Validation of a Reagent-free GC Lipid Derivatization Method Using an Enzymetic Microreactor

by

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Abstract

Derivatization of fatty acids to produce volatile methyl or ethyl esters (FAME or FAEE) prior to GC analysis is an indispensable procedure in lipid analysis. A lipase immobilized porous polymer monolith microreactor (PPMM) was developed and shown to achieve online and quantitative conversion of triglycerides to FAEE. When in use, a low flow of oil in ethanol is passed through the 15cm long microreactor. Full conversion of oil into FAEE is achieved during the passage of the solution through the enzymatic microreactor, so that the products can be collected for direct GC analysis. Here I describe the optimization and a validation of the first generation microreactor for reagent-free derivatization of vegetable oil samples with a range of fatty acid distributions. Results demonstrate: (i) artifact-free quantitative FAEE formation giving equivalent overall accuracy compared to AOCS method *Ce 1k-09* for FAME for fatty acid determination; (ii) the microreactor intermediate precision and reusability; (iii) improved times for conversion of TAG to FAEE. These attributes are required for a future automated system.

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List of Symbols, Nomenclature, or Abbreviations

C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
C19:0	Noadecanoic acid
C19:1	Noadecenoic acid
C20:0	Eicosanoic acid
C20:1	Eicosenoic acid
C22:1	Docosenoic acid
DAG	Diacylglycerol/ Diglyceride
EO	Ethyl oleate/ Ethyl ester of C18:1/ Oleic acid ethyl ester
ELSD	Evaporative light scattering detector
FAAE	Fatty acid alkyl ester
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas chromatography

GC/MS-NCI	Gas chromatography mass spectrometry – chemical ionization (negative mode)
HPLC	High performance liquid chromatography
MAG	Monoacylglycerol/ Monoglyceride
NP	Normal phased
RBD	Refined Bleached and Deodorized
SEM	Scanning electron microscope
SM	Silica monolith
PPMM	Porous polymer monolith microreactor
TAG	Triacylglycerol/ Triglyceride
TIC	Total ion current
TO	Trioleoylglycerol/ Triolein

Chapter 1 Introduction

1.1 Lipid Derivatization: General Concern

Lipids, including fatty acids and their derivatives (waxes, cholesteryl esters, acylglycerols, lipopolysaccharides, sphingolipids, lipoproteins, glycerophospholipids and glycoglycerolipids), are widely distributed in nature (Nakashima and Wada, 2005). In foods, the analysis of lipids has been of importance to both the research chemist and industrial chemist, mainly to evaluate the effect of dietary supplementation and the physical properties of fats and oil products (Lucci *et al*., 2009; Abeysecara *et al*., 2013; Esche *et al*., 2013; Kroll and Auell, 2013; Lay *et al*., 2013). For example, saturated fatty acids are desirable for stability and shelf life, but are reported to raise health risks such as heart attacks and strokes by increasing the level of bad cholesterol (LDL-C, short for low density lipoprotein-cholesterol). On the contrary, unsaturated fatty acids that are considered as healthy components increasing the level of good cholesterol (HDL-C, short for high density lipoprotein-cholesterol) by breaking down the LDL-C, are chemically unstable and may go rancid because of the easily oxidized double bonds. Thus, the level of saturated and unsaturated fatty acids in fats and oils is a concern among consumers, and analysis of saturated and unsaturated fatty acids has been developed on spectrometry, chromatography, mass spectrometry and electrophoresis to determine their content in labeling food products (Pitts and Thomson, 2003; Blin *et al*., 2013; Mueller *et al.,* 2013; Barra *et al*., 2013)

Generally, the analysis of lipids has been carried out by Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC). Both GC and HPLC require a chemical modification of the analytes to make the analytes more suitable for the analytical procedure. Such a modification process is known as derivatization. An example is in HPLC/fluorescence (FL) analysis, where analytes are required to have favorable functional groups called fluorophores to enable their direct monitoring by the FL detector (Nakashima and Wada, 2005). Derivatization reagents with specific functional groups are then required, such as 2-nitrophenylhydrazine that reacts with fatty acids to give highly fluorescent derivatives (Miwa *et al*., 1996; Miwa, 2002). In GC analysis, lipid analytes are normally derivatized in order to increase their volatility, decrease adsorption to the stationary phase, or increase sensitivity by increasing the molecular weight of short-chain fatty acids (Christie, 1989).

1.1.1 Transesterification of Triglycerides for GC

Esterification of free fatty acids (FFA) or transesterification of *O*-acyl lipids, particularly methylation producing fatty acid methyl esters (FAME), is by far the most common derivatization technique performed for lipid analysts. The procedure can be carried out on all lipid classes for fatty acid determination, including both simple lipids (triglycerides), and complex lipids (cholesterol esters, phospholipids and sphingomyelin) (Lepage and Roy, 1986). In some cases when short chain fatty acids are involved, higher alkyl esters are prepared, since short chain FAMEs are too volatile for manipulation. Examples include preparation of

isopropyl, *n*-butyl and decyl esters of fatty acids using isopropanpl/BF_3, *n*-butanol/BF_3 and decyl alcohol/HCl respectively (Biondi and Cagnasso, 1975; Lambert and Moss, 1972; Choudhary and Moss, 1976; Craig *et al*., 1963). In this study, we are focusing on transesterification of vegetable oils consisting of mainly medium to long chain triglycerides.

Triglycerides, present in vegetable oil or animal fat, can be transesterified into methyl/ethyl esters with the addition of methanol/ethanol and catalyst under heat. The transesterification reaction takes three consecutive reversible steps: triglyceride (TAG) into diglyceride (DAG), DAG into monoglyceride (MAG), and MAG into fatty ester and glycerol (Ma and Hanna, 1999). The overall reaction is depicted in Figure 1-1.

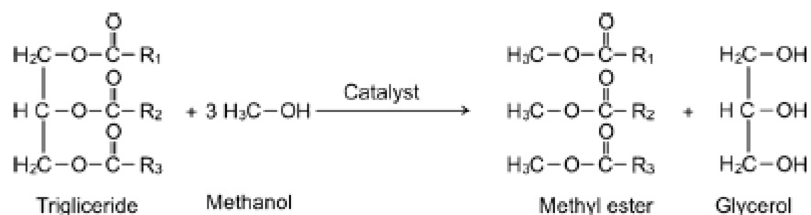


Figure 1-1 Chemistry of the Triglyceride Transesterification Reaction

Transesterification processes are usually catalytic reactions (American Oil Chemists' Society, 2012; Association of Official Analytical Chemists, 1965; International Organization for Standardization, 1978; International Union of Pure and Applied Chemistry, 1979). The catalysts can be either acid (e.g. HCl, H_2SO_4, BF_3 and BCl_3) or basic catalysts (e.g. NaOH, $NaOCH_3$ and KOH) for lipid transesterification.

1.1.2 Acid-catalyzed Transesterification

For the purpose of GC analysis, both FFA esterification and *O*-acyl lipids transesterificaion can be catalyzed by acidic catalysts. During the reaction, heat is also applied to speed up the reactions. The temperature often ranges from 60-90 °C (Liu, 1994). As shown in Figure 1-2, in transesterification reaction, the ester is firstly protonated, and then added with the exchanging alcohol to give the intermediate. The intermediate can be dissociated and form a new ester (Christie, 1993).

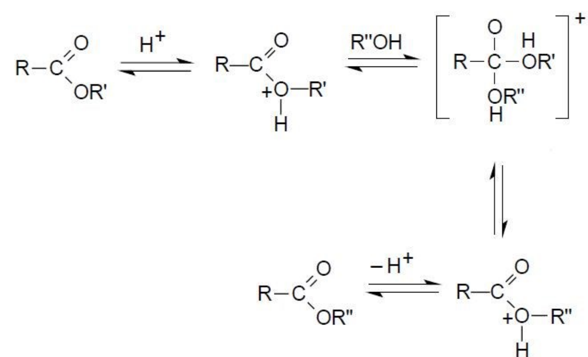


Figure 1-2 General Mechanism of Acid-Catalyzed Transesterification of Esters

A solution of 5% (v/v) HCl in methanol was used to transesterify fatty acids prior to GC analysis in 1959 (Stoffel *et al*., 1959). It has been reported to be the most common and mildest esterifying reagent (Christie, 1989). The HCl catalyzed reaction can be completed either under reflux for about 2h or in stoppered tube at higher temperature for a shorter period (Christie, 1989). Alternatively, a solution of 1-2% (v/v) H_2SO_4/MeOH was also used, and it should

be noted that the preparation of H_2SO_4/MeOH is very easy (AOAC, 1965). In 1961, the use of boron fluoride alcoholate, which is a strong Lewis acid, was reported to be effective in FAME preparation from FFA (Metcalfe and Schmitz, 1961). Later on, Metcalfe *et al.* (1966) described an improved BF_3/MeOH method combined with alkaline hydrolysis at the beginning to shortern the reaction time to 10 min, because alkaline hydrolysis frees the "bound lipids" into FFA form, on which BF_3/MeOH performs higher catalyzing efficiency. Since then, the BF_3/MeOH method has become the most commonly used FAME derivatization catalyst, and it is also adopted by the American Oil Chemists' Society (AOCS) as an official method *Ce 2-66* in 1969 (AOCS, 1969).

In acid-catalyzed transesterification or esterification, the concentration of catalyst must be well controlled within the documented range. Otherwise undesirable side reactions may occur, such as loss of unsaturated esters (Morrison and Smith, 1964). Additionally, the formed esters could react with water and undergo hydrolysis reaction, which is the reverse reaction of esterification and transesterification (Liu, 1994). Thus, the presence of water may prevent the reaction from going to completion (Eqn. 1-1).

$$R'\text{-CO-OH} + R\text{-OH} \overset{H^+}{\leftrightarrow} R'\text{-CO-OR} + HOH \qquad \text{(Eqn. 1-1)}$$

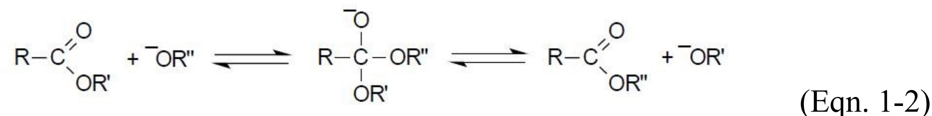
It also needs to be taken into consideration that the most commonly used BF_3/MeOH reagent is toxic and has very limited shelf life. Furthermore, the use of old or too concentrated reagent could result in undesired side-products and

affect the FA profiling results (Christie, 1989). For example, the use of BF_3/methanol has been reported to have several undesirable side reactions: 1) artefact peaks are observed late in GC chromatograms, resulting from the addition of methanol across the double bonds of unsaturated FAs, thus decrease the measurement of unsaturated FAs (Lough, 1964); 2) *cis-trans* isomerization of double bonds can occur in conjugated FAs (Christie, 1989); 3) BF_3 is reported to react with the antioxidant, butylated hydroxytoluene (BHT) commonly recommended in the heated transesterification reaction, producing interfering peaks on GC chromatogram (Christie, 1993).

1.1.3 Alkaline-catalyzed Transesterification

Compared to acid catalysts, alkali catalysts transesterify lipids at a much faster speed. However, unlike acidic catalysts that catalyze esterification and transesterification, alkaline catalysts are only effective on transesterification of *O*-acyl lipids (Christie, 1989).

The general mechanism of alkaline-catalyzed transesterification is shown in Equation 1-2. The ester will form such as an anionic intermediate in the presence of an alcoholate anion. The intermediate can dissociate back to the initial ester as a reverse reaction, or form a new ester. When the alcohol is in excessive amount, meaning the alcoholate anion is in excess as well, the intermediate will have the tendency to form a new ester. In this way, complete transesterification can be achieved using a large excess of alcohol (Christie, 1993).



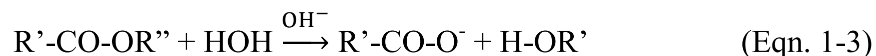
(Eqn. 1-2)

Among the alkaline catalysts, 0.5M sodium methoxide (or potassium methoxide) in anhydrous methanol has been used most frequently (Koohikamali *et al*., 2012; Seiceira *et al*., 2005; Xu et al., 2012; Velasquez-Orta *et al*., 2013). The $NaOCH_3$/ MeOH or $KOCH_3$/ MeOH reagent is prepared by simply dissolving sodium or potassium metal in anhydrous methanol. Besides the most commonly used $NaOCH_3$/ MeOH and $KOCH_3$/ MeOH catalyst, NaOH and KOH in MeOH also have some applications (Velasquez-Orta *et al*., 2013, Christie, 1989). However, NaOH and KOH are not as recommended as the methoxide reagents. This is because NaOH and KOH tend to catalyze the lipid hydrolysis reaction when there is little amount of water present, resulting in free fatty acid salts in the final product that cannot be esterified by alkaline catalysts (Glass, 1971; Hubscher *et al.*, 1960).

Nonetheless, in some classical methods, particularly those using BF_3, the hydrolysis catalyzing ability of NaOH and KOH catalysts can play a role in lipid derivatization (AOCS official method *Ce 1k-09*, 2012). The alkaline catalysts are used in combination with acid catalysts in these cases. The alkaline catalysts hydrolyze the fatty acid ester bonds in all lipid forms, including triglycerides and phospholipids, resulting in free fatty acid salts. Since acid catalysts exhibit more efficiency on esterification rather than transesterification, the following acid-

catalyzed esterification of the fatty acid salts is going to be faster than without performing the alkaline hydrolysis (Matcalfe *et al*., 1966).

The limitations of using alkaline catalysts in lipid derivatization include its inability to catalyze FFA. Additionally, the presence of water in the reactant solution will exhibit a larger effect on the alkaline-catalyzed reaction (Liu, 1994). As shown in equation 1-3, the formed ester will react with water and form carboxylate anion under alkaline condition. The carboxylate anion is in resonance stabilized state and shows tendency to react with Na^+ or K^+ in the reaction solution to form a stable salt, rather than react with alcohol (Eqn. 1-4). In this way, the presence of water will result in an irreversible side reaction producing salt. Therefore, the alkaline-catalyzed transesterification should be strictly controlled under anhydrous conditions.



$$\text{R'-CO-OR''} + \text{HOH} \xrightarrow{OH^-} \text{R'-CO-O}^- + \text{H-OR'} \qquad \text{(Eqn. 1-3)}$$

$$\text{R'-CO-O}^- + \text{Na}^+ \rightarrow \text{R'CO-ONa} \qquad \text{(Eqn. 1-4)}$$

1.1.4 Enzymatic Transesterification

The limitations of acid and alkaline catalysts mentioned above, as well as the hazard in using acidic or alkaline reagents, lead to the development of biocatalysts (enzymes). Enzymes are favored for the moderate reaction conditions they require. For example, an alkaline-catalyzed traneseterifcation often operates at high temperatures around 100°C, while enzymatic transesterification can be conducted at 40°C (Cerveró *et al.*, 2014). In transesterification reactions, enzymes

are reported to have the ability to work with a broad range of free fatty acid and water contents (Pratt and Cornely, 2004). Enzymes provide a reaction pathway from reactants to products, which requires lower activation energy than the uncatalyzed reaction. Thus, reacting molecules more easily reach the transition state per unit time, and more products are formed per unit time (Pratt and Cornely, 2004). Biocatalysts in this case are lipases isolated from a variety of bacteria, yeast or fungus species, with the ability of catalyzing transesterification reactions. The common lipase sources include *Pseudomonas fluorescens, Pseudomonas cepacia, Rhizopus oryzae, Candida rugosa, Candida antarctic*a, *Thermomyces lanuginosus* and *Rhizomucor miehei* (Vasudevan and Briggs, 2008).

Many enzymatic transesterification systems were reported as alternative to acid or alkaline catalyzed systems (Nelson *et al*., 1996; Breivik *et al.*, 1997; Shimada *et al*., 1998; Maruyama *et al*., 2000; Park *et al*., 2000). Nelson *et al*. (1996) used R. *miehei* lipase for transesterifying several oils and fats. They found that >95% TAGs were transesterified into fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE) in reactions using either MeOH or EtOH. In their study, the transesterification catalyzing efficiency of *Candica antarctica* lipase was tested as well, which was also claimed by them to be suitable for MeOH and EtOH transesterification of TAGs (Nelson *et al*., 1996). The *Candica Antarctica* lipase was further applied for non-selective ethanolysis of TAGs by Breivik *et al.* (1997). The lipase was reported to have similar catalyzing efficiency on poly-unsaturated fatty acids (PUFA) as on other constituent fatty acids, resulting in fully conversion of PUFA-rich oil (e.g. fish oil, sunflower oil) into FAEEs

(Breivik *et al.*, 1997). These efficient conversions indicate that enzymatic transesterification is comparable with classical acid or alkaline-catalyzed reactions, avoiding the use of hazardous acidic or alkali reagents at the same time.

