Area_{MonoC12} / M_{MonoC12} \quad \text{(Equation 3)}$$

$$RF_{DiC12} = Area_{DiC12} / M_{DiC12} \quad \text{(Equation 4)}$$

$$RF_{TriC12} = Area_{TriC12} / M_{TriC12} \quad \text{(Equation 5)}$$

The mass percentage of mono-, di- and triglycerides of C12 and C14 was calculated:

$$M_1 = (A_{Mono1} / RF_{MonoC12}) \times (100/m) \quad \text{(Equation 6)}$$

$$D_1 = (A_{Di1} / RF_{DiC12}) \times (100/m) \quad \text{(Equation 7)}$$

$$T_1 = (A_{Tri1} / RF_{DiC12}) \times (100/m) \quad \text{(Equation 8)}$$

Where

$RF_{MonoC12}$, RF_{DiC12}, RF_{TriC12} are response factors of C12 MG, DG, TG respectively;

$Area_{MonoC12}$, $Area_{DiC12}$, $Area_{TriC12}$ are peak areas of external standards C12 MG, DG, TG;

M_1, D_1, T_1 are the concentration of C12 and C14 mono-, di- and triglycerides

A_{Mono1}, A_{Di1}, A_{Tri1} are the sums of the peak areas of C12 and C14 MG, DG, and TG

m is the weight of sample (mg)

For mono-, di- and triglycerides of carbon chain C16 and above,

$$M_2 (\% \text{ m/m}) = (A_{Mono2} / A_{MonoC19}) \times (M_{MonoC19}/m) \times 100 \quad \text{(Equation 9)}$$

$$D_2 (\% \text{ m/m}) = (A_{Di2} / A_{DiC19}) \times (M_{DiC19}/m) \times 100 \quad \text{(Equation 10)}$$

$$T_2 (\% \text{ m/m}) = (A_{Tri2} / A_{TriC19}) \times (M_{TriC19}/m) \times 100 \quad \text{(Equation 11)}$$

Where

M_2, D_2, T_2 are the concentration of mono-, di- and triglyceride of carbon chain C16 and above in the sample respectively;

A_{Mono2}, A_{Di2}, A_{Tri2} are the sums of the peak areas of the mono-, di-, and triglycerides of carbon chain C16 and above in the sample respectively;

$A_{MonoC19}$, A_{DiC19}, A_{TriC19} are the peak areas of internal standards C19 MG, DG, TG;

$M_{MonoC19}$, M_{DiC19}, M_{TriC19} are the weight (mg) of internal standards C19 MG, DG, TG;

m is the weight of sample (mg)

3.6.3 Total Glycerin determination

The total glycerin in the sample (GT) in % (m/m) is calculated as below:

$$GT = G + 0.255\ M + 0.146\ D + 0.103\ T \qquad \text{(Equation 12)}$$

3.7 Method validation

3.7.1 Linearity

A series of reference standard solutions were prepared and analysed by the same GC conditions as per samples analysis. The linearity curve of each reference standard was plotted the signal area as a function of concentration. A high correlation coefficient (r^2 >0.95) is often recommended as evidence of goodness of fit. The concentration range for the reference standards are glycerol standards solutions (0.005 – 0.05 % wt), C12 MG, DG and TG standards solutions (0.1 – 2.0 % wt) represent medium chain glycerides (C12 – C14), and C18:1 MG, DG and TG standards solutions (0.1 – 2.0 % wt) represent long chain glycerides (C16 and above).

3.7.2 Precision

3.7.2.1 Repeatability

Duplicates of identical sample solutions were injected on the same day under constant operating conditions with the same apparatus. The standard deviation (SD) and relative standard deviation (RSD) of the two test results were determined.

3.7.2.2 Reproducibility

Duplicates of identical sample solutions were injected on two different day under constant operating conditions with the same apparatus. The standard deviation (SD) and relative standard deviation (RSD) of the mean of test results from two independent test on different day were determined.

3.7.3 Limit of detection (LOD)

The LOD is based on the blank value (x_{bl}) determined from 5 injections of solvent plus three times the standard deviation of the blank ($x_{bl} + 3SD_{bl}$).

3.7.4 Limit of quantification (LOQ)

The LOQ is based on the blank value (x_{bl}) determined from 5 injections of solvent plus 10 times the standard deviation of the blank ($x_{bl} + 10SD_{bl}$). Then, the value is confirmed by injecting the concentration of the calculated LOQ and it should fit into the the linearity curve at correlation coefficient ($r^2 > 0.95$).

4.0 RESULT AND DISCUSSION

4.1 Fast GC method optimization

4.1.1 Column selection

A metal column (MET-Biodiesel column) with dimensions of 14m length x 0.52mm internal diameter x 0.16μm film thickness was used instead of the commonly used fused silica column. The metal capillary column able to withstands higher oven temperatures (can be programmed up to 430℃), offering a more robust and reliable long-term analysis. Additionally, the integrated guard column acts as a retention gap, minimizing peak broadening as well as extending the analytical column life with a leak-free connection. The integrated design of guard and analytical column in one continuous piece of tubing eliminates the physical connection between both columns, hence reducing leaks and breakage.

In fact, in terms of thermal stability, the metal column has similar durability as a polyimide coated fused silica column (such as ZB-5HT Inferno) that available in the market. It was designed specifically for high-temperature analysis and also programmable up to 430℃. However, the shorter column length and thicker film thickness of the selected MET-Biodiesel column is more suitable to this study objective. The goal was to obtain the good resolution with shorter GC run time.

4.1.2 GC run time optimization

Referring to the EN14105 standard method, the total GC run time for a single analysis of free and total glycerin in FAME sample is about 36 min. The total run time for a single analysis was shorten to 16 min after modifying the carrier gas flow rate and

oven heating program as shown in Table 4.1. It is more than 2 times reduction of the analysis time.

The initial oven temperature was increased to 80 ℃ to speed up the elution of solvent peak. The overall temperature programming rate was also increased, especially the methyl esters elution zones, since the methyl esters peaks are not the interest of this study. Figure 4.1(a) and (b) show the comparable resolution of analyte peaks of a PSME sample analysed with EN 14105 standard method using 5HT (15 m x 0.32 mm ID x 0.10 μm) polyimide coated fused silica column compared to fast GC method using MET-Biodiesel (14 m x 0.53 mm ID x 0.16 μm) metal column.