Nonetheless, enzymes still face constraints for application in transesterification processes, because of the high cost of the enzyme and the enzyme deactivation that occurs in organic solvents (Bajaj *et al.*, 2010). Specifically, methanol as the most common lipid GC derivatization reagent producing FAMEs, has been reported to deactivate lipase (Torres and Otero, 1996). Loss of enzyme activity happens when the molar ratio of methanol to oil exceeds 1.5: 1 (Shimada *et al.*, 1999). One solution to this obstacle is methanol step-addition strategy suggested by Shimada *et al*. (2002). This approach kept the concentration of methanol at a lower percentage, helping to preserve the function of lipase. For immobilized lipase, a pretreatment procedure using $CaCl_2$ and $MgCl_2$ salt solution was introduced (Lu et al., 2010). By immersing the immobilized lipase in the salt solution at 4 °C for 24 h, the salts can incorporate into the lipase and prevent its conformational change due to organic solvent in the environment, resulting in 50-60% improvement on transesterification yield in a batch system. Besides the solutions to enzyme deactivation, there are two approaches for lowering the enzyme cost as well: improving the lipase production process, such as using cheap agro-industrial byproducts to produce lipase; and optimizing the reaction condition to extend the life of lipase, such as step addition of organic solvents.

1.2 New Lipid Derivatization Technique: Flow-through Microreactor with Immobilized Lipase

1.2.1 Flow-through Transesterification Reactors

The conventional acid and alkaline-catalyzed transesterification methods mentioned above require large quantities of sample for product recovery. However in an analytical study, the sample is usually analyzed in microscale (Mugo and Ayton, 2010). The use of a microreactor is an approach to achieve laboratory-scale conversion. A microreactor is normally a flow-through platform, and is originally designed for integrating analytical or chemical processes including sample preparation, derivatization, separation and detection into a single platform (Watts and Wiles, 2007).

An automatic flow-through reactor producing FAME derivatives from different oils for GC determination was developed as early as 1993 (Ballesteros *et al*., 1993). A sample solution containing 10-120 mg oil in 100 mL *n*-hexane, and a stream of 5% acetyl chloride in methanol (v/v) was continuously introduced into a 500 cm long reaction coil (0.5 mm ID) through a segmenter. The coil was heated to 80 °C to allow the derivatization reaction to proceed. Complete conversion was seen within 15 min inside the reaction coil. After that, a stream of water was inserted into the product solution coming out of the reaction coil, in order to remove the excessive acetyl chloride in methanol from FAME products. A 5μL fraction of FAME was then introduced into GC port for fatty acid determination. The proportions of the fatty acids in different types of oil, including virgin olive

oil, sunflower-seed oil, and codfish-liver oil, were consistent when using conventional AOAC saponification and BF_3 esterification method and the automatic flow-through method (Ballesteros et al., 1993). However, this method used a large amount of oil solution (100 mL), thus requiring large quantities of methanol and acid catalysts. The reactor was also equipped with segmenter, coil water bath and injection valve to separate water/ methanol phase and ester phase, which made it complicated to assemble or move. The introduction of water into the system could be a potential hazard to GC equipment as well.

Another flow-through transesterification microreactor (much smaller sample size of 1 mL) showing simpler operation and milder reaction condition was developed in 2009. The microreactor was packed with alkaline metal hydroxide (NaOH and KOH) as catalyst, and was claimed to complete the transesterification of plant seed oil for GC analysis in less than 1 min (Kaewkool *et al*., 2009). However, due to the hydrolysis catalyzing capability of alkaline metal peroxides, the FAME products were likely to be hydrolyzed into FFA. A similar problem was also observed on a high FAME yield KOH-catalyzed transesterification microreactor for unrefined rapeseed and cottonseed oil GC analysis, resulting in decreased FAME yield (Sun *et al*., 2009). In addition, the reusability of these microreactors was not reported.

1.2.2 Enzymatic Esterification/ Transesterification Microreactors

Enzymatic transesterification microreactors are expected to avoid side reactions including hydrolysis and saponification. However, as ethanol has shown a weaker inhibitory effect over enzymes than methanol, it could be more suitable for the enzymatic reaction to produce FAEE (Fjerbaek *et al*., 2009; Chen and Wu, 2003; Cerveró *et al.*, 2014). Several studies on enzymatic transesterification microreactors have reported good conversion from oils to FAEE using ethanol. In 2011, a flow-through lipase-catalyst microreactor consisting of silica micro structured fiber (MSF) for use in canola oil transesterification was reported to achieve 90% conversion into monoacylglycerols, showing comparable lipase activity to that obtained under conventional conditions (Anuar *et al*., 2011). More remarkably, another lipase immobilized silica monolith microreactor (SM) was developed by Anuar *et al.*, using the same lipase from *Candida antarctica*, but giving quantitative conversion of vegetable oils into FAEE derivatives. It was also proved to be reusable for up to 8 runs (Anuar *et al.*, 2013).

More recently, a lipase-catalyzed flow-through microreactor made of poly(GMA-*co*-EDMA) monolith was developed for esterification of pure lauric acid (C12:0 FA) with lauryl alcohol (Mugo and Ayton, 2013). The reusability of the enzymatic polymer microreactor was also studied, being 15 times used with lauryl laurate yield remaining more than 97%. The transesterification of castor oil into FAEEs was also tested using the enzymatic microreactor, but the conversion was observed to be incomplete. Mugo *et al*. (2014) then improved this

microreactor on its transesterification function by optimizing the enzyme immobilization condition, as well as the reaction flow rate. The improved enzymatic microreaction system can complete the transesterification of camelina oil into FAEE within the continuous flow, and could be reused up to 4 times.

Benign operation conditions and mild reaction requirements are the most notable advantages of enzymatic microreactors. Furthermore, immobilized lipases in a microreactor can also improve enzymatic activity in comparison to free lipase because its active sites become more effective (Iso *et al.*, 2001; Mateo *et al.*, 2007; Liu *et al.*, 2012). An active site is a part of an enzyme that directly binds with the substrate molecule, and it contains amino acids that promote formation and degradation of chemical bonds (Ganjalikhany *et al.*, 2012). Lipases have two conformations with very different activity: inactive closed form and active open form (Figure 1-4). The closed form isolated the active sites of lipase from the reaction medium, while the open form fully exposes the active sites to the reaction medium (Brady *et al.*, 1990; Brozozowski *et al.*, 1992). For example, *Candica antarctica* lipase B (CALB) has an active site containing catalytic serine that can bind with fatty acids, and the lipase has to be in open form for the fatty acid substrates to be accessible to the active sites inside (shown in green in Figure 1-4 a).

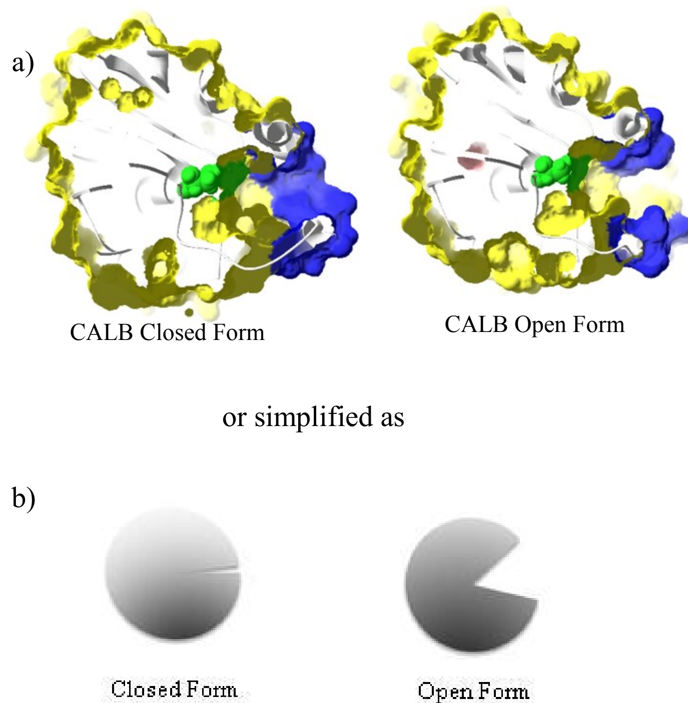


Figure 1-3 Two Conformations of Lipase a) closed and open form of *Candina antarctica* lipase B, green part is the serine active site (Picture from Ganjalikhany *et al.*, 2012 with Public Library of Science open permission); b) simplified enzyme closed and open form (Picture from Rodrigues *et al.*, 2013 with permission of Royal Society of Chemistry)

The immobilization process can be a tool to fix the lipase in its open form, by a means of adsorbing the enzyme onto hydrophobic support in a low ionic strength environment (Fernández-Lafuente *et al.*, 1998), or by crosslinking the lipase onto the support in the presence of a detergent (Fernández-Lorente *et al.*, 2006). For example, the immobilization of lipase onto a poly(GMA-*co*-EDMA) support increases the enzyme activity via the process shown in Figure 1-4. Lipases in open form have a tendency to form bimolecular aggregates, with their active sites interfacing each other and therefore show decreased enzyme activity (Palomo *et al.*, 2003). A large surface area poly(GMA-*co*-EDMA) support, which

is highly hydrophobic (Zhou *et al*., 2007), tends to adsorb the lipase onto the surface, thus cleaving the enzyme dimers resulting in the open form of lipase (Palomo *et al.*, 2003). As a result, the lipase activity is increased.

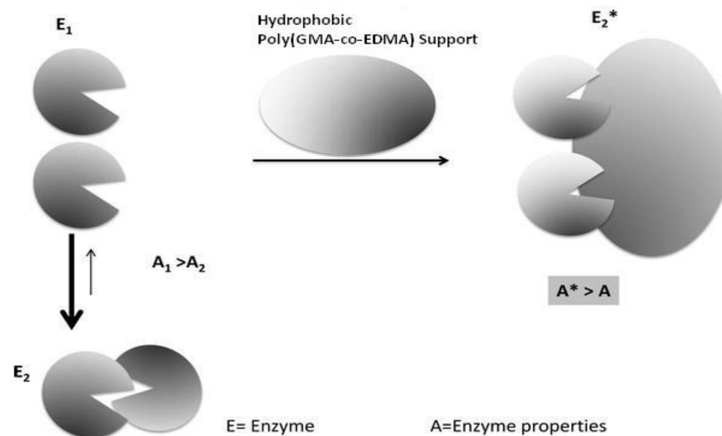


Figure 1-4 Increased Lipase Activity on Hydrophobic Support (Picture from Rodrigues *et al.*, 2013 with permission of Royal Society of Chemistry)

1.2.3 Fabrication of Enzymatic Microreactors

Fabrication techniques for microreactors are mostly photolithography, wet etching, powder blasting, hot embossing, injection molding and laser micromachining (McCreedy, 2000). Compared to these microfabrication techniques, the use of polymer sol-gel transformation in existing capillary microreactors mentioned above are favored for producing microreactors because of the simpler preparation work.

For enzyme immobilization onto microreactors, choosing the immobilization support is crucial, as it affects the chemical, biochemical

mechanical and kinetic properties of the interaction between support medium and the enzyme (Sheldon, 2007). Porous monoliths have been applied in protein, peptide and nucleic acid reaction microreactors, and are being developed for applications in lipid studies as well (Verpoorte 2003; Krenková and Foret 2004; Girelli and Mattei 2005; Kawakami et al., 2005). These applications are possible due to the low backpressure while passing reactants through and controllable porosity that can be achieved in porous monoliths (Urban et al., 2006; Peterson, 2005). Commonly used monolith supports for enzyme bonding include Cordierite monoliths, silicon alkoxides monolith, epoxy resins, silica monoliths, silica microstructured optical fibers, and porous polymer monolith (Mugo and Ayton, 2013). A porous polymer monolith that has previously been applied in lipid transesterification microreactors was adopted as the lipase support in this thesis research. Many materials can be used to generate polymer monoliths, such as styrenes, methacrylates, acrylates, vinylpyridines, vinylpyrrolidones, polyurethanes, acrylamides and norbornene (Gibson *et al.*, 2008). Poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) [poly(GMA-*co*-EDMA)] monolith is one of the polymer monolith materials (Fig. 1-5). It exhibits good reproducibility and stability, as well as easy functionalized catalyst attachment (Wen and Feng, 2007). Most commonly, the monolith is attached to a silica capillary with an internal diameter of 300 - 500 µm.

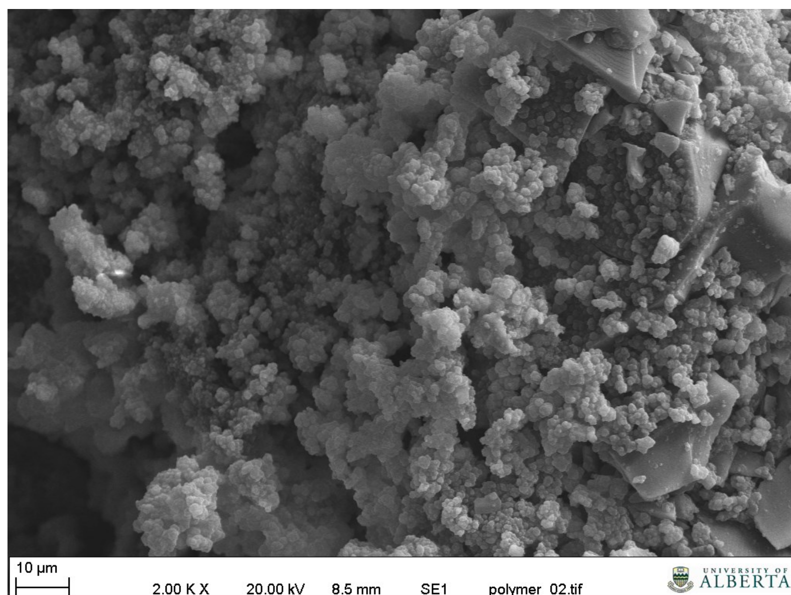


Figure 1-5 Scanning Electron Microscopy Image (SEM) of a Porous Poly(GMA-*Co*-EDMA) Monolith Support

1.3 Hypothesis and Objectives

1.3.1 Hypothesis of the thesis

It is hypothesized that lipase immobilized porous poly(GMA-*co*-EDMA) microreactors can be made, and retains full catalyzing efficiency for transesterification of oils by ethanol with reduced reaction and collection times. Additionally, the microreactor is hypothesized to be an equivalent method as standardized official methods for fatty acid determination of vegetable oils, and also reproducible and reusable.

To verify this hypothesis, two main area of study were carried out as described in Chapters 2 and 3.

1.3.2 Objectives of Chapter 2

a) To demonstrate the successful manufacture of lipase immobilized porous poly(GMA-*co*-EDMA) microreactors by scanning electron microscopy imaging, in addition to tests of lipase immobilization and porosity.

b) To reduce the transesterification reaction and collection time of the microreactor to give rapid full conversion of a triolein TAG standard.

1.3.3 Objectives of Chapter 3

After confirming that polymer monolith microreactor gives full conversion from triolein to ethyl oleate, the second study described in Chapter 3 focused on validating the performance of the microreactor on vegetable oils. This validation includes the following objectives:

a) To demonstrate the good agreement of the fatty acid profile of three vegetable oils between the lipase immobilized porous poly(GMA-*co*-EDMA) microreactor/ GC-FID method and the AOCS official method *Ce 1k-09*.

b) To determine the reproducibility and reusability of the microreactor using a standard TAG mixture solution.

Chapter 2 The Performance of the Enzyme Immobilized Polymer Monolith Microreactor on Pure Triacylglycerol Transformation Using Ethanol

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2.1 Introduction

Microreactors were initially designed for integrating several chemical or analytical processes into one platform to simplify the operation (Watt and Wiles, 2007). A continuous flow-through microreactor offers many advantages including: shorter reaction time due to large surface area to volume ratio of the microreactor material (Mason et al., 2007); controllable reaction process (Mason et al., 2007); lower sample usage (Lu et al., 2004) and simple product separation. In the past decade, microreaction technology has become increasingly popular among clinical diagnostics for immunoassay, analytical and synthetic chemistry (Watt and Wiles, 2007; Mason et al., 2007).

The microreaction technique has been now applied to lipid transformations using immobilized lipase. Compared to the conventional acid or alkali catalyzed transesterifications in batch systems, the use of an enzymatic flow-through microreactor on lipid derivatization has many advantages: simple sample preparation, no post-reaction work-up, no use of hazardous acid or alkaline, mild reaction condition at room temperature, and fewer byproducts (glycerine).

Previously, Mugo and Ayton (2011) developed a flow-through bio-catalyst poly(GMA-*co*-EDMA) monolith (PPMM) microreactor, and tested its performance for esterifying lauryl alcohol and lauric acid to lauryl laurate, as well as in the transesterification of triolein and crude canola oil. *C. antarctica* lipase B was used for a 700μm ID, 28cm long silica capillary. It gave up to 97% conversion for synthesis of lauryl laurate via esterification, at a flow rate of 10μL/min of a mixture of 0.1M in both lauric acid and lauryl alcohol. They also reported that the microreactor can be reused at least 15 times over a 1 month time period, stored at room temperature, with minimal to no reduction in the enzyme activity. Newer research by Mugo et al. (2014) further tested the performance of a smaller ID (320 μm) poly(GMA-*co*-EDMA) column with immobilized *Candida antarctica* lipase B. The microreactor converted pure triolein and triacylglcerols in camelina oil completely into ethyl ester derivatives at a flow rate of oil/ ethanol solution of 0.3μL/min. The monolith microreactor was reusable up to 5 times with minimal or no lipase activity loss.