The use of megabore column (i.e. wide internal diameter column) allows high carrier gas velocity. However, large ID column was found to reduce the retention of analyte and hence loss of resolution; but, it has been compromised with thicker film thickness.

In addition, cryogenic (liquid nitrogen) cooling for quick column cool down can be used to accelerate returning to the initial oven temperature. This can shorten the interval stabilization time between each analysis.

Table 4.1: Comparison of GC configuration settings of standard method and fast GC method

	EN 14105 standard method				**Fast GC method**			
Oven program	**Temp. (℃)**	**Rate (℃/min)**	**Hold (min)**	**Total (min)**	**Temp. (℃)**	**Rate (℃/min)**	**Hold (min)**	**Total (min)**
	50	-	1.00	1.00	80	-	0.00	0.00
	180	15	0.00	9.67	230	30	0.00	5.00
	230	7	0.00	16.81	250	10	0.00	7.00
	370	10	5.00	35.81	380	30	4.67	16.00
Total run time	35.81 min				16 min			
Column flow	11.60 psi (constant pressure)				4 mL/min (constant flow)			

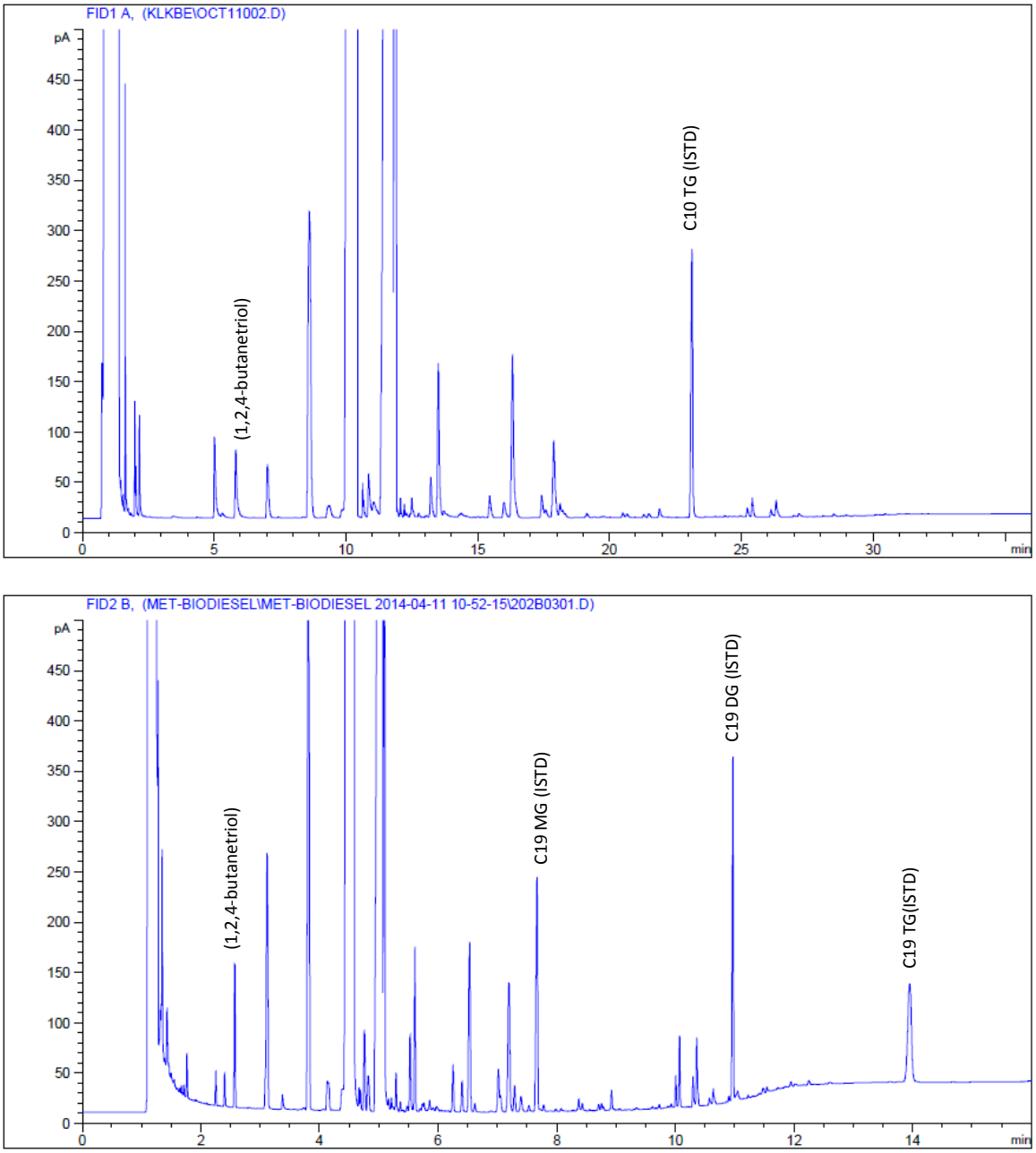


Figure 4.1: GC chromatogram of PSME analysed with (a) EN 14105 method using ZB-5HT column (15 m x 0.32 mm ID x 0.10 µm), and (b) fast GC method using MET-Biodiesel column (14 m x 0.53 mm ID x 0.16 µm).

4.1.3 Calibration module

Basically, the calibration module used was referred to the EN 14105:2011 standard method, except for the medium chain (C12 to C14) glycerides quantification. It was found that the response of medium chain glycerides are quite different with long chain (C16 and above) glycerides compounds. Therefore, an external standard calibration was used for medium chain glycerides quantification, whereas the long chain glycerides were based on the response of C19 MG, DG, and TG internal standard.

4.2 Biodiesel composition identification and quantification

Individual standard of methyl esters, mono-, di-, and triglycerides that represent the possible compounds in the palm-based biodiesel were injected under the same GC condition to identify the retention time of respective peak (Figure 4.2). The elution time of glycerides peaks are overlapping to each other, especially medium chain glycerides compounds. By considering the convenient in data integration compromising with the insignificance impact of the small peak contributed by glyceryl decanoate (C10 MG), it was decided to be excluded in total glycerin quantification. Nevertheless, glyceryl dodecanoate (C12 MG) and glyceryl tetradecanoate (C14 MG) that eluted in between long chain methyl esters peaks were integrated because of the considerable amount of these medium chain monoglycerides might present in the palm-based biodiesel, especially PKME. The integration range for respective MG, DG and TG group were shown in Figure 4.4.

The peaks integration range for the glycerides compounds were not only includes the long chain glycerides. In contrary, it covers whole range of medium to long chain (C12 and above) of glycerides compounds, as well as some phytosterol derivatives and steryl glucosides that might carry over from the palm oil raw material.

These minor components are naturally present in vegetable oils. The steryl glucosides can be converted from the transesterification process of acylated steryl glucosides. The presence of steryl glucosides contaminant in biodiesel will cause filter plugging problem in vehicles (Lacoste *et al.*, 2009). Therefore, it is not desired and can be distilled off together with the glycerides heavies during refinery process to produce B100 biodiesel. Figure 4.3 shows the retention time of steryl glucosides.

Three types of biodiesel samples were studied in this research. There are palm stearin methyl esters (PSME), palm kernel methyl esters (PKME), and grade B100 biodiesel of palm stearin base. The results of samples tested with EN14105 method and fast GC method were compared in Table 4.2. Comparable result of free glycerin contents were obtained using both methods and the result is within the EN 14214 specification.

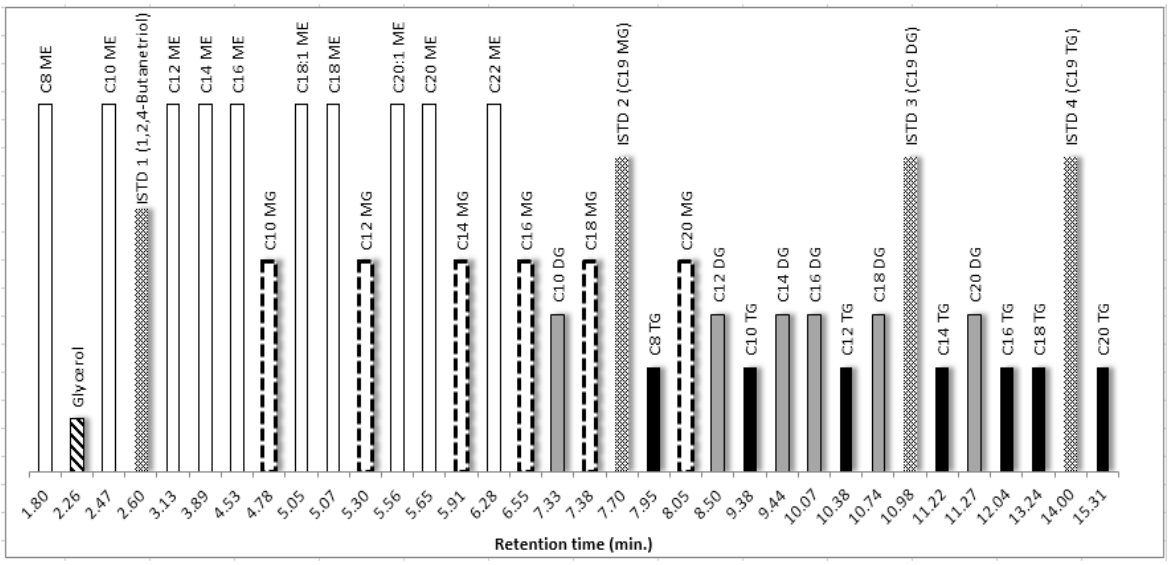


Figure 4.2: Retention times of calibrated compounds using fast GC method.

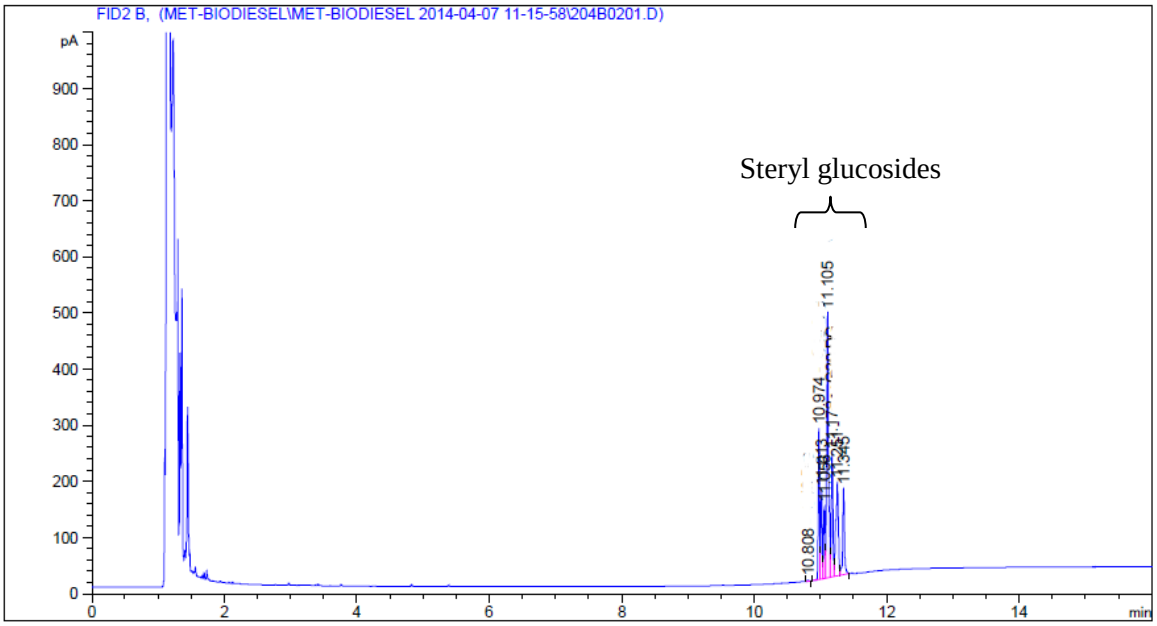


Figure 4.3: GC chromatogram to show retention time of steryl glucosides.