Although the study on lipase immobilized PPMM microreactor demonstrated promising results, the total operation time, including infusion and collection, was quite long (5 h). As described in Chapter 1, this is much longer than most of the standardized official acid or alkaline-catalyzed transesterification, which can last from a minimum several minutes to 2 h. Therefore, the PPMM microreactor will be more advantageous if the operation time can be improved.

The objectives of this study were 1) to characterize a 320µm ID, 15cm long lipase immobilized porous poly(GMA-*co*-EDMA) monolith (PPMM) microreactor to demonstrate successful manufacturing; and 2) to achieve the transesterification reaction with a reduced total flow-through time. The transesterification was performed on a TAG standard: triolein, using ethanol to produce ethyl oleate (EO) at room temperature. The reaction products were analyzed by Normal Phase (NP) HPLC-ELSD to detect if there were any triacylglycerol residues. NP/HPLC can separate lipid classes, such as TAG, diglyceride (DAG), monoglyceride (MAG) and fatty acid ethyl esters (FAEE), thus this method allows one to detection any TAG residues, or any DAG and MAG as intermediate products indicating incomplete transesterification. GC-FID was used to identify and quantify the individual FAEE products, which can be resolved in GC according to the different carbon chain lengths and different degree of unsaturation. With the assurance of full transesterification efficiency of the PPMM microreactor in an optimized operation time, it can then be validated for wider use with oils, as explained in Chapter 3.

2.2 Experimental Procedures

2.2.1 Materials

Fused silica capillary (ID: 320µm, 15cm) was obtained from Polymicro Technologies (Pheonix, AZ, USA). A Harvard Model '11 Plus syringe pump was from Harvard Apparatus (Holliston, MA, USA). Lipase from *Candida antarctica*

was obtained from Sigma-Aldrich Ltd (St. Louis, MO, USA). Pure Triolein (>99%), ethyl oleate (EO) standards were purchased from Nu-Check (Elysian, MN, USA). All organic solvents were HPLC analytical grade from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 Preparation of Poly (GMA-co-EDMA) Monolith Capillary

The polymer monolith (PM) was made following the procedure described in detail earlier (Mugo *et al*., 2013) and adjusted during the experiment. The procedure was also outlined in Figure 2-1. A 15 cm fused silica capillary was cut by a ceramic cutter, and then connected to a 5 mL plastic syringe by a microtight. The capillary was flushed with 1mL 1.0M NaOH solution, capped, and allowed to stand overnight. Twenty percent g-methacryloxyproplymethoxysilane (γ-MAPS), 30% glacial acetic acid and 50% D.I. water (v/v/v) were mixed and votexed as anchoring site solution. The mixture (1 mL) was injected into the silica capillary at a flowrate of 5-10 μL/min, and was left in the capped capillary overnight at room temperature. To get rid of the excess anchoring site solution, 1mL acetonitrile was manually pushed through the capillary from a syringe. The capillary was then dried by passing a stream of air through it for 2min. The pre-polymer sol was prepared by mixing the monomers glycidyl methacrylate (GMA, 24%) and ethylene dimethacrylate (EDMA, 8%) with two long chain alcohols cyclohexanol (40%) and 1-dodecanol (18%), which act as pore generating solvents (v/v) (Mozo et al., 2009). The mixture was added with 1% 4,4'-azobis (4-cyanovaleric acid) (w/v). After the preparation of pre-polymer sol, the mixture

was injected slowly inside the silica capillary at 5μL/min without tilting, avoiding any air bubbles. The filled capillary was then sealed at both ends, wrapped with aluminum film and cured in an 80 °C oven overnight. GMA-*co*-EDMA polymer monolith was then formed inside the silica capillary.

2.2.3 Lipase Immobilization onto the Polymer Monolith Support

The polymer monolith capillary was first flushed with 1mL 50% acetonitrile at 1 μL/min before immobilization, followed with 1 mL 0.1M sodium phosphate buffer (pH 7.2) at a flow rate of 1 μL/min. Another 1 mL of the buffer solution was filtered before dissolving 8mg lipase enzyme. The 8mg/mL lipase solution was filled into the monolith capillary at 0.5 μL/min (four times lower than the reported manufacturing procedure). After the injection, the enzyme loaded polymer monolith capillary was left at room temperature immobilization efficiency. The monolith's morphology, attachment to the capillary, porosity and enzyme loading were measured, in order to confirm that the microreactors produced in this study are physically comparable to those reported by Mugo *et al*. (Mugo *et al*., 2014).

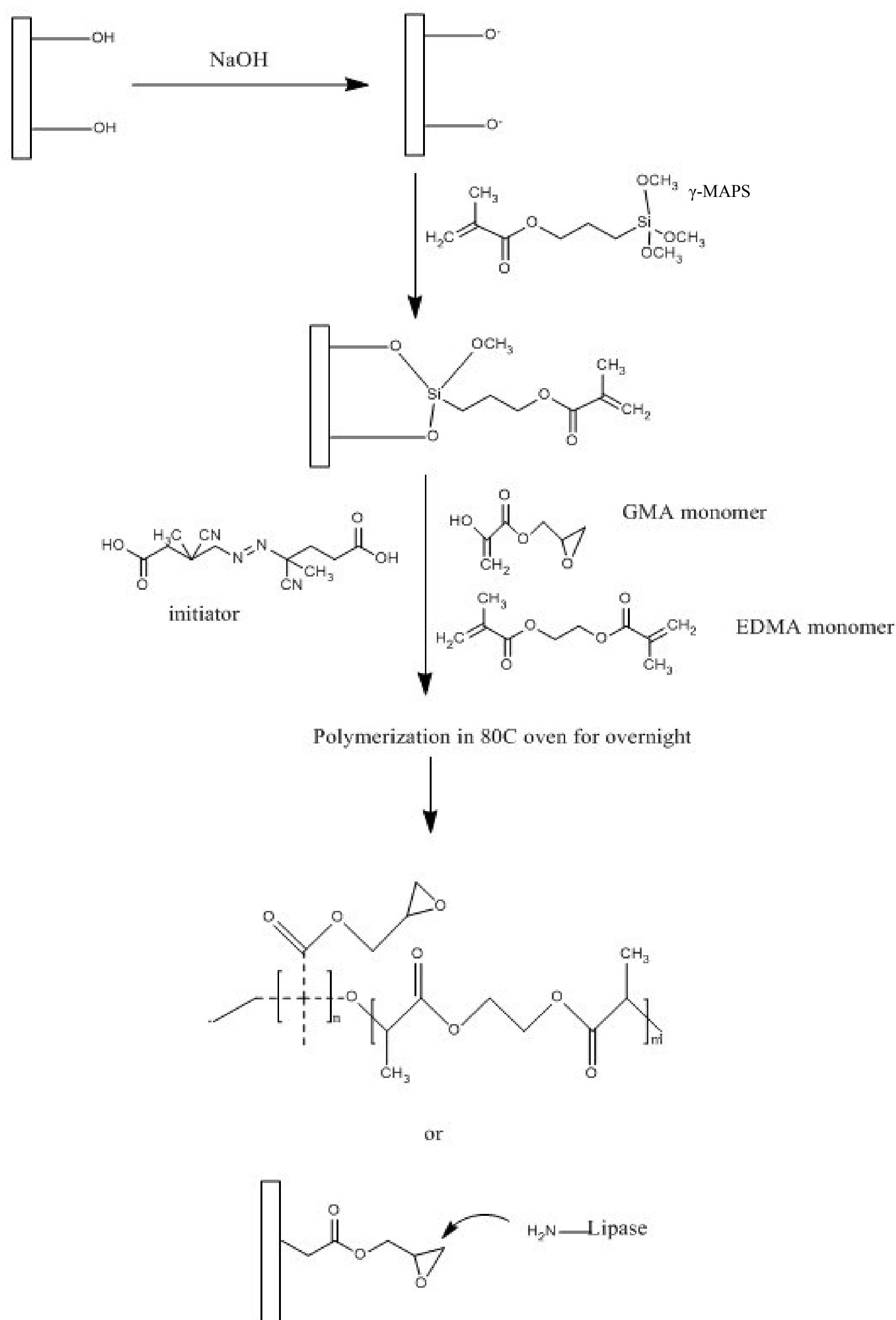

Figure 2-1 Production and structure of poly(GMA-co-EDMA) monolith inside a silica capillary

2.2.3.1 Scanning Electron Microscopy

A scanning electron microscopy (SEM) with LaB6 crystal source (Zeiss EVO MA 15; Carl Zeiss Microscopy; 2-17 Earth Sciences Building, University of Alberta) was used for imaging the polymer monolith. The SEM was equipped with a Bruker Silicon Drift Detector for Energy Dispersive X-Ray analysis/mapping with a peak resolution of 125 eV. The enzyme immobilized polymer monolith microreactor was first cut into a 1cm piece using a ceramic cutter. The cleaved piece was then attached to a SEM plate for vacuum drying. The specimens were gold coated with a Nanotech SEM Prep 2 DC sputter coater. Images were taken at both ×425 and ×5000 magnification.

2.2.3.2 Porosity Determination

The porosity of the polymer monolith was estimated based on the difference of PPM microreactor weight before and after filling it with water. The calculation (Eqn. 2-1) was also used by other researchers (He *et al*., 2010).

$$\epsilon = \frac{(M_M/\rho)}{(M_T/\rho)} = \frac{V_M}{V_T} \qquad \text{(Eqn. 2-1)}$$

In the equation, M_M is the mass of water that fills the 15cm polymer monolith microreactor, measured by the difference between the weight of microreactor with and without water; M_T is the mass of water that fills a 15cm empty silica capillary, the same as the one used for microreactor manufacturing; ρ

stands for the density of water at room temperature. Three porosity measurements were performed on three separate microreactors, and the average of the three measurements were calculated as the porosity of the PPMM microreactor.

2.2.3.3 Biuret Protein Assay

The amount of lipase loaded on the polymer monolith was determined by the Biuret protein assay, based on the concentration difference between the lipase solution introduced into the column and that eluted from the column. The Biuret reagent was freshly made before the test, and prepared with 0.60g sodium potassium tartrate and 0.15g copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) in 50mL water, together with 30mL 10% (w/v) sodium hydroxide. The volume of the mixture was made up to 100mL with water, and the final solution was used as the Biuret reagent. The calibration curve was established with serial diluted bovine serum albumin (BSA) solutions, covering concentrations from 0.1mg/mL to 2mg/mL. Biuret reagent (2mL) was added to 0.5mL lipase eluent and 0.5mL initial lipase solution respectively, in 4mL quartz cuvettes. The blank was made by 0.5mL sodium phosphate buffer and 2mL Biuret reagent. The absorbance of blank and the lipase solutions were read at 540nm using a V-530 UV/Vis Spectrophotometer (Jasco. Inc., Japan).

2.2.4 Catalyzing Efficiency of the Polymer Microreactor

The performance of the enzyme immobilized polymer monolith microreactor was evaluated by the transesterification of triolein with ethanol.

Before the reaction, the microreactor was flushed with ethanol for 1 h in order to remove the aqueous buffer as well as any unbound lipase. Triolein (TO, 0.50 mg/mL) was prepared in ethanol: hexane (4:1, v/v). The mixture was vortexed until complete dissolution and then transferred to a 1mL glass syringe (Hamilton Company, Reno, USA). The reaction solution was injected into the PPMM microreactor continuously at 0.3 μL/min using a Harvard Model '11 Plus syringe pump (Harvard Apparatus, Holliston MA) at room temperature. After 1.5h, the product solution was collected into a GC glass vial, and was further diluted by 30 μL 0.10 mg/mL C19:1 FAME in ethanol for HPLC/ELSD and GC/FID analysis. The transesterification for triolein was performed for three times on different microreactors using prepared triolein solution, and the average of the transesterification rate in each run calculated by GC/FID was measured. The microreaction device for this offline reaction is shown in Figure 2-2.

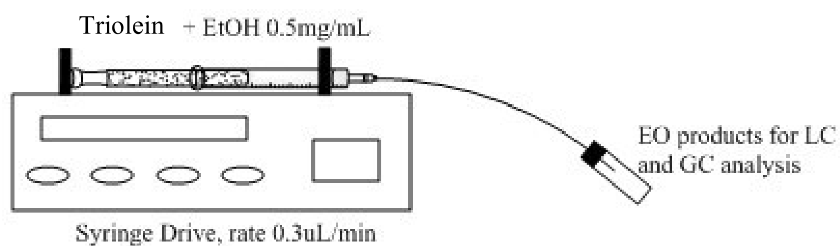

Figure 2-2 Microreaction Device of Triolein Transesterification Reaction Using the PPMM Microreactor

2.2.5 Reversed Phase Liquid Chromatography / Evaporative Light Scattering Detection (HPLC/ELSD)

The lipid classes present in the product solution were analyzed by non-aqueous reversed phased high performance liquid chromatography (HPLC) using

Agilent 1200 HPLC system coupled with evaporative light scattering detector (ELSD) model 1260 HPLC Infinity (Agilent Technologies, Santa Clara, CA, USA). The column was an Agilent Zorbax HT C18 column (4.6 × 50mm, 1.8um, Agilent Technologies). The separation condition was the same as previously reported (Anur *et al*., 2011). The mobile phase consisted of A, 100% methanol and B, isopropanol: hexane (5:4), with initial gradient 25% B, then increased to 95% B at 5 min, before returning to 25% B at 5.1min and held for 2.9 min (t = 8 min) to equilibrate the column. Evaporative light scattering detector (ELSD) drift tube temperature was set to 33°C, with computer-controlled N_2 gas flow of 3 L/min at pressure of 2.0 bars.

2.2.6 Gas Chromatography/ Flame Ionization Detector (GC/FID)

An 7890 GC system (Agilent Technologies) coupled with a flame ionization detector (FID), autosampler and spilt/ splitless injector was used for quantification. The column was a SP-2560 column 100 m × 0.25 mm × 0.2 µm GC column (Agilent Technologies). The external calibration curve for ethyl oleate (C18:1 FAEE) was constructed at concentrations of 0.0007, 0.00175, 0.0035, 0.007, 0.0175, 0.035, 0.07, 0.175 and 0.35 mg/mL, with 0.03 mg/mL C19:1 FAME internal standard prepared in EtOH. All data were collected using Agilent Chemstation software (version G1701EA). The GC system was set at: 2 µL injection volume, split ratio 20:1, H_2 as FID carrier gas at 2 mL/min, inlet temperature 250 °C, detector temperature 280 °C, He as make-up gas. The temperature program was as previously reported (Anur *et al*., 2013): 140 °C (hold

for 5 min), 8 °C/min to 180 °C (0 min), 4 °C/min to 210 °C (0 min), 20 °C/min to 270 °C (hold for 7 min). All reaction products were diluted with 30 μL 0.1 mg/mL C19:1 FAME in EtOH prior to GC/FID analysis, which partly helps make up to an adequate volume for analysis using an autosampler.

2.3 Results and Discussion

2.3.1 Evaluation of the Polymer Microreactor

The SEM was used to image the cross-sections of the microreactor. From the image shown in Figure 2-3 a) and c), the poly(GMA-*co*-EDMA) monolith formed near the capillary wall does not show any gaps. This indicates the strong attachment of monolith to the column capillary wall, which prevents any detachment with use as a flow-through microreactor, and also ensures the contact of reactants with lipase on inside monolith channel. This strong attachment is benefited by the anchoring sites provided by γ-MAPS (Gibson and Mugo, 2008). The highly packed monolith also explains the back pressure encountered while trying to increase the reaction flow rate. The morphology of the monolith appears to be identical to the ones reported by Mugo *et al*. (2013). From Figure 2-3 b, it is clearly seen that the microreactor consists of a micro-porous and monolithic network, which provides a large surface area, and a relatively low back pressure with applied flow. Figure 2-3 d) show the lipase immobilized microreactor after one use (with buffer wash afterwards). The microreactor still retains much of the porosity and monolithic structure, as well as remaining attached to the capillary

wall. Any loss of microreactor porosity after use might isolate the lipase from contact with substrates and could be one of the reasons why microreactor has decreasing efficiency over time.

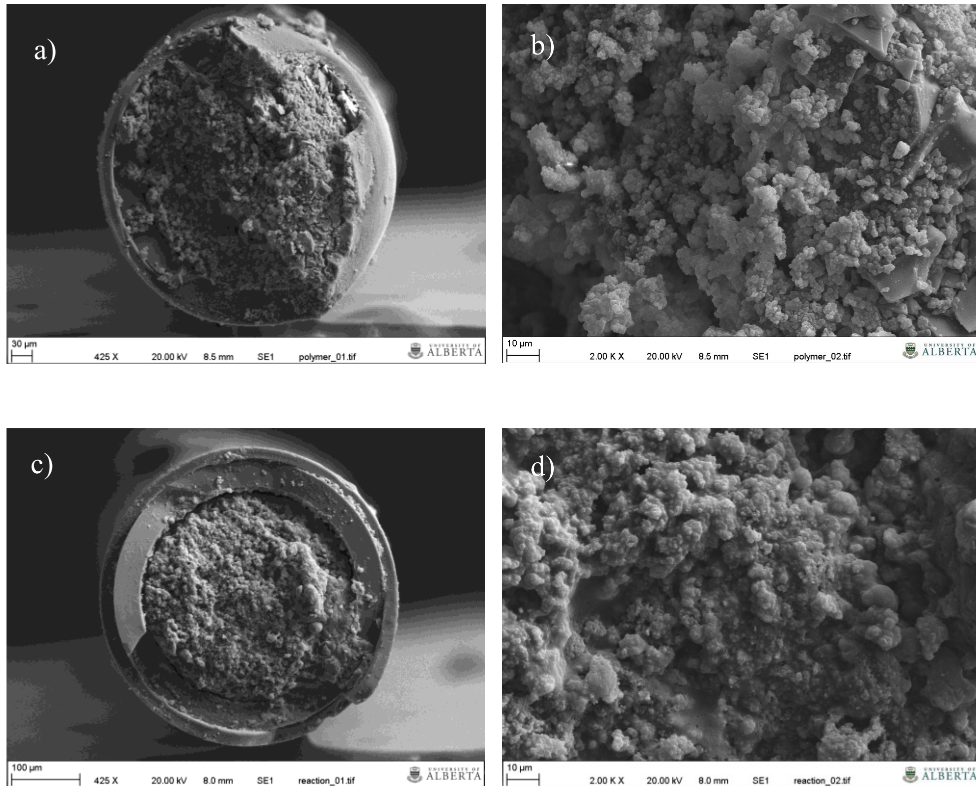

Figure 2-3 SEM Image of the Cross-section of: a) a polymer monolith capillary at ×425 magnification, b) same capillary at ×2000 magnification, c) a polymer monolith capillary after triolein transesterification reaction at ×425 magnification, d) same capillary after one use at ×2000 magnification

It has been reported that the surface area of the porous polymer monolith can be represented by its porosity (He *et al*., 2010). Here, porosity measurements were carried out in triplicate (see Eq. 2-1) and the average was determined to be

0.81 ± 0.03 (Table A2-1), which is in good agreement with the reported porosity of the PPMM microreactor: 0.80 ± 0.02 (Mugo *et al*., 2013).

The poly(GMA-co-EDMA) monolith has epoxy groups at the end that are available to directly react with the amine groups on an enzyme (Liu et al., 2012). Thus, an activation process, like the APTES treatment used with silica monolith microreactors (Anur *et al*., 2013) is not necessary. Prior to immobilization, a 50% acetonitrile wash was used to remove the excess polymer sol gel. An 8 mg/mL lipase dissolved in pH 7.2 buffer was then pumped through the column. This flow through process improves the mass transfer ratio, therefore gives better immobilization yields than the static protocol, where the microreactor was filled with lipase solution to react (Monzo et al., 2007). The concentration of lipase solution before and after being pumped through the microreactor was measured using a spectrometer. The calibration curve was established using BSA protein standard according to Biuret method. Biuret protein assay is among the most common approaches for protein concentration measurement. The absorption of the violet-color product at 540nm is in a linear relationship with the concentration of total protein. Thus, a standard curve is created to calculate the concentration of unknown sample. The resulting BSA standard curve obtained here exhibits a linear relationship with a correlation coefficient (R^2) of 0.9994 (Figure 2-4).

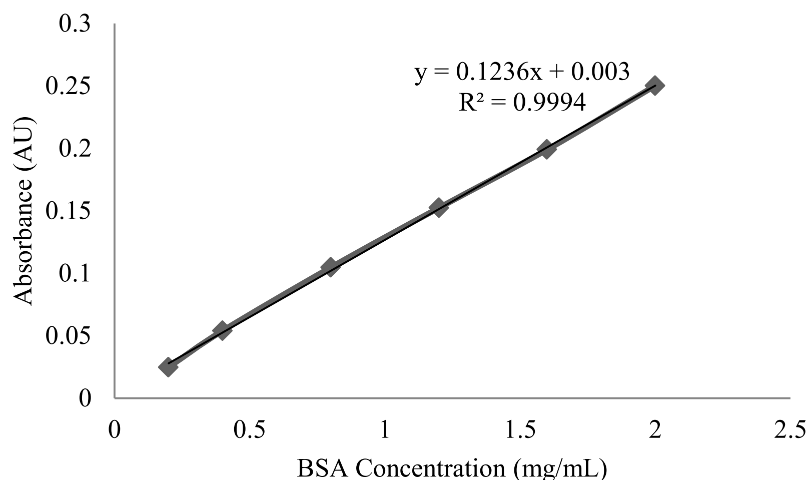

Figure 2-4 BSA Calibration Curve at 540nm Wavelength

From the equation of UV absorbance to protein concentration, the amount lipase immobilized onto the 15 cm polymer microreactor was calculated to be 2.14 mg from 1 mL of 8 mg/mL lipase flow (Table A2-2). Hence, the immobilization yield was then calculated to be 0.14 mg/cm PPMM microreactor. Comparing to the silica monolith microreactor also designed for transesterification reaction (Anuar *et al*., 2011), which has an immobilization yield of 0.42 mg/cm microreactor, PPMM microreactor has a much lower enzyme-bonding efficiency.

To optimize the lipase immobilization onto epoxy groups, there are many approaches. 1) Increase the reaction time: the linking between lipase and epoxy groups is a multipoint interaction. This interaction is a time-consuming and time-dependent process, because it needs to align the groups on enzymes and on supporting surface correctly (Poppe *et al*., 2013). 2) Optimize the polymer

composition: the porogenic solvents that generate pores and the monomer (GMA and EDMA) ratio both affect the pore radius, pore distribution and thus the inside surface area of the PPMM microreactor (Monzo *et al*., 2007). It has been reported that an enzyme can be immobilized well within pores 3 to 9 times larger than the size of the enzyme itself, and the size of the pore influences the immobilization substantially (Liu *et al.*, 2013).

However, in previous batch *Candida antarctica* lipase B catalyzed transesterification systems, 20 wt% lipase (based on oil weight) usage can already achieve highest conversion rate (Li *et al*., 2006; Nie *et al*., 2006). This means that for a 0.50mg/mL triglyceride reaction solution used in this study, more than 2 mg immobilized lipase with 1U/mg activity would be adequate for catalyzing the transesterification reaction.

Based on these evaluations, the microreactors manufactured in this project were expected to have comparable catalyzing efficiency with those reported by Mugo *et al*. (2013).

2.3.2 Catalyzing Efficiency of the PPMM Microreactor

The PPMM microreactor is able to achieve complete conversion from 0.50mg/mL triglyceride in ethanol to ethyl esters with a total collection time of 5 hours (includes infusion and collection) at a flow rate of 0.3μL/min (Mugo *et al*., 2013). However, the reaction time should be shorter than the reactant solution

residence time inside the microreactor (15 min), which is the time required for a 0.3μL/min flow to pass through a 15 cm long, 320μm internal diameter microreactor. The long collection time used in the previous study was only for collecting sufficient product volume for analysis. Since the reaction is completed before coming out of the microreactor, the reaction time can be shortened till adequate amount of product can be collected. A shorter flow-through time of 1.5h was selected in this project. Theoretically, with a flowrate of 0.3 μL/min, 27 μL product solution can be collected after 1.5 h flow. However, considering the dead volume of the connector of microreactor and syringe, the collected volume was approximately 20 μL only. This way, collected product solution could be diluted with 30 μL 0.10 mg/mL C19:1 FAME solution to makeup to approximately 50 μL solution for reliable GC/FID quantification and HPLC/ELSD analysis, remaining sufficient signal to noise ratio. If the GC autosampler was able to inject from a much smaller volume, the flow-through time of the triglyceride in ethanol solution can be reduced even further.

Normal Phase HPLC (NP-HPLC) is a useful technique for lipid class separation. In this experiment, NP-HPLC is used to separate TAG from FAEE as well as from monoacylglyceride (MAG) or diacylglyceride (DAG), if they are present as intermediate products formed during transesterification. It is observed on the HPLC/ELSD chromatogram that triolein (TO) was completely converted into ethyl oleate (EO) according to the retention time (Figure 2-5) which matches that of an EO standard. From Figure 2-5 a) and c), the peak area of TO is much greater than that of EO at the same concentration, which means that TO and EO

have different response factors in HPLC/ELSD. This explains the much lower peak area of the EO product compared to the initial TO peak area. Also, the relationship of the EO peak area to its concentration is not linear in the HPLC/ELSD chromatogram. Thus, to exactly quantify the EO products for evaluating the conversion rate of the microreactor, another method needs to be applied.

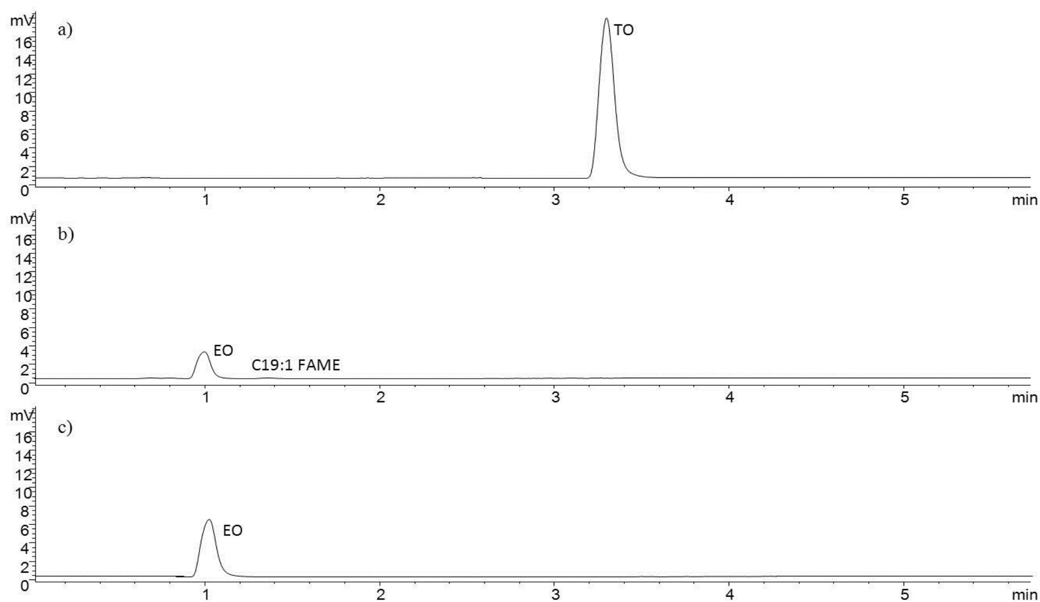

Figure 2-5 HPLC/ELSD Traces for a) 0.50 mg/mL Triolein in EtOH/Hexane 4:1, v/v starting material; b) 0.50 mg/mL ethyl oleate in EtOH/Hexane 4:1, v/v, reacted in microreactor for 1.5 h with C19:1 FAME standard; c) 0.50 mg/mL ethyl oleate standard.

GC/FID was used to quantify the concentration of EO in the collected solution. It also gave more confidence on identification of the product by comparing the retention time with that of EO standard. Unlike NP-HPLC, different FAEEs are well separated in GC, including some of their isomers. In the

GC/FID chromatogram in Figure 2-6, the product trace resembles that of the EO standard, which confirms the presence of EO in the product.

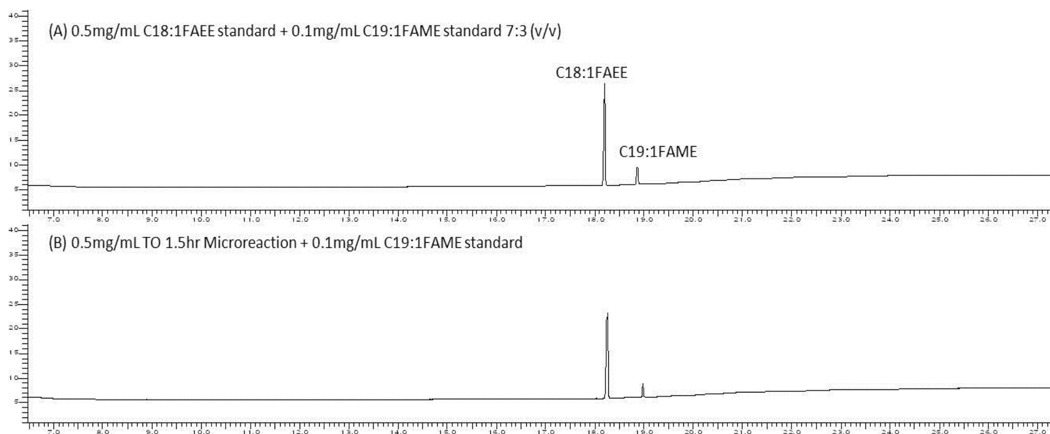

Figure 2-6 GC/FID Chromatogram for (A) 0.50 mg/mL C18:1 FAEE standard and 0.10mg/mL C19:1 FAME standard mixture 7:3, v/v; (B) 0.50 mg/mL Triolein in ethanol reacted on PPMM microreactor for 1.5h, with 30 µL 0.10mg/mL C19:1 FAME standard before analysis

A GC auto-injector ideally requires more than 40µL sample size to ensure reliable injection volumes. However, 1.5 h reaction on the PPMM microreactor only generates less than 30 µL product, and the exact volume is unknown. An internal standard solution of 0.10 mg/mL C19:1 FAME solution in ethanol was prepared both for diluting the collected product to meet the minimum GC injection volume requirements, and for calculating the dilution factor. The EO concentration in the diluted solution was obtained from an external calibration curve. The dilution factor was indicated by the decrease of the internal standard C19:1 FAME concentration after addition (Eqn. 2-2). The two values then revealed the real EO concentration following PPMM microreaction. This number was then compared with the theoretical EO concentration that should be obtained

from quantitative transesterification of the starting solution of 0.50mg/mL TO (Eqn. 2-3).

$$\text{Dilution Factor } d = \frac{V_p}{V_p + V_{IS}}$$
$$= 1 - \frac{V_{IS}}{V_p + V_{IS}}$$
$$= 1 - d_{IS}$$
$$= 1 - \frac{conc_{IS\ after}}{conc_{IS\ initial}} \qquad \text{(Eqn. 2-2)}$$

where: d is the dilution factor of the collected product from PPMM microreactor
V_p is the volume of collected product from PPMM microreactor
V_{IS} is the volume of internal standard added, which is 30μL
d_{IS} is the dilution factor of the internal standard after added to the collected microreactor product solution
$conc_{IS\ after}$ is the concentration of C19:1 FAME internal standard in the diluted solution, which is calculated by C19:1 FAME external calibration curve;

$conc_{IS\ initial}$ is the initial concentration of C19:1 FAME before added to the PPM microreaction product, which is 0.1mg/mL.

$$Conversion\ rate = {}^{conc_{exp}}/_{conc_{theo}}\ \%$$

$$= \frac{{}^{conc_{GC}}/_{d}}{conc_{TO} \times CF_{TO\ to\ EO}}\ \% \qquad \text{(Eqn. 2-3)}$$

where: $conc_{GC}$ is the concentration of EO in the diluted solution, which is calculated by EO external calibration curve;

d is the dilution factor of PPM microreaction product;

$conc_{TO}$ is the initial concentration of TO as reactant, which is

0.5mg/mL;

$CF_{TO\ to\ EO}$ is the conversion factor from TO to EO based on molar ratio 1: 3, which is 1.0519 based on mass

The concentration of EO produced by PPM microreactor as well as its conversion rate compared to the theoretical value are shown in Table 2-1.

Table 2-1 Conversion Rate of 0.50 mg/mL Triolein to Ethyl Oleate on PPMM Microreactor after 1.5 h Reaction

	Conc. EO (mg/mL)	Conversion rate
RUN 1	0.5156	98.04%
RUN 2	0.5135	97.65%
RUN 3	0.5091	96.80%
Average	**0.51**	**97%**
RSD%	0.65%	

For this experiment, three individual PPMM microreactors were used. The conversion rate obtained using these 3 microreactors were consistent, and the average conversion was 97% of the theoretical value (0.53mg/mL FAEE production). Any impurities in the initial TAG reactant and any mass transfer loss inside the PPMM microreactor could contribute to the loss of EO yield, thus decrease the conversion rate. However, the decrease would be consistent for each component, so that can be compensated for when using a TAG internal standard before reaction to quantify the fatty acids in an oil sample (see Chapter 3). The closeness of the PPMM microreaction conversion to the theoretical value, the absence of TAG peak or intermediate MAG and DAG peaks on HPLC/ELSD trace, and the sole EO peak on both HPLC and GC chromatogram together indicate a complete conversion of triolein to ethyl oleate by the PPMM

microreactor. On the basis of the quantitative transesterification capability of the PPMM microreactor, further validation on its conversion of oil samples will be conducted.

2.4 Conclusion

In conclusion, the Porous Polymer Monolith Microreactor (PPMM) containing immobilized *C. antarctica* lipase was demonstrated to be fabricated successfully according to the reported procedure. The monolith morphology, porosity, attachment to the wall, and lipase loading were measured and shown to agree with the previously reported microreactors (Mugo *et al*., 2014).