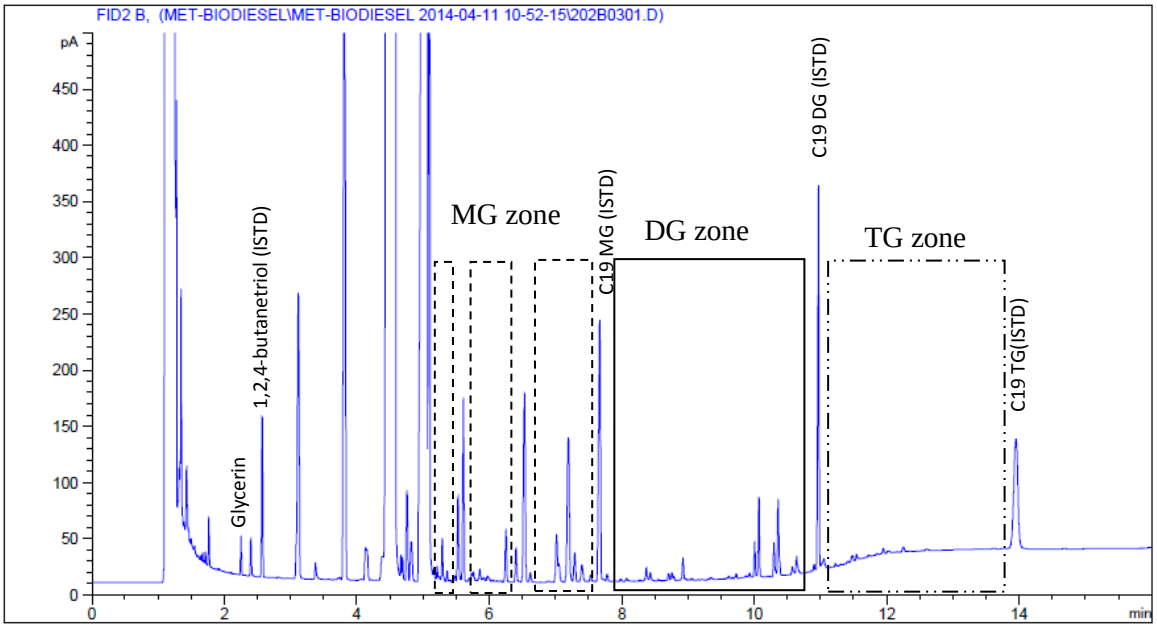
Nevertheless, the mono-, di-, and triglycerides contents were much higher than the result obtained by EN 14105 method. Moreover, the result (Table 4.2) shows the total glycerin and the respective glycerides content do not meet the EN14214 specification when tested with fast GC method. This is due to the different in calibration and quantification method used. Referring to EN14105 method, the area integration only covers the long chain (C16 to C18) glycerides peaks. Thus, lower total glycerin contents were obtained. On the other hand, the whole range of glycerides content, steryl glucosides, and also other non-methyl esters impurities were quantified in this study. Therefore, the quantification module used in this study might not fulfill the EN14214 standard. However, all these high molecular weight substances will be removed during refining process, and B100 biodiesel that meet quality specification of EN 14214 will be produced (Table 4.2). A thorough quantification is important to estimate the heavy residue that would be discharged in the refining step, and hence can monitor the process throughput.

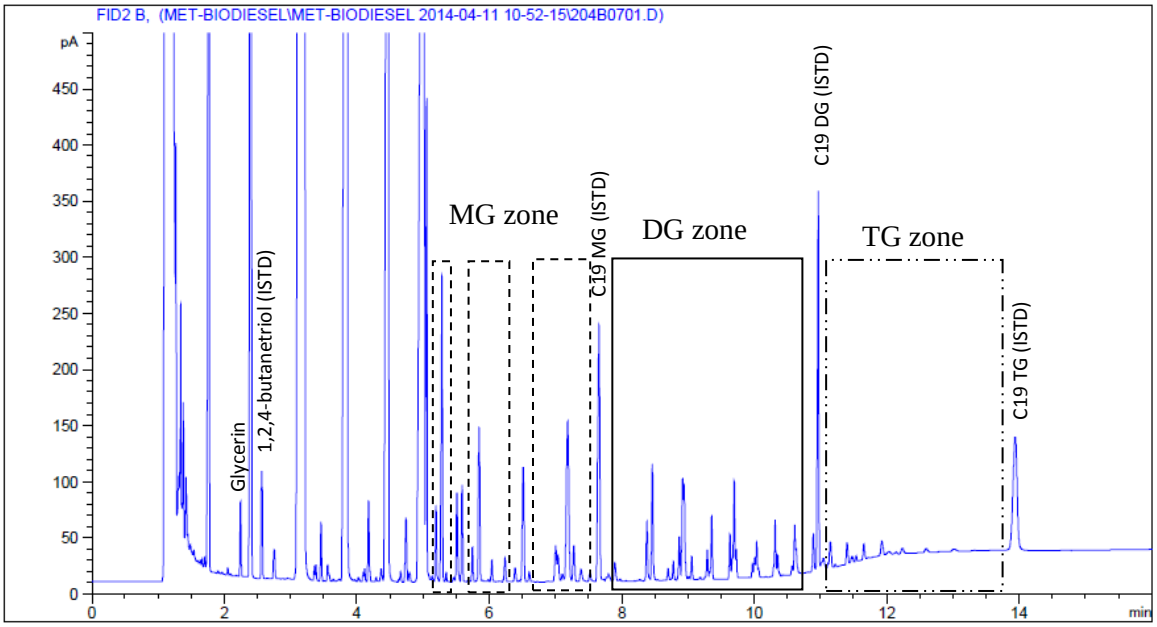
The result also shows that PKME contains higher mono-, di-, and triglycerides compared to PSME because of the composition of palm kernel oil. The presence of medium chain glycerides residues after transesterification have the boiling point quite close to the methyl esters make it hard to be separated in the distillation process. Consequently, more glycerides were carried over into the PKME product. The typical chromatograms for PSME, PKME and B100 samples were shown in Figure 4.4.

Table 4.2: Comparison of result analysed by fast GC method and EN 14105 method.