In addition, the PPMM microreactor was demonstrated to complete sample solution infusion, transesterification reaction within flow, and product collection process within 1.5 hours, which was reduced from 5 hours (Mugo *et al*., 2014). With the shortened collection time, the PPMM microreactor was still observed to consistently and completely convert the 0.50 mg/mL triolein in ethanol into ethyl oleate, without producing any intermediate products (i.e. diglyceride, monoglyceride). The reduced collection time provides an additional advantage of PPMM microreactor method over conventional acid or alkaline-catalyzed methods, besides its benign operation environment and simple operation.

Chapter 3 Validation of the Enzyme Immobilized Polymer Monolith Microreactor for Application on Vegetable Oil

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3.1 Introduction

In lipid GC analysis, lipids are normally derivatized into fatty acid alkyl esters (FAAE), in order to increase their volatility, decrease the adsorption to GC stationary phase, or increase the sensitivity of short-chain fatty acids by adding molecular weight (Christie, 1989). An enzyme immobilized flow-through transesterification microreactor producing fatty acid ethyl esters (FAEE) from lipid samples, containing a porous poly(GMA-*co*-EDMA) monolith (PPMM for short), was developed by Mugo *et al*. (2013), as an alternative to conventional GC derivatization processes. In chapter 2, it was demonstrated that the PPMM microreactors can achieve complete conversion of triolein in an ethanol/hexane solution into ethyl oleate, without the use of other reagents. This chapter extends the application of PPMM into real-world lipid samples, as illustrated by a range of natural TAG vegetable oils. In order to demonstrate that methods employing the lipase immobilized PPMM could realistically substitute for the widely adopted derivatization methods currently used to prepare FAME or FAEE, a method validation was performed.

The analytical derivatization process can be either transesterification or esterification of lipid samples by reaction with alcohol. Conventional methods

derivatize lipids into fatty acid methyl esters (FAME) using acid or alkaline catalysts in methanol, such as HCl, BF_3, BCl_3, $NaOCH_3$, NaOH and KOH (Stoffel *et al*., 1959; AOAC, 1965; AOCS official methods, 2006; Christie, 1989; Koohikamali *et al*., 2012; Seiceira *et al*., 2005; Xu et al., 2012; Velasquez-Orta *et al*., 2013). The reasons why FAME is preferable include its lower molecular weight thus higher volatility compared to other FAAE derivatives, and the robust and reproducible chromatographic data obtained (Christie, 1993; Ballesteros *et al.*, 1993; Xu et al., 2012). However, hazardous acidic or alkali reagents are required in these methods, separation of the FAAE products from catalysts needs to be conducted, and interfering side-reactions easily occur if caution is not taken while using the catalysts (described in Chapter 1).

The enzyme immobilized PPMM microreactor can avoid the disadvantages of conventional derivatization methods mentioned above, especially the multiple sample manipulation required by the conventional methods. This makes the microreactor ideal for automated lipid GC analysis. To validate the use of the polymer monolith microreactor method for fatty acid determination, the method accuracy, intermediate precision and PPMM reusability were tested. In the present context, accuracy is demonstrated by the closeness between the fatty acid measurement for vegetable oils using 1) PPMM microreactor and 2) AOCS official method using BF_3/MeOH. The intermediate precision is defined here by the consistency of results obtained from different microreactors under the same condition. The PPMM reusability is demonstrated

by how many times a microreactor can be used to obtain quantitative conversion. These are described in detail in Section 3.3.

Boron trifluoride, one of the most common Lewis acid catalysts for lipid methylation, is adopted in AOCS official methods following an additional alkaline hydrolysis process using NaOH/MeOH (AOCS *Ce 1k-09*, 2012). This BF_3/MeOH derivatization method is used in this project as a reference to the microreactor method. The hydrolysis process can 1) release bound fatty acids into the free fatty acid salt form to enable faster reaction rate of acid-catalyzed esterification in the following reaction (Metcalfe *et al*., 1966), involving in conversion from fatty acid salts into FAMEs; and 2) transesterify some lipids into FAME during hydrolysis step because of the catalytic property of the alkaline reagent (Liu, 1994).

Oils and fats, consisting of triglyceride mixtures with chains of fatty acids with different degrees of unsaturation (Emmanuel and Mudiakeoghene, 2008), play very important functional and sensory roles in food products (Dauqan *et al*., 2011). The triglyceride and fatty acid composition largely affect the physical and chemical properties of an oil or fat sample: short chain fatty acids have lower melting point and are more soluble in water compared to long chain fatty acids; unsaturated fatty acids have lower melting point than saturated ones with similar chain length (Chayanoot *et al*. 2005). Furthermore, the fatty acid components in oil and fats can have different effects on health. For example, omega-3 fatty acids have a beneficial role in brain and cardiovascular health (Perica and Dalas, 2011);

monounsaturated fatty acids are reported to reduce blood cholesterol levels (Kris-Etherton, et al., 1999); whereas erucic acid (C21:1 ω9) carries the concern of lipidosis problems in animals (Charlton *et al*., 1975). Thus, the fatty acid composition of TAG mixtures dictates the dietary benefits and physical properties of oil and fats.

The lipase immobilized PPMM can be a TAG derivatization method prior to GC analysis of oils and fats. The flow-through microreactor has the potential to be coupled with GC injection port, so that original TAG samples are infused into the PPMM microreactor, and FAAE derivatives are injected into GC after the sample's passage through the reactor. This proposed automated lipid analysis system would be safe, simple, and would require minimal sample handling. It could be proposed as an alternative method to conventional lipid analysis methods that require hazardous acidic and alkaline derivatizing reagents and considerable number of sample manipulations, once it has been validated to show reliable measurements.

In this study, the use of the PPMM microreactor on fatty acid determination will be validated, including an accuracy assessment, precision measurement, and reusability test according to a pre-established validation plan. This is to demonstrated that the PPMM microreactor can be an equivalent derivatization method compared to conventional methods for GC analysis of lipids.

3.2 Experimental Procedures

3.2.1 Materials

Fused silica capillary (ID: 320µm, 15cm) was obtained from Polymicro Technologies (Pheonix, AZ, USA). A Harvard Model '11 Plus syringe pump was from Harvard Apparatus (Holliston, MA, USA). Sodium sulfate, sodium hydrogen carbonate, sodium chloride and lipase from *Candida antarctica* were obtained from Sigma-Aldrich Ltd (St. Louis, MO, USA). Food grade canola oil, camelina oil and refined, bleached and deodorized (RBD) palm olein were purchased from a local grocery store. 14% BF_3/MeOH solution was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pure triolein (>99%) and all C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C19:0, C20:0, C20:1, C22:1 ethyl ester (FAEE) standards; C19:0, C19:1 methyl ester (FAME); GLC FAME standard mixture No.714, containing C8:0, C10:0, C12:0, C13:0, C14:0, C16:0, C16:1, C17:0, C18:0, C18:2, C18:3, C20:0, C20:1, C21:0, C20:4, C20:3, C20:5, C22:0, C22:1, C23:0, C22:5, C24:0, C22:6 and C24:1, distributed evenly by weight; C19:0 TAG standard; GLC TAG standard mixture No.406, containing 4.0%C16:0 TAG, 2.0%C18:0 TAG, 61.0%C18:1 TAG, 21.0%C18:2 TAG, 9.0%C18:3 TAG, 1.0%C20:0 TAG, 1.0%C20:1 TAG and 1.0%C22:1 TAG (% by weight), were purchased from Nu-Check (Elysian, MN, USA). All organic solvents were HPLC analytical grade from Sigma-Aldrich (St. Louis, MO, USA).

3.2.2 Direct transesterification using PPMM Microreactors

Separate solutions of the GLC 406 TAG standard mixture, canola oil, camelina oil and refined, bleached and deodorized (RBD) palm olein were prepared, each at 0.50 mg/mL in ethanol: hexane 4: 1 (v/v). To 1mL of each oil solution was added 200 μL of 0.50mg/mL C19:0 TAG in hexane as the internal standard (IS). This gave final solutions containing 0.42 mg/mL TAG and 0.08 mg/mL C19:0 TAG IS in each case. The mixtures were vortexed vigorously until oil and TAG standard were dissolved completely. The solution was then infused through the enzymatic PPMM microreactor at room temperature at a flowrate of 0.3 μL/min for 1.5 h using a Harvard Model '11 Plus syringe pump. The eluent was collected in a GC vial, and diluted with 30 uL 0.10 mg/mL C19:1 methyl ester IS in EtOH prior to analysis by HPLC/ELSD, GC/FID and GC/MS.

3.2.3 AOCS official method for FAME preparation

AOCS official method *Ce 1k-09* "Direct Methylation of Lipids in Food for the Determination of Total Fat, Saturated, *cis*-Monounsaturated, *cis*-Polyunsaturated, and *trans* Fatty Acids by Gas Chromatography" was used as the reference method (AOCS, 2012).

To prepare the internal standard and test portion, 5.0mg/mL C19:0 TAG internal standard was dissolved in chloroform. The internal standard solution (2 mL) was transferred into a 50 mL round flat-bottom reaction flask and was dried with nitrogen to remove solvent. The amount of the C19:0 TAG internal standard

was recorded. 100 mg sample was weighed into the reaction flask containing internal standard. Boiling chips were then added before methylation reaction.

Alkali catalyzed methyl ester preparation was selected for the vegetable oils. A 5 mL 0.5M NaOH/methanol was added to the flask first, with a condenser attached. The reactor was heated to 70°C and was refluxed for 15min after boiling begins. Another 5 mL 14% BF_3/MeOH was added to the boiling flask and the mixture was refluxed for additional 2 min.

After the reaction was completed, the reaction flask was cooled to room temperature. Cooled solution was washed with enough saturated sodium chloride, and dried over sodium sulfate. A portion of the washed solution was transferred into a 2mL autosampler vial and ready for GC analysis. This reaction was done three times for each sample, and the relative standard deviation (RSD) and average of each sample was obtained.

3.2.4 Reversed Phase Liquid Chromatography / Evaporative Light Scattering Detection (HPLC/ELSD)

The lipid classes present in the product solution was analyzed by a non-aqueous reversed phased high performance liquid chromatography (HPLC) using Agilent 1200 HPLC system coupled with an evaporative light scattering detector (ELSD) model 1260 HPLC Infinity (Agilent Technologies, Santa Clara, CA, USA). The column was an Agilent Zorbax HT C18 column (4.6 × 50mm, 1.8 µm, Agilent Technologies). The mobile phase consisted of A, 100% methanol and B,

2-isopropanol: hexane (5:4), with a gradient of 25% B, increasing to 95% B in 5 min, before returning to 25% B at 5.1 min and holding for 2.9 min (t = 8 min) to equilibrate the column. The ELSD drift tube temperature was set to 33°C, with computer-controlled N_2 gas flow of 3 L/min at pressure of 2.0 bar.

3.2.5 GC/FID

For FAEE quantification after PPMM microreaction, the GC/FID method was previously optimized method (Anuar *et al*., 2011). An Agilent 7890 GC system (Agilent Technologies) coupled with a flame ionization detector (FID), autosampler and spilt/ splitless injector was used. The column was a SP-2560 column 100 m × 0.25 mm × 0.2 µm GC column (Agilent Technologies). All data were collected by Agilent Chemstation software (version G1701EA). The GC system was set at: 2 µL injection volume, split ratio 10:1, H_2 as FID carrier gas at 2 mL/min, inlet temperature 250 °C, detector temperature 280 °C, He as make-up gas. The temperature program was set as: 140 °C (hold for 5 min), 8 °C/min to 180 °C (0 min), 4 °C/min to 210 °C (0 min), 20 °C/min to 240 °C (hold for 15 min).

For FAME quantification after the official AOCS methylation method, AOCS method *Ce 1h-05* was adjusted and applied according to the procedure described on lipid derivatization method *Ce 1k-09*. The same SP-2560 column was used under an isothermal temperature program at 181 °C. Injection volume

was 1 μL. The split ratio was 100:1, H_2 was FID carrier gas and the flow rate was 1mL/min. Both injector and detector temperatures were 250 °C.

All GC/FID measurements of the FAAE components contained in both PPMM microreaction products and AOCS official methylation products were corrected with Theoretical Correction Factors (TCF), relative to the internal standard C19:0 FAEE (C19:0 FAME for AOCS method) as described in AOCS *Ce 1h-05*. The GC/FID was also tested for its accuracy before each run, by evaluating the difference between the Experimental Correction Factor (ECF) and TCF, with a relative difference within 10% was considered as an accurate GC run.

3.3 Validation Plan

3.3.1 Purpose of Validation

The purpose of this validation is to demonstrate that the lipase immobilized poly(GMA-*co*-EDMA) microreactor (PPMM) is an effective platform for fast and simple triglyceride transesterification. The validation should demonstrate that fatty acid quantification using FAEE produced via the PPMM is equivalent to fatty acid quantification using official FAME methods.

3.3.2 Scope of Validation

The validation plan is limited to the use of PPMM microreactors manufactured according to Chapter 2 and their function in transesterifying natural

plant oils into fatty acid ethyl esters, under the optimized reaction conditions described in section 3.2.2.

3.3.3 Validation Tests

In Chapter 2, it was demonstrated that the lipase immobilized PPMM microreactor could quantitatively generate ethyl oleate from triolein. In this chapter, a validation of the PPMM was conducted to demonstrate the accuracy and intermediate precision of fatty acid analysis as well as the reusability of the microreactor under the optimized conditions.

3.3.3.1 Accuracy

Association of Official Analytical Chemists (AOAC) defines “accuracy” as the closeness of tested value to true or accepted value, and the difference between the reported value and the accepted value is the bias of the method under reported conditions (AOAC, 2002). The true value can be obtained in several ways, including conducting an established reference method. In this work, the accuracy of the PPMM microreactor method was defined as the closeness of the fatty acid (FA) determination between the use of PPMM microreactor and a reference method. The fatty acid determination included both the total amount of fatty acids per mg of sample ($\mathbf{W}_{FA}$) and the individual fatty acid weight percentages (%FA). The accuracy measurement was performed by comparing: 1) results from microreactor ethylation of the test oils and 2) the same oils quantified using AOCS methylation method *Ce 1k-09*. Four samples were selected for the

accuracy assessment: GLC 406 TAG standard mixture (containing C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C22:1 TAGs), canola oil, camelina oil and RBD palm olein. All the measurements using the two methods were performed in triplicates.

The determination of total FA weight per mg lipid sample ($\mathbf{W}_{FA}$) for GLC 406 standard was represented by total FAEE product weight per mg lipid sample. Conversion of the GLC 406 standard TAG mixture into FAEE results in a small change in weight due to the replacement of a glycerol residue by 3 ethanol residues, indicated by the conversion factor from TAG to FAEE ($CF_{TAG\ to\ FAEE}$):

$$\mathrm{CF_{TAG\ to\ FAEE}} = \frac{3 \times \mathrm{Mw_{FAEE}}}{\mathrm{Mw_{TAG}}} \qquad \text{(Eqn. 3-1)}$$

The theoretical (reference) and experimental weights of FAEE produced from GLC 406 TAG are:

$${}^{\mathrm{GLC}}\mathbf{W}_{\mathrm{FA\ ref}}\ (mg\ FAEE/mg\ GLC) = \frac{\mathrm{GLC\ m_{FAEE\ theo}\ (mg)}}{\mathrm{GLC\ m_{TAG}\ (mg)}}$$

$$= \mathrm{CF_{TAG\ to\ FAEE}} \qquad \text{(Eqn. 3-2)}$$

Where: $\mathrm{CF_{TAG\ to\ FAEE}}$ is the conversion factor from TAG to FAEE

GLC $\mathrm{m_{FAEE\ theo}}$ (mg) is the theoretical weight of FAEE products from initial GLC TAG standard

GLC $\mathrm{m_{TAG}}$ (mg) is the initial GLC 406 standard weight in 1mL 0.42mg/mL GLC/ethanol/hexane solution passing through the PPMM microreactor

$$^{\text{GLC}}\mathbf{W}_{\text{FA exp}}\ (mg\ FAEE/mg\ GLC) = \frac{\text{GLC m}_{\text{FAEE exp}}\ (\text{mg})}{\text{GLC m}_{\text{TAG}}\ (\text{mg})} \qquad \text{(Eqn. 3-3)}$$

Where: GLC $m_{\text{FA exp}}$ (mg) is the weight of FAEE produced from initial GLC TAG standard using PPMM microreactor, the calculation was described in Eqn. 3-8

For the oil samples, $\mathbf{W}_{\text{FA}}$ was indicated by the total weight of free fatty acid (FFA) per mg oil sample determined. Different from the GLC 406 standard with known TAG content, the reference values for oils were determined using AOCS official method *Ce 1k-09*. Since the official method is a methylation reaction producing FAME, both the FAEE and FAME quantification were converted to the equivalent FFA amount in order to compare them. The $\mathbf{W}_{\text{FA}}$ for oil samples was calculated as the amount of FFA (mg) produced by 1 mg oil:

$$^{\text{oil}}\mathbf{W}_{\text{FA ref}}\ (mg\ FFA/mg\ oil) = \frac{\text{oil total m}_{\text{FAME}}\ (\text{mg}) \times \text{CF}_{\text{FAME to FFA}}}{\text{oil weight (mg)}} \qquad \text{(Eqn. 3-4)}$$

Where: $\text{CF}_{\text{FAME to FFA}}$ is the conversion factor from FAME to FFA

oil total m_{FAME} (mg) is the total weight of FAME produced from each oil sample using AOCS reference method, the calculation is described in Eqn.3-7

oil weight is the initial weight of oil (100.0mg) added to the flask before performing AOCS official methylation method

$$^{oil}\mathbf{W}_{FA\ exp}(mg\ FFA/mg\ oil) = \frac{\text{oil total } m_{FAEE}\text{ (mg)} \times CF_{FAEE\ to\ FFA}}{\text{oil weight (mg)}} \quad \text{(Eqn. 3-5)}$$

Where: $CF_{FAEE\ to\ FFA}$ is the conversion factor from FAEE to FFA

oil total m_{FAME} (mg) is the total weight of FAEE produced from each oil sample using PPMM microreactor, the calculation is described in Equation3-7

oil weight is the initial weight of oil in 1mL 0.42mg/mL oil/ethanol/hexane solution passing through the PPMM microreactor

Therefore, the accuracy of the total fatty acid quantification measured using the PPMM is:

$$\text{Error of } \mathbf{W}_{FA} \text{ Determination} = \frac{\mathbf{W}_{FA\ exp} - \mathbf{W}_{FA_{ref}}}{\mathbf{W}_{FA_{ref}}} \times 100\% \quad \text{(Eqn. 3-5)}$$

The accuracy of the determination of individual FA weight percentages (%FA) was to be assessed for the GLC 406 standard and the three vegetable oils. For the standard, the closeness of the FAEE product distribution produced by the PPMM microreactor can be compared to the known reference distribution, given in the manufacturers' product specification (Nuchek Prep Inc). For the test vegetable oils, the closeness of the FFA weight percentages determined using the PPMM method can be compared to those determined using AOCS reference method *Ce 1k-09*:

$$\text{Absolute Error of \%FA determination} = \%FA_{exp} - \%FA_{ref} \quad \text{(Eqn. 3-6)}$$

Where: $\%FA_{exp}$ is the PPMM experimental value of each FAEE weight percentage based on total FAEE weight for GLC 406 standard, and is the PPMM experimental value of each FFA weight percentage based on total FFA weight for the three vegetable oils

$\%FA_{ref}$ is the corresponding FA weight percentage from the reference method (product information for GLC 406 TAG standard mixture, and corresponding FFA weight percentage determined using AOCS official method)

The target for the accuracy test was that the total total fatty acid weight (W_{FA}) determined should agree between methods within ±5.0%, and the individual fatty acid weight percentages (%FA) absolute differences should be less than ±3.0%. For accuracy, both of these conditions should be met.

3.3.3.2 Intermediate Precision

AOAC describes the precision of an analytical method as the degree of agreement between the repeated values determined in the same or different laboratories (AOAC, 2002). Precision is considered by AOAC at three levels: repeatability precision, reproducibility precision and intermediate precision. In order to evaluate the consistency of PPMM microreactors from different batches made on separate days, intermediate precision is tested, as AOCS has defined the intermediate precision as the degree of agreement of repeated determinations in a single laboratory but not simultaneously, i.e., on different days, with different batch of equipment, by different operators, etc. (AOAC, 2002). For the intermediate precision test, 5 sets of replicate reactions on the same test materials are the minimum (AOAC, 2002).

GLC 406 TAG standard mixture was used for evaluating the intermediate precision. Transesterification of the sample was performed on 5 different PPMM microreactors, and the Relative Standard Deviation (RSD) of total FAEE product weight (W_{FAEE}) in each run was calculated to represent the intermediate precision. The calculation of $\mathbf{W}_{FA}$ was the same as that described in accuracy assessment. RSD less than ±2.0% is considered to be precise.

3.3.3.3 Reusability

Lipase in the PPMM microreactor can denature or deactivate with repeated use. Multiple reactions were performed using the GLC 406 TAG standard mixture under the same described conditions on the same microreactor. The products of each run were analyzed by HPLC/ELSD in order to examine any TAG residues. The absence of TAG peaks in HPLC/ELSD traces indicates an acceptable PPMM microreaction run. The number of acceptable runs indicates how many times a PPMM microreactor can be reused.

3.3.4 Summary of Validation Criteria

The accuracy and precision assessment are considered to be acceptable within the ranges described in table 3-1.

Table 3-1 Acceptance Criteria for PPM Microreactor Validation

Parameters		Calculation	Target Acceptance Criteria
Accuracy	Total FA amount/mg sample determination (**W_{FA}**)	**$W_{FA\ exp}$** (mg FAEE/mg GLC) measured by PPMM method, compared to the theoretical **$W_{FA\ ref}$** (mg FAEE/mg GLC) (%Error)	Within ±5.0%
		$W_{FA\ exp}$ (mg FFA/mg oil) measured by PPMM, compared to **$W_{FA\ ref}$** (mg FFA per mg oil) measured by AOCS *Ce 1k-09* methylation method (%Error)	
	Individual FA weight percentage determination (%FA)	$\%FAEE_{exp}$ measured by PPMM microreactor, compared to known $\%FAEE_{ref}$ given by GLC 406 TAG standard product information (%Error)	Within ±3%
		$\%FFA_{exp}$ measured by PPMM microreactor, compared to $\%FFA_{ref}$ obtained by AOCS method (%Error)	
Precision		**W_{FAEE}** of GLC 406 TAG standard mixture measured over 5 different microreactors (RSD)	Within ±2.0%
Reusability		Number of runs a PPMM microreactor can achieve acceptable production of FAEE from GLC 406 TAG standard mixture	No TAG or byproduct peaks in HPLC/ELSD results

3.4 Results and Discussion

3.4.1 Identification of the Transesterification Products

Four test samples, including GLC 406 TAG standard mixture, canola oil, camelina oil and RBD palm olein, were converted into FAEEs using the lipase immobilized PPMM microreactors under the conditions described in section 3.2.2.

The consumption of TAGs was monitored by HPLC/ELSD in a way to show the transesterification products FAEE. HPLC is one of the most common tools used to separate and quantify nonvolatile lipids. Normal Phase HPLC (NP-HPLC) is normally used for lipid class separation based on the considerable differences in their polarity (Firestone and Mossoba, 1997), which means that different lipid classes including TAG, monoglyceride (MAG), diglyceride (DAG) and FAEEs can be well resolved from each other. Reverse Phase HPLC (RP-HPLC) based on partition chromatography where analytes show different affinities to mobile and stationary phases, is useful in separating individual components that belong to one lipid class (Firestone and Mossoba, 1997). For example, free fatty acids (FFA), geometrical and positional FAME isomers of octadecenoic acid (Svensson *et al.*, 1982), and monosaturated TAGs in vegetable oils (Dionisi *et al*., 1995) have been determined on RP-HPLC. In the present case, the application of HPLC/ELSD was for the examination of TAG residue, or MAG and DAG intermediates. Since the separation of individual lipid species within

each class is not necessary in this case, NP-HPLC is a better option and was used here.

It was found that in all four test samples, TAG were completely converted into FAEE after passing through the PPMM microreactor, without producing any MAG or DAG byproduct that could be observed by ELSD detector (Figure 3-1, A-H). This shows that the PPMM is an efficient method to quantitatively convert TAG into FAEE. As described above, different molecular species within one lipid class might have similar retention times in the NP-HPLC system, resulting in co-eluting peaks. Therefore, fewer peaks in FAEE product NP-HPLC/ELSD traces were observed than the number of actual FAEE constituents seen on GC/FID traces (Figure 3-2).

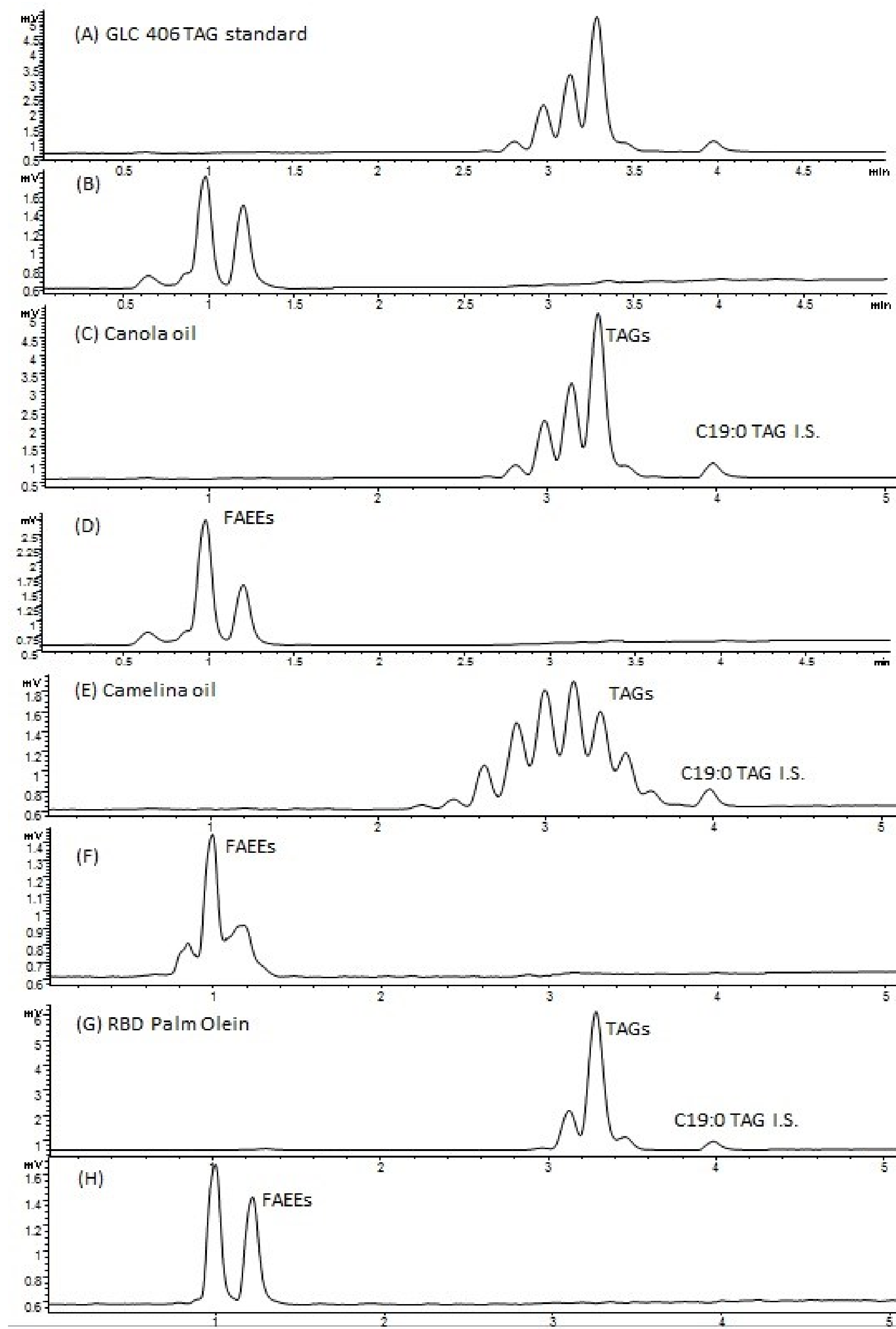

Figure 3-1 NP-HPLC/ELSD Traces for: (A) & (B) GLC 406 TAG Standard; (C) & (D) canola oil; (E) & (F) camelina Oil; (G) & (H) RBD palm olein before and after 1.5 h PPMM microreaction

The major FAAE produced from GLC 406 TAG standard mixture, canola oil, camelina oil and RBD palm olein were then identified by GC/FID. GC is a powerful separation technique that can completely separate common fatty acids as FAME or FAEE. This is normally realized on a highly polar GC column (e.g. biscyanopropyl siloxane in this experiment), where both boiling point and polarity affects the retention of the analytes (Rodríguez-Estrada *et al*., 2002). The highly polar column is able to resolve FAAE with different chain length and degree of saturation, as well as some of the isomers (Firestone and Mossaba, 1997). According to AOCS GC/FID analytical method for fatty acid derivatives *Ce 1h-05*, by direct comparison of the retention times with FAAE standards, a preliminary identification can be made. For example, Figure 3-2 shows the GC/FID peaks of FAEE derivatized using PPMM microreactor transesterification, with correspondent retention times to FAEE commercial standards. The FAEE product peaks in Figure 3-2 (B), (C), (D), (E), obtained from reaction of GLC 406 TAG standard mixture, canola oil, camelina oil and RBD palm olein, respectively, could be identified by directly comparing their retention times to those of commercial standards, shown in Figure 3-2 (A).

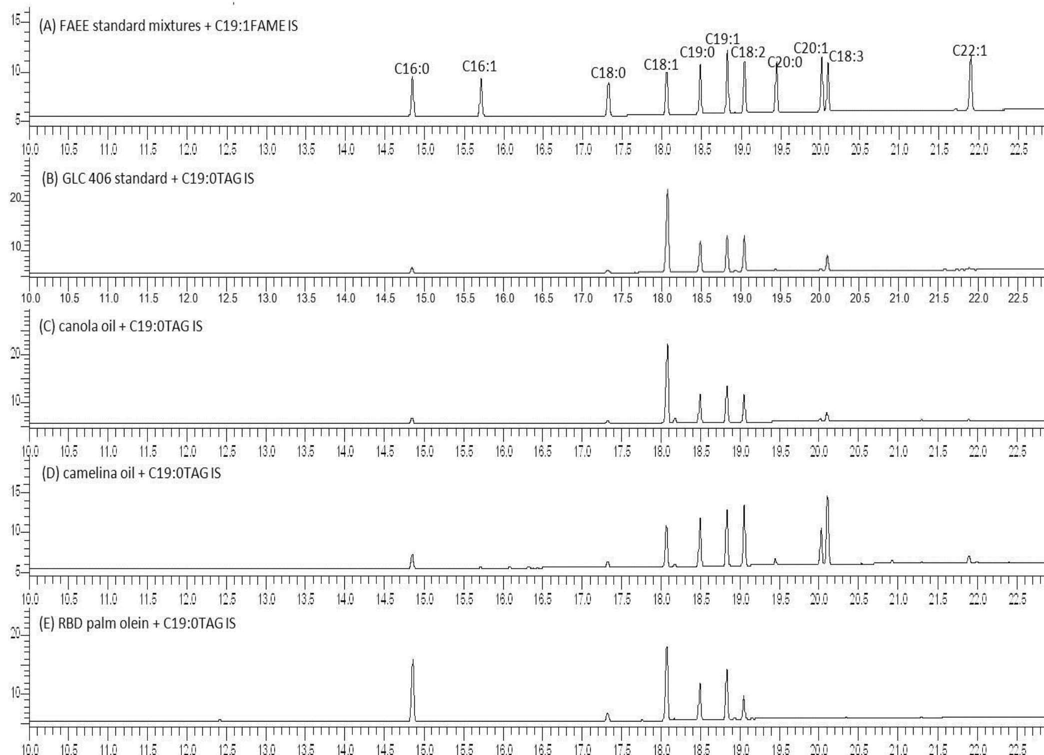

Figure 3-2 GC/FID Traces of (A) standard mixture of FAEE with addition of C19:1 FAME as internal standard; standards with C19:0 TAG internal standard transesterified by microreactor after 1.5 h: (B) GLC 406 TAG standard mixture; (C) canola oil; (D) camelina oil; (E) RBD palm olein

From the chromatograms, it can be seen that C19:0 FAEE is well resolved from all other peaks, and is also mid-range in retention time among the FAEE of interest. These indicate C19:0 TAG a suitable internal standard for the transesterification reaction. It is also observed in Figure 3-2 (E) that RBD palm olein contains much more saturated fatty acid (mainly C16:0) than canola and camelina oils. Saturated fatty acids usually have lower solubility in alcohol, resulting in an interface resistant to mass transfer. In this way, an organic solvent might be required as co-solvent in order to enhance the oil solubility, inducing a larger contact of substrates thus greater reaction rate. This was demonstrated by RBD palm olein, which did not dissolve completely in pure ethanol, but with the

addition of some hexane, the RBD palm olein dissolved into a homogenous phase in ethanol and hexane mixture, as described below.

A solvent that dissolves oils as well as being miscible with ethanol can be used as a co-solvent in the transesterification reaction. Hexane is one of the most common co-solvents used in enzymatic transesterification of oils (Caetano *et al*., 2012; Anur *et al*., 2011), because of its lower tendency to deactivate lipases compared to other suitable co-solvents, such as toluene (Fu and Vasudevan, 2010). In optimizing the ratio of ethanol and co-solvent mixture, RBD palm olein was chosen as the test sample. Ethanol was added with hexane in three different ratios: 2:1, 3:1 and 4:1 (ethanol/ hexane, v/v). The ethanol and hexane mixture was then used as the solution for oils while they passed through the PPMM microreactor. Although the sample was fully dissolved in the solvent, the HPLC/ELSD trace in Figure 3-3 (A) indicated incomplete conversion from RBD palm olein to FAEE when using ethanol/hexane 2:1, v/v, by showing the TAG residue peaks. In contrast, transesterification was found to be complete with an ethanol to hexane ratio of either 3:1 or 4:1 (v/v), from the HPLC/ELSD results in Figure 3-3 (B) and (C). The reason for the incomplete conversion when ethanol to hexane was 2:1 (v/v) might be the inhibitory effect of hexane on lipase catalytic activity when larger quantity was used. Thus, even though the co-solvent can help to enhance the conversion for oils containing high content of saturated fatty acids, its concentration should be minimized. Considering this, ethanol/ hexane 4:1, v/v was chosen as an appropriate solvent for oils, to ensure complete dissolution and

transesterification, especially for samples consisting of high content of saturated FA.