	% (m/m) ± SD (n = 4)					
Sample	**PSME**		**PKME**		**B100**	**(% max)**
Method	**Fast GC**	**EN14105**	**Fast GC**	**EN14105**	**Fast GC**	**EN14214 Spec**
Free Glycerin	0.01 ± 0.00	0.02	0.03 ± 0.00	0.03	0.00 ± 0.00	0.02
Monoglycerides	1.10 ± 0.03	0.77	1.48 ± 0.03	0.59	0.42 ± 0.03	0.80
Diglycerides	0.45 ± 0.03	0.21	1.23 ± 0.05	0.2	0.08 ± 0.02	0.20
Triglycerides	0.06 ± 0.00	0.04	0.14 ± 0.01	0.07	0.01 ± 0.01	0.20
Total glycerin	0.36 ± 0.01	0.25	0.60 ± 0.01	0.22	0.12 ± 0.01	0.25
Total unreacted oil[1]	1.61 ± 0.05	1.02	2.85 ± 0.07	0.86	0.50 ± 0.03	1.20

[1] Total unreacted oil is the sum of mono-, di-, and triglycerides content.


FID2 B, (MET-BIODIESEL\MET-BIODIESEL 2014-04-11 10-52-15\202B0301.D)
pA
Glycerin
1,2,4-butanetriol (ISTD)
MG zone
C19 MG (ISTD)
DG zone
C19 DG (ISTD)
TG zone
C19 TG(ISTD)
min


FID2 B, (MET-BIODIESEL\MET-BIODIESEL 2014-04-11 10-52-15\204B0701.D)
pA
Glycerin
1,2,4-butanetriol (ISTD)
MG zone
C19 MG (ISTD)
DG zone
C19 DG (ISTD)
TG zone
C19 TG (ISTD)
min

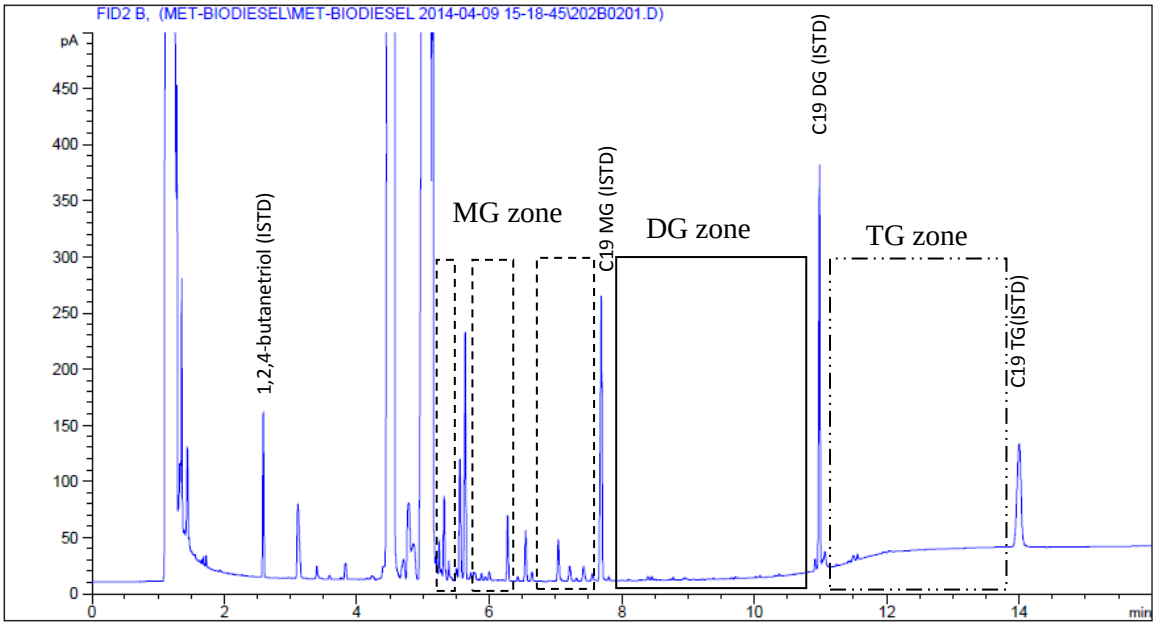


Figure 4.4: GC chromatograms (a) PSME, (b) PKME and (c) B100 sample.

4.3 Method validation

4.3.1 Linearity

A serial concentration of glycerin, lauryl (C12) mono-, di-, triglycerides that represent medium chain glycerides, and oleoyl (C18:1) mono-, di-, triglycerides that represent long chain glycerides were tested to plot the linearity curves (Appendix D). Good linearities ($r^2 > 0.95$) were obtained for each compound as tabulated in Table 4.3.

Table 4.3: Correlation coefficients of certain compounds.

Compound standard	**correlation coefficients (r^2)**
Free Glycerin	0.9858
C12 MG	0.9883
C12 DG	0.9937
C12 TG	0.9933
C18:1 MG	0.9888
C18:1 DG	0.9886
C18:1 TG	0.9771

4.3.2 Precision

Repeatability refers to the degree of agreement of results when conditions are maintained as constant as possible with the same analyst, reagents, equipment, and instruments performed within a short period of time. It usually refers to the standard deviation (SD) and relative standard deviation (RSD %). Each sample was injected twice within the same day using the same instrument and the SD and RSD were determined.

Reproducibility precision refers to the degree of agreement of results when operating conditions are as different as possible. In this study, the reproducibility only tested on different analyst and different day's variation.

The repeatability and reproducibility results (Table 4.4) shows acceptable values with SD (0 – 0.7) and RSD (1 – 8 %) for analysis conducted on the same day by same analyst; and SD (0 – 0.04) and RSD (0.2 – 7%) for analysis conducted on different day by different analysts.

4.3.3 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ are based upon the variability of the blank. The LOD was determined by calculation by multiplying 3 times of the SD of blank injections. The LOD of free glycerin was about 0.003% m/m, and the LOD for mono-, di-, and triglycerides were about 0.01% m/m respectively.