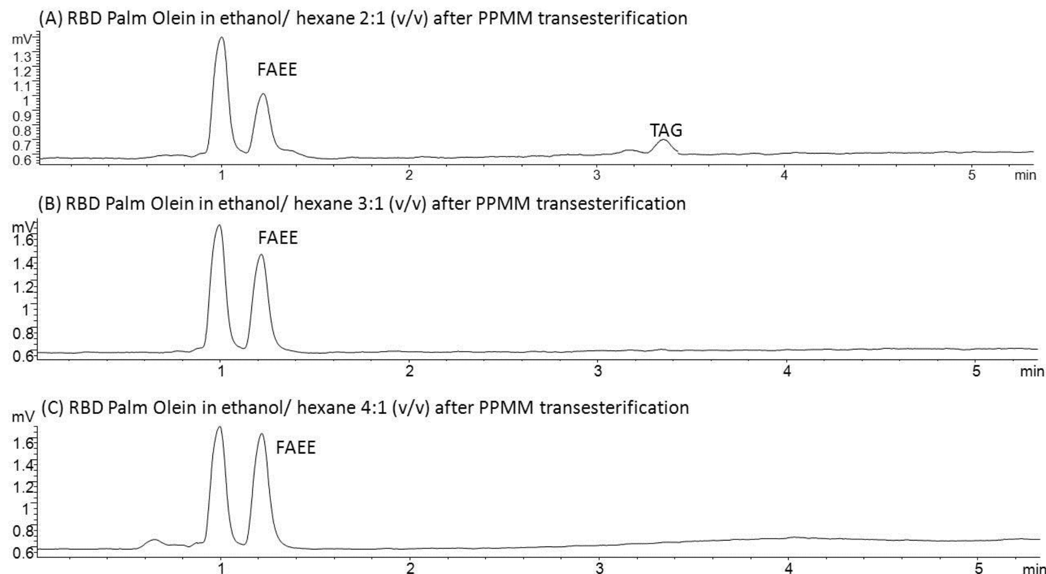

Figure 3-3 HPLC/ELSD Traces of the Optimization of the Ratio of Ethanol and Hexane as Solvent for RBD Palm Olein Transesterification on PPMM Microreactor

3.4.2 Validation of the Transesterification Property of Microreactor

3.4.2.1 Accuracy in determination of total fatty acid amount per mg sample

In Chapter 2, the complete conversion of 0.50 mg/mL triolein into ethyl oleate was demonstrated on the porous polymer microreactor (PPMM), at a reactant flow rate of 0.3 μL/min at room temperature for 1.5 h. To measure the accuracy of fatty acid determination using PPMM microreactor derivatization method, three vegetable oils and one commercial GLC TAG standard mixture (0.42mg/mL in final reactant solution), with 0.08 mg/mL internal standard C19:0

TAG were transesterified by passing through the microreactor. Another derivatization method, AOCS official method *Ce 1k-09* using BF_3 as catalyst, was adopted as a reference method for the oil samples, to compare with the use of the PPMM (AOCS, 2012).

As mentioned in the previous section, PPMM is a micro-scale ethylation method producing FAEE derivatives for GC, while AOCS official method *Ce 1k-09* is a larger-scale (100.0mg oil sample) methylation method generating FAME derivatives for FA GC analysis. The GC method used to analyze FAEE products from the PPMM was a gradient program on a SP-2560 column. The FAME products from AOCS official method were analyzed by the GC method described in AOCS *Ce 1h-05* recommended for vegetable oils analysis, which was also on a SP-2560 column. The derivatives from both methods were called fatty acid alkyl esters (FAAE) in general in the following context, representing for FAEE collected from PPMM microreactor method and FAME obtained from AOCS derivatization method *Ce 1k-09.*

The FAAE derivatives from both methods were quantified according to the numerical method outlined in AOCS method *Ce 1h*-05 (AOCS, 2012). However, in this work the C21:0 internal standard was replaced with C19:0 internal standard, in terms of quantification (Eqn. 3-7). A theoretical correction factor (TCF), indicating the relative response between the analyte and internal standard on FID detector, was calculated for each compound for optimum accuracy, with C19:0 FAAE as reference (Eqn. 3-8).

$$W_{FAAE_x} = \frac{A_x \times W_{IS} \times CF_{IS} \times TCF_x}{A_{IS}} \qquad \text{(Eqn. 3-8)}$$

Where: A_x is the area counts for Fatty Acid x
W_{IS} is the weight of C19:0 TAG I.S. added to the test portion
CF_{IS} is the conversion factor of I.S. from TAG to FAEE or FAME
TCF_x is the TCF for FAAEs relative to C19:0 FAAE I.S.
A_{IS} is the area counts for C19:0 FAAE I.S.

$$TCF_x = \frac{MW_x}{(N_x - 1) \times 12.011 \times TCF_{IS}} \qquad \text{(Eqn. 3-9)}$$

Where: MW_x is the molecular weight of FAAE x
N_x is the number of carbon in FAAE x
12.011 is the atomic weight of carbon
TCF_{IS} is 1.3592 for C19:0 FAEE and 1.3694 for C19:0 FAME

An empirical correction factor (ECF), indicating the actual response factor between FAAE and internal standard, was also calculated. The variation of ECFs for both FAEE and FAME standards is shown in Figure 3-4 and Figure 3-5. The percentage difference between ECF and TCF for major components (C16:0, C18:0, C18:1, C18:2 and C18:3, which make up more than 90% of the FA content in the four samples) are all less than 3%. Other components contributing less than 3% in vegetable oil composition also have a ECF/TCF %difference less than 6%. The ECF/TCF %difference implies the closeness between the experimental response factor of FAAE molecules and the theoretical response factor for FID detector. Hence, a small ECF/TCF %difference indicates that the chromatography, including injection, separation and detection is near ideal.

Although this is not required in AOCS method *Ce 1h-05*, the test was done with the GC/FID run for samples to illustrate the variation of the results calculated by TCFs, since deviation between ECF and TCF may result in significant errors.

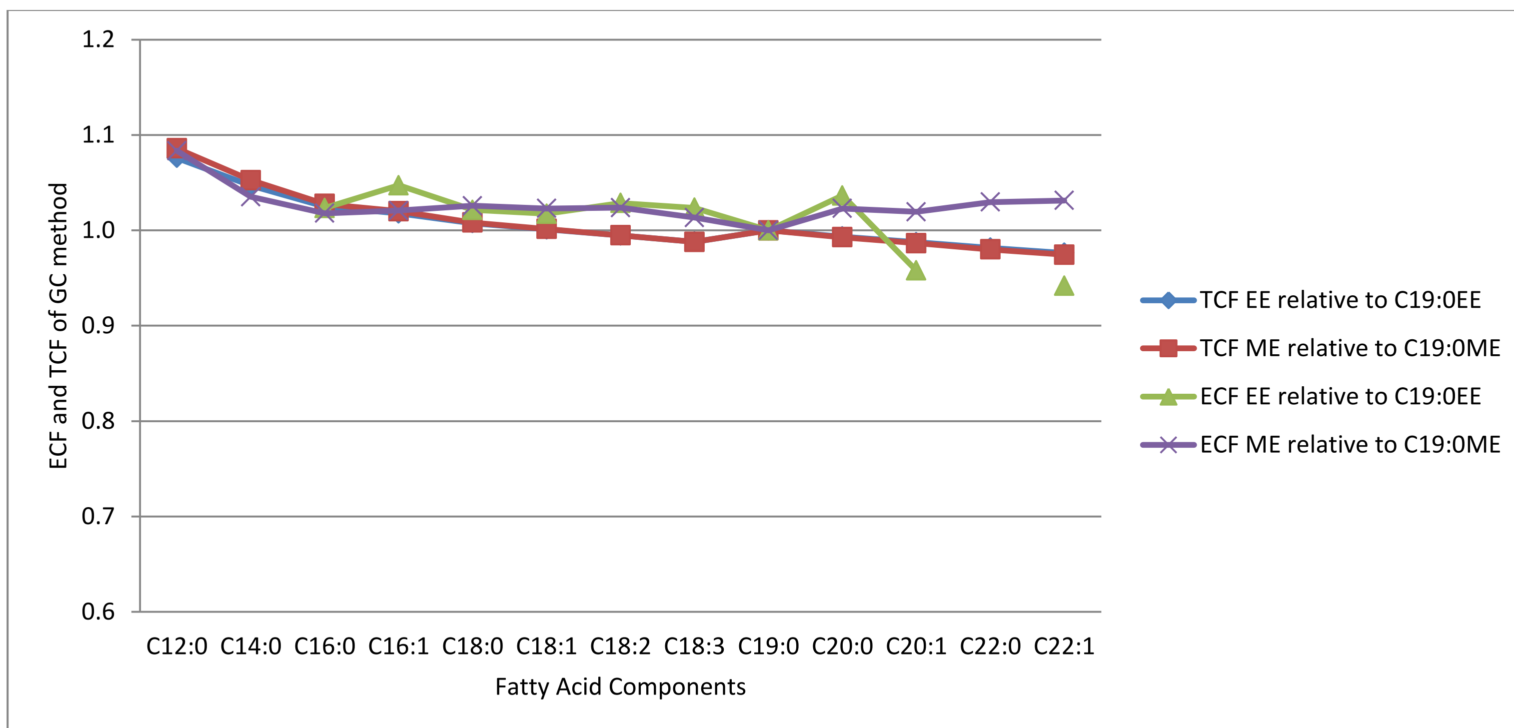

Figure 3-4 TCF and ECF Relative to C19:0 Internal Standard for FAEE and FAME Standards Using GC/FID (GC condition described in section 3.2.6)

Note: The trend lines are added as a visual aid only.

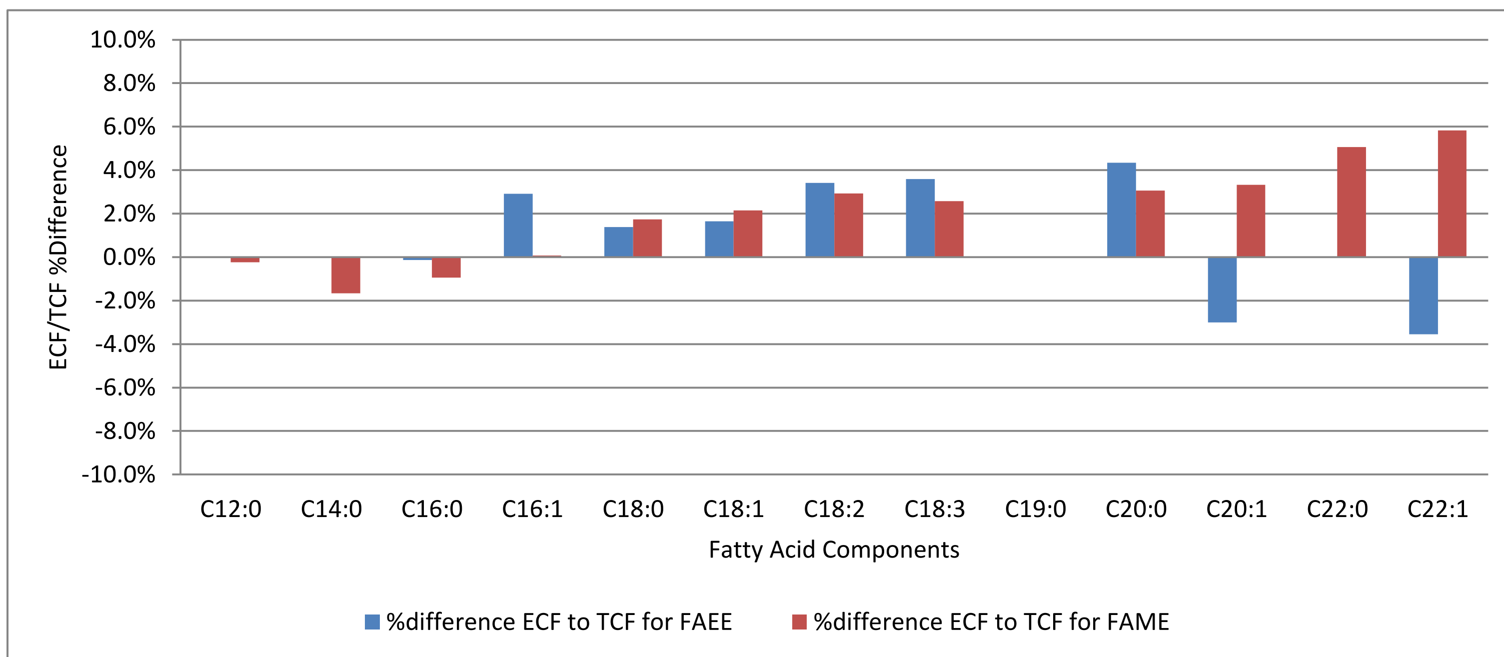

Figure 3-5 Difference of ECF to TCF Relative to C19:0 Internal Standard for Both FAEE and FAME on GC/FID (GC condition described in section 3.2.6)

As described in the validation plan, the accuracy of using the microreactor for fatty acid determination includes the accuracy of total FA weight per mg of sample measurement ($\mathbf{W}_{FA}$) compared to the reference value, and the accuracy of individual FA weight percentage determination (%FA). The PPMM method derivatized TAG into FAEE for quantification by GC/FID, and the results were converted to the equivalent FFA amount (mg FFA/mg sample). The reference method using BF_3/MeOH reagent transesterified TAG into FAME for GC/FID analysis, and the amount of FAME was converted to equivalent FFA amount (mg FFA/mg sample) for comparison as well. However, for the GLC 406 TAG standard sample, whose FA content is already known, FAEE was quantified and used directly in comparison with the known value. Table 3-2 shows the results of $\mathbf{W}_{FA}$ measurement and the relative error of the numbers to reference value.

Table 3-2 Total FA Weight per mg of Sample Measurement Accuracy using the PPMM Microreactor

Accuracy Accuracy of Total FA Weight per mg of Sample Measurement				
		$W_{FA\ exp}$ (mg FAEE/ mg sample)	$W_{FA\ ref}$ (mg FAEE/mg sample)	Relative Error (%)
GLC 406 TAG standard	Run 1	0.9983	1.0520	-5.1%
	Run 2	0.9931		-5.6%
	Run 3	1.0134		-3.7%
	Average	**1.0016**		**-4.8%**
		$W_{FA\ exp}$ (mg FFA/ mg sample)	$W_{FA\ ref}$ (mg FFA/ mg sample)	Relative Error (%)
Canola Oil	Run 1	1.0981	1.0584	3.8%
	Run 2	1.0955	1.0627	3.1%
	Run 3	1.0977	1.0607	3.5%
	Average	**1.0971**	**1.0606**	**3.4%**
Camelina Oil	Run 1	1.0771	1.0356	4.0%
	Run 2	1.0802	1.0248	5.4%
	Run 3	1.0760	1.0205	5.4%
	Average	1.0777	1.0270	**4.9%**
RBD Palm Olein	Run 1	1.1101	1.1077	0.2%
	Run 2	1.1515	1.1121	3.5%
	Run 3	1.1062	1.0848	2.0%
	Average	**1.1226**	**1.1016**	**1.9%**

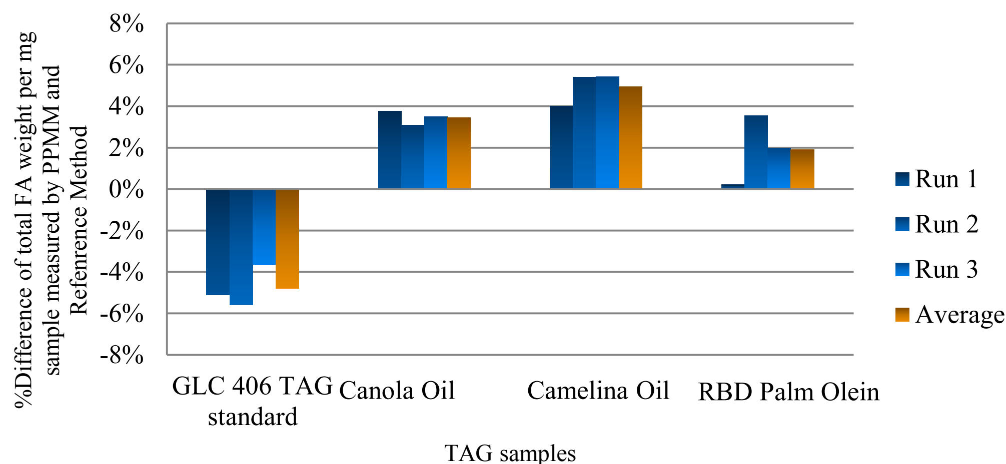

Figure 3-6 Accuracy of Total FA Weight per mg Sample Measurement of the PPMM Microreactor

As shown in Table 3-2 and Figure 3-6, the total FA amount per mg of sample determined by PPMM method was lower than the theoretical value (GLC 406 TAG standard determination), but is higher than the reference results obtained from the established methylation method using BF_3 catalyst (canola oil, camelina oil, RBD palm olein determination).