For LOQ, it also can be estimated by multiplying 10 times of the SD of blank injections. But, it must be verified by analysing the calculated amount to check the fitness into the linear curve. By calculation, the LOQ shall be 0.01 % m/m for free glycerin, and 0.03 % m/m for mono-, di- and triglycerides. But, when injected the 0.05% of mono-, di-, and triglycerides into the same GC condition, the r^2 reduce as shown in Figure 4.5. Thus, the LOQ were determined as 0.1% m/m as per the minimum concentration of calibration.

Table 4.4 Repeatability and reproducibility of PSME and PKME samples analysis.

	Repeatability (n = 2)				Reproducibility (n = 2)			
Sample	**PSME**		**PKME**		**PSME**		**PKME**	
Compound	**SD**	**RSD %**	**SD**	**RSD %**	**SD**	**RSD %**	**SD**	**RSD %**
Free Glycerin	0.00	3.54	0.00	1.72	0.00	3.31	0.00	1.46
Monoglycerides	0.03	2.49	0.03	1.73	0.00	0.21	0.01	0.78
Diglycerides	0.03	6.81	0.05	4.05	0.03	5.68	0.04	2.92
Triglycerides	0.00	6.24	0.01	7.86	0.00	6.68	0.00	0.75
Total glycerin	0.01	2.93	0.01	1.99	0.00	0.84	0.00	0.43
Total unreacted oil[1]	0.05	3.17	0.07	2.53	0.02	1.17	0.02	0.81

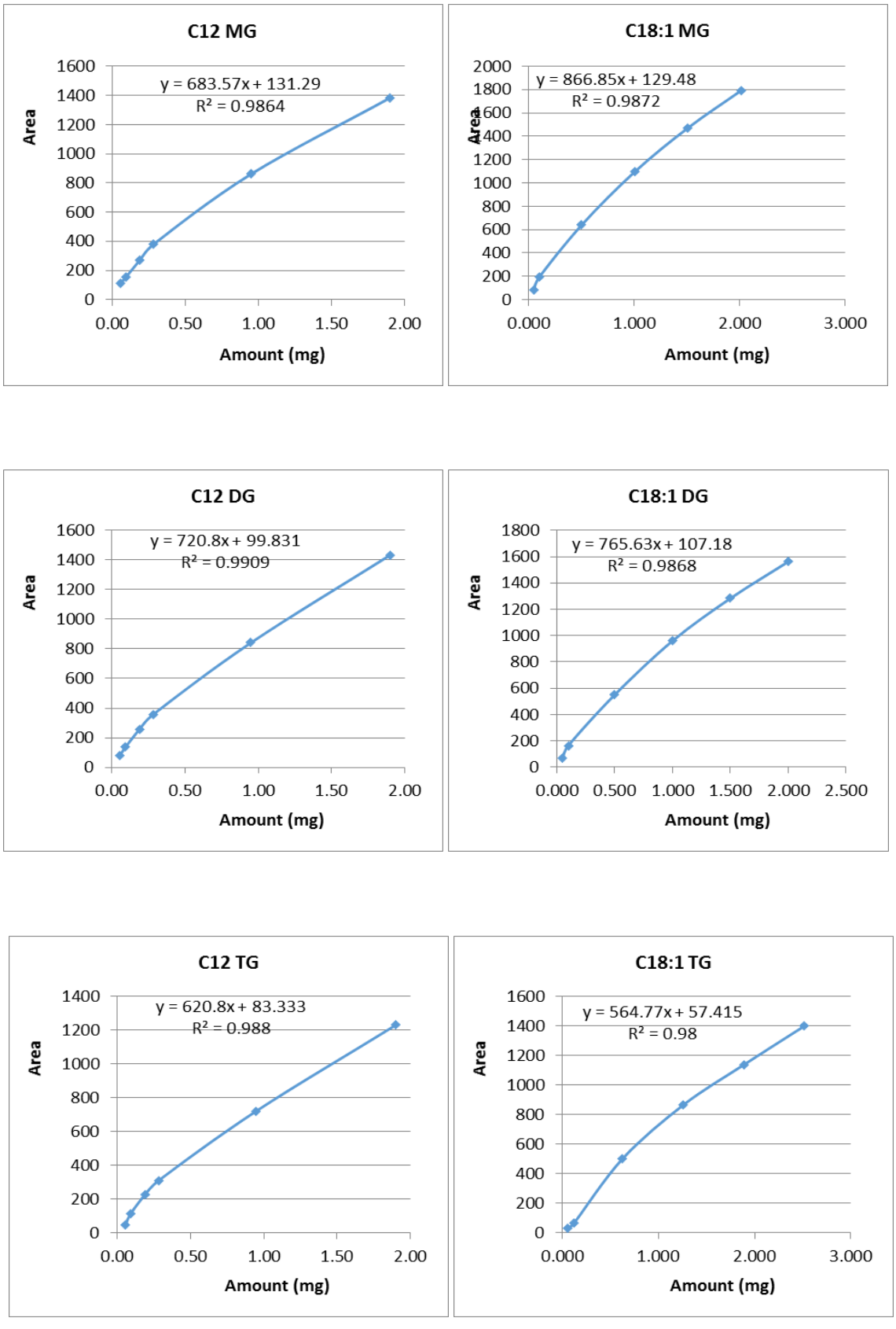


Figure 4.5 Linearity curves show the reduction of linear regression at point 0.05% m/m.

5.0 CONCLUSION

A fast GC method was developed to reduce the analysis time from 36 minutes to 16 minutes without significant loss of separation efficiency. The linearity and repeatability of the developed method demonstrated excellent system integrity, which makes the system ideally suited for the analysis of free glycerin and total glycerin, as well as mono-, di-, and triglyceride content in biodiesel. The metal column used in this study not only has the advantage of integrated guard with a leak-free connection. It also provides good peak shape and resolution for all glyceride impurities of interest.

In addition, the method developed covers not only for FAME derived from long carbon chain oils, but also suitable for medium chain glycerides oils, such as palm kernel oil. Different calibration modules were used to calibrate medium chain glycerides and long chain glycerides to avoid loss of accuracy due to discriminative response of these compounds. However, extending the peaks integration range increases the mono-, di-, triglycerides and also the total glycerin contents in the biodiesel samples, especially in PKME sample. Anyway, this is expected and these contaminants can be removed in the refined B100 biodiesel samples.

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Appendices

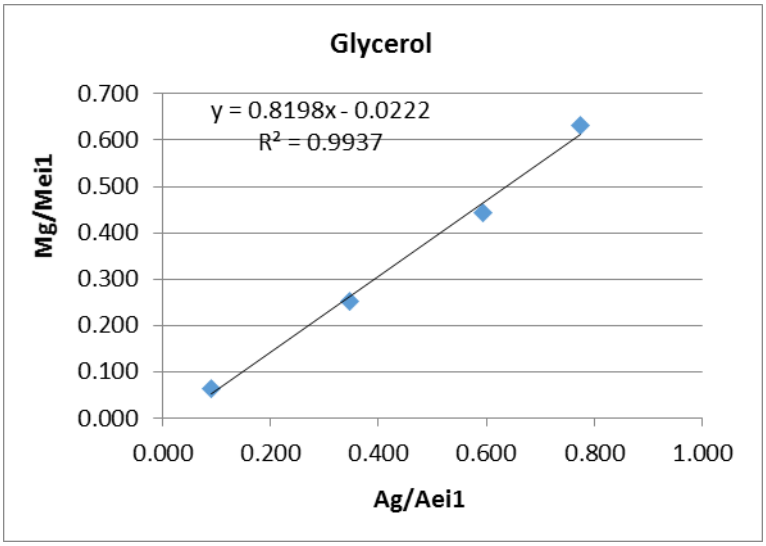
Appendix A: Retention time follow the elution order of each identified compounds

RT (min.)	Compound	Compound group
1.8	C8 ME	ME
2.263	Glycerin	Free glycerin
2.467	C10 ME	ME
2.602	1,2,4-butanetriol	ISTD 1
3.132	C12 ME	ME
3.893	C14 ME	ME
4.529	C16 ME	ME
4.783	C10 MG	MG
5.046	C18:1 ME	ME
5.065	C18 ME	ME
5.304	C12 MG	MG
5.556	C20:1 ME	ME
5.651	C20 ME	ME
5.911	C14 MG	MG
6.281	C22 ME	ME
6.554	C16 MG	MG
7.329	C10 DG	DG
7.384	C18 MG	MG
7.697	C19 MG	ISTD
7.946	C8 TG	TG
8.052	C20 MG	MG
8.496	C12 DG	DG
9.376	C10 TG	TG
9.436	C14 DG	DG
10.073	C16 DG	DG
10.384	C12 DG	TG
10.735	C18 DG	DG
10.982	C19 DG	ISTD
11.221	C14 TG	TG
11.271	C20 DG	DG
12.035	C16 TG	TG
13.241	C18 TG	TG
13.998	C19 TG	ISTD
15.313	C20 TG	TG

Appendix B: Calibration data and curve of free glycerin

Calibration solution	M_{gly}	M_{is1}	M_{gly}/M_{is1}	A_{gly}	A_{is1}	Ag/A_{is1}
1	0.005	0.08	0.063	13.97372	156.0847	0.090
2	0.020	0.08	0.253	52.96374	152.3184	0.348
3	0.035	0.08	0.442	82.37624	138.8085	0.593
4	0.050	0.08	0.632	105.15321	135.9226	0.774

ag	0.8198
bg	-0.0222

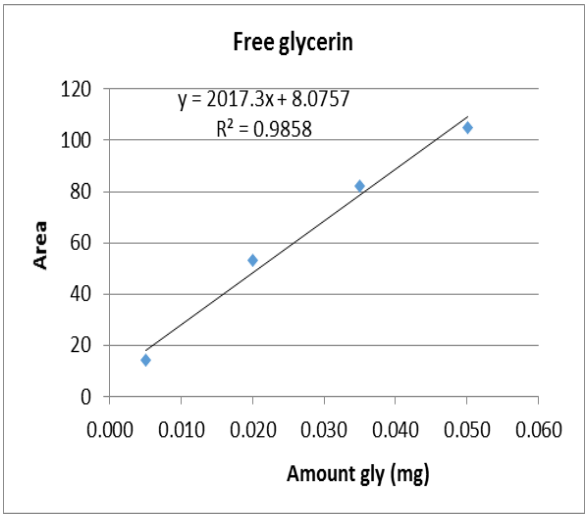


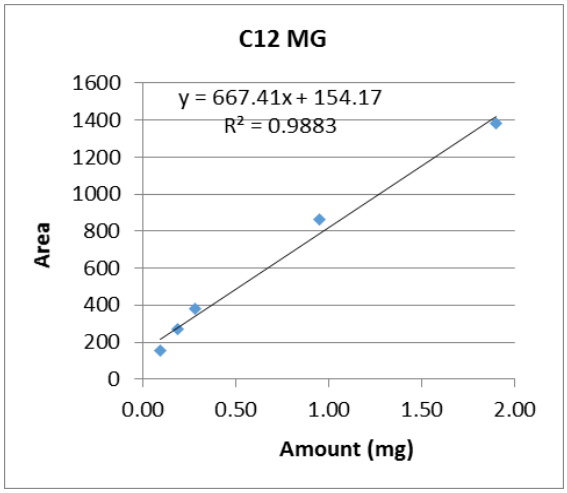
Appendix C: Calibration data of medium chain glycerides using C12 MG, DG, and TG.

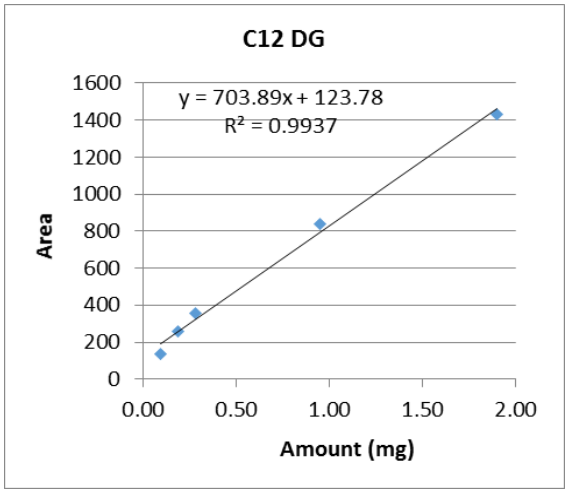
External standard calibration:

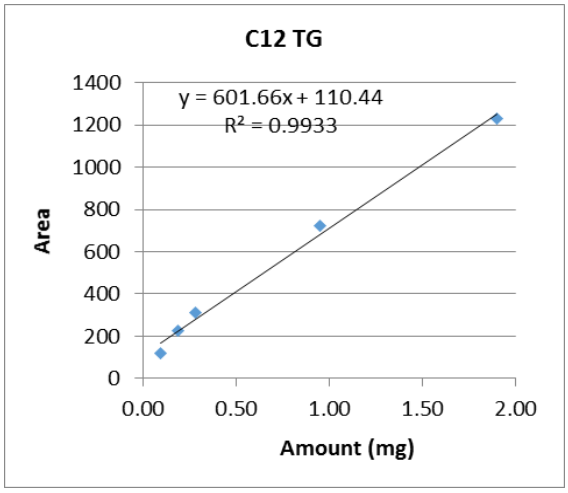
C12 MG	Amount (mg)	Area	RF	Average RF
1	0.09	155.39	1636.952	
2	0.19	271.34	1429.226	
3	0.28	380.67	1336.755	1207.70
4	0.95	860.97	906.997	
5	1.90	1383.21	728.581	
C12 DG	**Amount (mg)**	**Area**	**RF**	**Average RF**
1	0.09	136.11	1433.916	
2	0.19	256.00	1348.444	
3	0.28	357.62	1255.809	1135.87
4	0.95	841.21	886.184	
5	1.90	1433.33	754.982	
C12 TG	**Amount (mg)**	**Area**	**RF**	**Average RF**
1	0.09	117.41	1236.863	
2	0.19	226.68	1194.003	
3	0.28	311.73	1094.642	986.87
4	0.95	722.28	760.893	
5	1.90	1230.14	647.955	

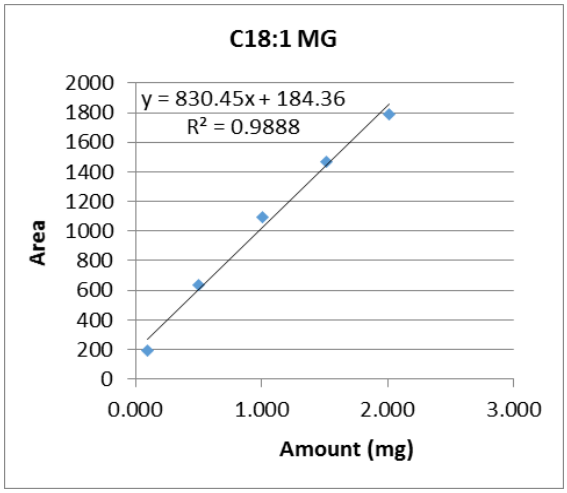
Appendix D: Linearity curves of free glycerin, C12 MG, DG, TG and C18:1 MG, DG, and TG.

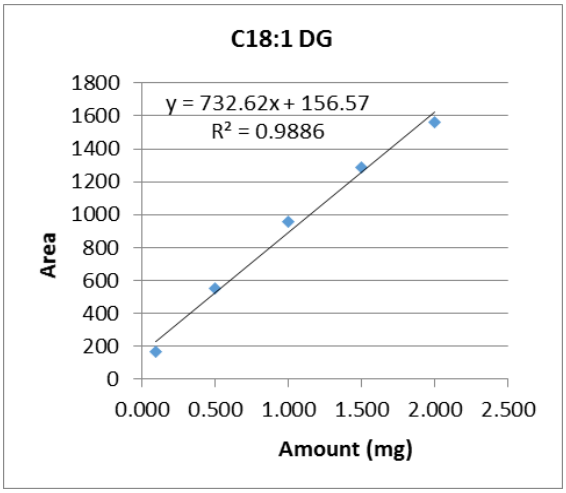


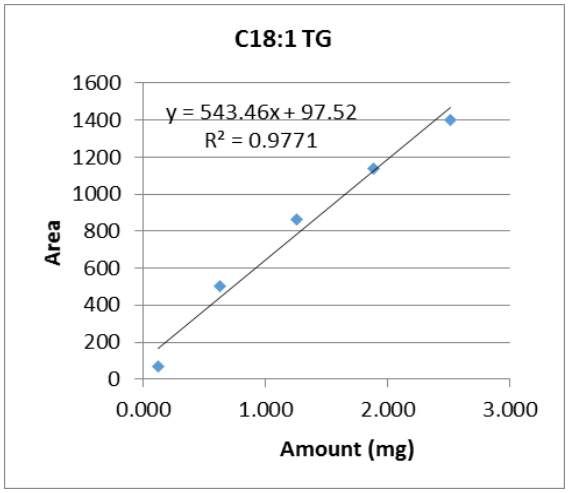







C18:1 MG
y = 830.45x + 184.36
R² = 0.9888
Area
Amount (mg)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
0.000
1.000
2.000
3.000


C18:1 DG
y = 732.62x + 156.57
R² = 0.9886
Area
Amount (mg)
0
200
400
600
800
1000
1200
1400
1600
1800
0.000
0.500
1.000
1.500
2.000
2.500


C18:1 TG
y = 543.46x + 97.52
R² = 0.9771
Area
Amount (mg)
0
200
400
600
800
1000
1200
1400
1600
0.000
1.000
2.000
3.000

APPENDIX E: Raw data of LOD and LOQ determination by calculation

	Area				
	Glycerol	**C12 MG**	**C18 MG**	**C18 DG**	**C18 TG**
Blank 1	2.98922	4.41323	4.70195	1.85553	7.7854
Blank 2	4.40195	1.84132	1.77365	1.20845	8.46205
Blank 3	5.95347	6.97801	8.12156	7.01528	5.36865
Blank 4	1.70982	2.35488	1.5752	6.36404	6.6138
Blank 5	7.20574	5.52271	7.20913	8.71055	6.25677
Average:	4.45	4.22	4.68	5.03	6.90
SD:	2.21	2.15	3.01	3.31	1.23
3 x SD:	6.62	6.45	9.04	9.94	3.69
10 x SD:	22.08	21.49	30.14	33.15	12.31
LOD (mg):	0.003	0.008	0.009	0.012	0.006
LOQ (mg):	0.010	0.028	0.032	0.040	0.021