The reason why GLC 406 standard sample has on average 4.8% lower measured total FA amount per mg sample value than the theoretical value may include: (i) the purity of the sample when calculating the theoretical conversion; (ii) any error in measurements of weights (the use of an analytical balance could make a ±1% contribution to the total error when the weights are sufficient for the balance); (iii) any loss during the PPMM transesterification procedure. Therefore, a deviation lower than 5% compared to the theoretical value can be accepted.

The rise of higher determined total FA weight per mg sample using PPMM method compared to conventional reference method might result from any undesirable side reactions in BF_3 catalyzed method. As described in Chapter 1, the BF_3/MeOH transesterification has been reported to result in undesired artifacts, by adding methanol across the double bonds of unsaturated fatty acids (Lough, 1964). This side reaction thus reduces the quantification of total unsaturated fatty acids. In the GC/FID chromatogram for FAME obtained from the AOCS BF_3/MeOH method (shown in Appendix Figure A3-2), more baseline peaks are observed compared to the chromatogram for FAEE obtained from the PPMM method. It is possible that these peaks are the unsaturated fatty acid artifacts produced during BF_3/MeOH transesterification, and caused reduction in total FA weight quantification. In this way, the less than 5% higher total FA amount per mg sample measured by PPMM method with no undesirable side reactions can be explained, and can still be considered accurate.

Although a shorter GC program was used for microreactor FAEE products quantification than that used for FAME obtained by the conventional method, the gradient temperature program instead of an isothermal program could compensate for that in providing adequate resolution. Thus, the difference in GC program used in the two methods may not affect the quantification results.

In summary, the relative difference between the total FA weight per mg sample ($\mathbf{W}_{FA}$) determination (expressed as mg FA/mg sample) using the PPMM method versus the reference value is within ±5%, which is accurate according to the validation criteria.

3.4.2.2 Accuracy in determination of individual fatty acid weight percentage

In addition to demonstrating accurate measurement of the total fatty acid content per mg sample, it is essential to also show that the weight percentage of each FA component (%FA) is determined accurately by the test method. Hence, the FAAE product distributions were determined using test and reference methods as a ratio against the C19:0 FAAE internal standard (Table 3-3 and Figure 3-7). For each reaction, three separate runs were processed, and the standard deviation (SD) of each component percentage was also obtained (shown in Table 3-3). The SD values indicate the consistency of the triplicates, thus demonstrate any possible variance of the FA distribution percentage result.

Table 3-3 Accuracy of Determination of Individual FA Weight Percentage Using PPMM Method

		Accuracy												
		%FA (% by wt.)[1]												**Range of Absolute Error**
		C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0	C20:1	C18:3	C22:0	C22:1	
GLC 406 TAG standard average	Microreactor			3.37 ±0.06		1.82 ±0.01	61.96 ±0.25	20.48 ±0.13	0.90 ±0.01	1.09 ±0.03	9.25 ±0.12		1.13 ±0.03	**-0.63% to 0.96%**
	AOCS method			4.00		2.00	61.00	21.00	1.00	1.00	9.00		1.00	
	Absolute Error			-0.63		-0.18	0.96	-0.52	-0.10	0.09	0.25		0.13	
Canola Oil average	Microreactor			4.42 ±0.01	0.26 ±0.01	2.30 ±0.01	66.71 ±0.07	17.56 ±0.02	0.79 ±0.00	2.16 ±0.04	5.79 ±0.03			**-1.08% to 0.38%**
	AOCS method			4.31 ±0.02	0.24 ±0.00	1.92 ±0.02	66.52 ±0.33	17.30 ±0.05	1.04 ±0.19	1.79 ±0.01	6.87 ±0.10			
	Absolute Error			0.11	0.02	0.38	0.19	0.26	-0.25	0.37	-1.08			
Camelina Oil average	Microreactor			6.18 ±0.02		2.93 ±0.01	19.30 ±0.06	22.79 ±0.20	2.16 ±0.01	14.24 ±0.06	27.75 ±0.07	0.65 ±0.00	4.01 ±0.03	**-0.59% to 0.72%**
	AOCS method			6.74 ±0.05		2.90 ±0.02	19.89 ±0.26	23.09 ±0.09	2.15 ±0.10	13.51 ±0.04	27.60 ±0.09	0.44 ±0.00	3.67 ±0.02	
	Absolute Error			-0.56		0.03	-0.59	-0.29	0.00	0.72	0.14	0.20	0.34	
RBD Palm Olein average	Microreactor	0.25 ±0.03	0.89 ±0.02	38.02 ±0.71		4.52 ±0.08	44.85 ±0.78	11.01 ±0.23	0.46 ±0.01					**-1.42% to 1.35%**
	AOCS method	0.32 ±0.01	1.07 ±0.01	39.44 ±0.39		4.25 ±0.05	43.50 ±0.52	10.99 ±0.12	0.43 ±0.02					
	Absolute Error	-0.06	-0.19	-1.42		0.27	1.35	0.03	0.02					

Note:[1]. %FA for GLC 406 sample represents for FAEE products distribution by weight, and is FFA distribution by weight for oil samples

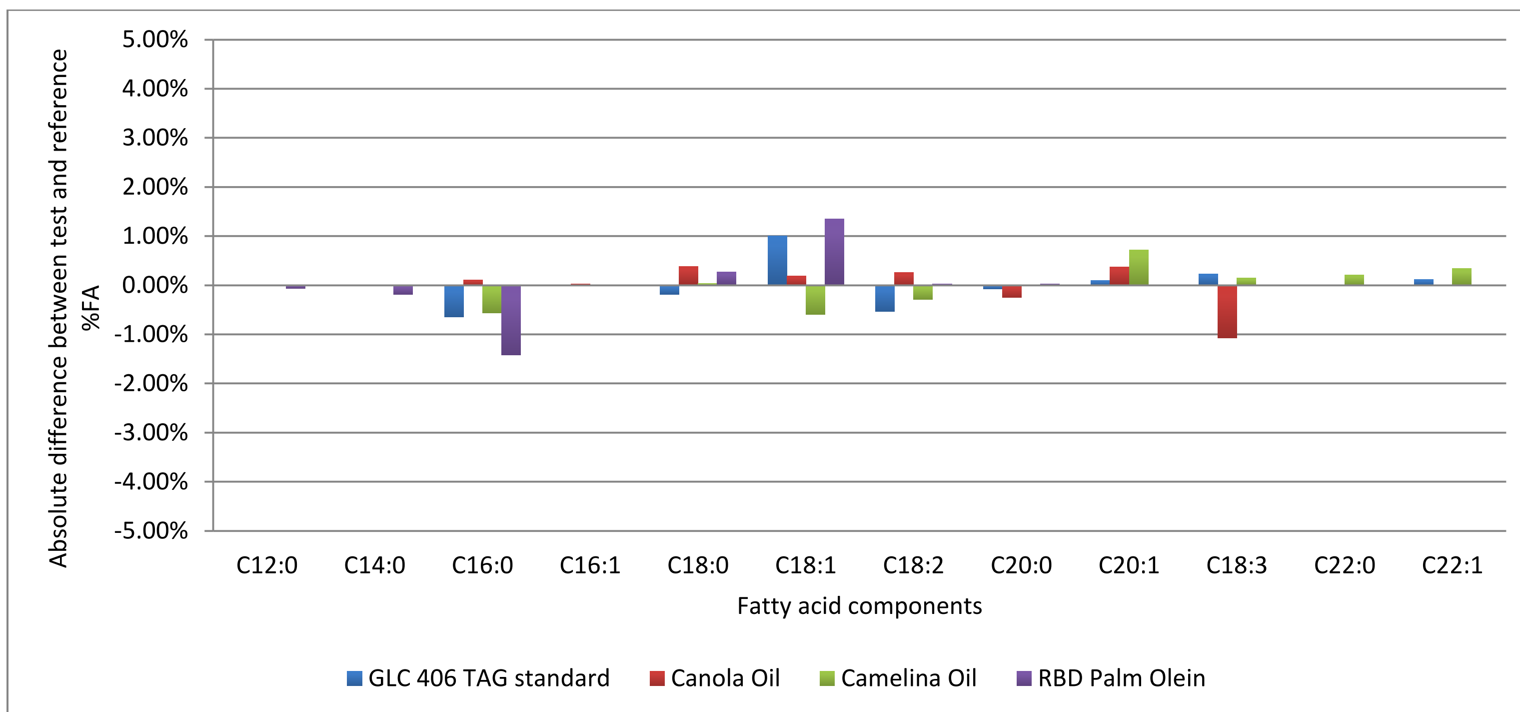

Figure 3-7 Accuracy of Determination of Individual FA Weight Percentage Using PPMM Microreactor

The side-reaction occurring between methanol and unsaturated fatty acids during AOCS BF_3/MeOH transesterification mentioned above potentially affects the quantification of individual unsaturated FA components, thus affecting the overall FA distribution determination. Specifically, the AOCS methylation measurement of unsaturated FA weight percentages would be lower than the real value, while that of saturated %FA would be higher. This may result in a positive error in PPMM measured unsaturated %FA (i.e. C18:1, C20:1 shown in Figure 3-7), and a negative error in PPMM measured saturated %FA (i.e. C12:0, C14:0, C16:0, C20:0 shown in Figure 3-7).

In addition, variations in the experimental response factors for the fatty acid derivatives will affect the individual fatty acid peak areas and hence the quantification of FA species. This is indicated by the difference between empirical correction factor of a FAAE compound relative to C19:0 internal standard (ECF) and the corresponding theoretical correction factor (TCF). The TCF is the theoretical FID detector response, which is proportional to the relative percentage of carbons in a molecule, while the experimental ECF takes into account the instrumental deviations. In the ECF assessment performed prior to the GC/FID analyses of FAEE and FAME, it was found that the percentage differences between ECF and TCF were less than 6% for all compounds (Figure 3-4). This means that if individual FAAE actually have a difference in FID response compared to the TCF value, it would contribute a maximum error of ±6% to the quantification of individual FA. However, considering the different weight percentages of each FA in the samples, it is assumed that any error resulting from

any ECF/TCF difference will be lower in the measured FA distribution (%FA). For the more abundant components such as C18:1 in the GLC 406 standard, canola oil and camelina oil, and C16:0 for RBD palm olein, any error in %FA determination resulting from ECF/TCF differences will likely be larger than for the less abundant components. This assumption agrees with the results in Figure 3-6. For example, consider the determination of C18:1 in GLC 406 TAG standard using the PPMM microreactor. The C18:1 FA makes up 61% of the GLC sample, and the ECF/TCF difference for C18:1 FAEE is approximately +2% (Figure 3-4). Thus, the experimental determination of the %C18:1 FA could vary +1.2% due to the instrument deviation, and this could be considered acceptable. As shown in Table 3-3, the error of determination of %C18:1 FA in the GLC 406 TAG standard using PPMM microreactor is +0.96%, which is within the acceptable range. In fact, the variations of the determination of weight %FA for other components are all within ±1.5% according to Table 3-3.

Thus, considering both the possible side-reaction in the conventional methylation method, and GC/FID instrumental variation, a ±1.5% error range in determining FA weight percentage in oils using PPMM microreactor can be accepted as accurate.

3.4.2.3 Intermediate Precision in Determination of Total Fatty Acid Amount

As for the intermediate precision of determining total fatty acid weight per mg sample using PPMM microreactors, a stock solution of 0.42mg/mL GLC 406

TAG standard with 0.08mg/mL C19:0 TAG internal standard in ethanol and hexane (4:1, v/v) was prepared. The TAG reactant solution in each run was then taken from the same stock solution. Five microreactors were manufactured separately on different days so that they reflect likely variations arising from their fabrication. The recovery corrected total amount of FAEE products per mg sample (W_{FAEE}) produced from the GLC 406 TAG standard in the 5 reactions is shown in Table 3-4.

Table 3-4 Intermediate Precision for 5 Different PPMM Microreactors

	Precision
	GLC 406[1] $\mathbf{W}_{FAEE}$ (mg FAEE/mg sample)
PPMM 1	0.9983
PPMM 2	0.9931
PPMM 3	1.0060
PPMM 4	1.0134
PPMM 5	1.0061
RSD (%)	**0.70%**

Note: [1] The GLC 406 TAG standard solution with C19:0 TAG internal standard was prepared as stock solution, and was used for all the five microreactions. The precision here only stands for the precision of microreactor conversion itself.

The Relative Standard Deviation (RSD) of the total recovered FAEE from the conversion of a single TAG solution by 5 different microreactors was low as 0.70%. It needs to be noted that the precision stands for the precision of the microreactor conversion efficiency only. The sample was prepared as a stock solution for universal use, thus preventing any experimental error during

measurement or transferring. Therefore, the low RSD indicates that the PPMM microreactor works consistently in converting TAGs into FAEEs giving high precision in FA determinations.

3.4.2.4 Reusability of the PPMM Microreactor

The lipase immobilized on PPMM microreactor is expected to have decreasing activity with use, especially when hexane was added as a co-solvent since hexane is reported to alter the conformation of the enzyme thus decrease its enzymatic activity (Liu, 1994). In order to demonstrate the reusability of the microreactor, transesterification of the GLC 406 TAG standard mixture was performed on a single microreactor under same conditions described in section 3.2.2. Six consecutive experiments were conducted, with a sodium phosphate buffer wash (pH 7.2) to restore the enzyme, an ethanol wash to remove water and an air drying step between each run. Figure 3-8 shows the analysis of the starting TAG standard mixture by normal phase HPLC/ELSD. It can be seen that only TAG is present in the starting materials. The products from the 6 PPMM conversions are also shown in Figure 3-8. The major product in all 6 runs was found to be the FAEE. The absence of TAG, MAG or DAG in the traces for runs 1 to 4 demonstrates that the microreactor can quantitatively convert TAG to FAEE without any loss in conversion efficiency over 4 runs. Small peaks of unreacted TAGs were observed in the 5th and 6th runs. Hence, under the conditions used in this validation, a single PPMM microreactor could be reused up to 4 times for quantitative TAG conversion to FAEE.

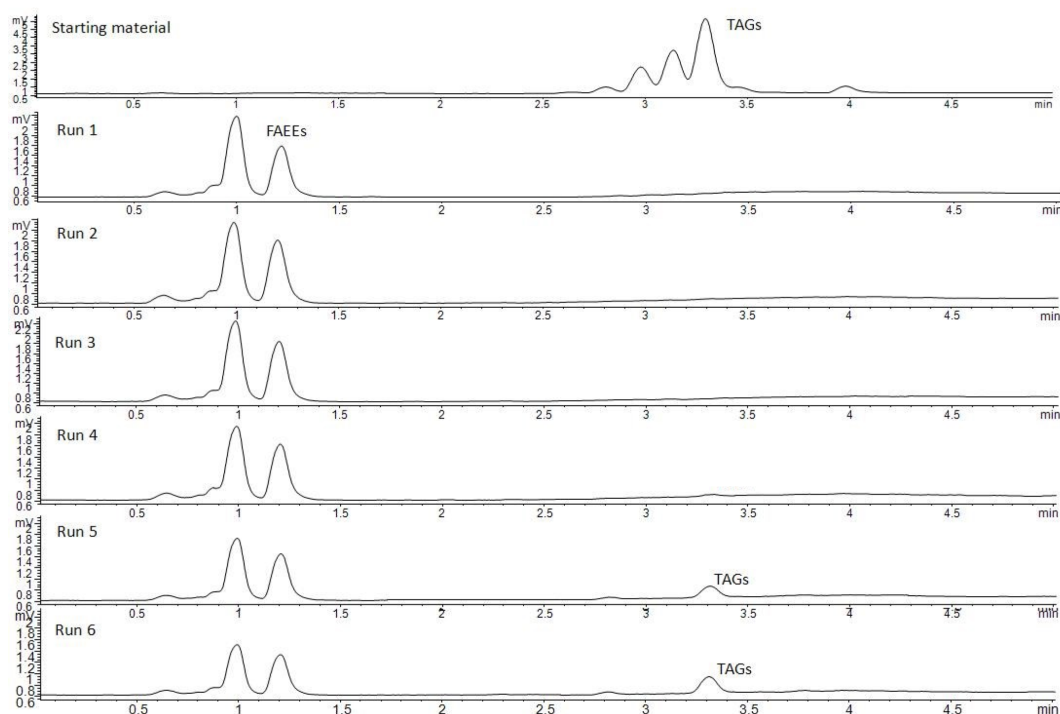

Figure 3-8 HPLC/ELSD Chromatograms for GLC 406 TAG Standard Transesterification Products in 6 Repeated Runs

The reusability of the PPMM microreactor may have benefitted from the reduced infusion and collection time, as optimized in Chapter 2, since the exposure of lipase to deactivating organic solvents was greatly shortened. To further extend the reusability of the PPMM microreactor, minimizing the damage of organic solvent to the lipase may be the key point. This can be achieved by: 1) test and find a co-solvent that is more benign to lipase than hexane, and optimize its ratio to ethanol; 2) pretreat the immobilized lipase with $CaCl_2$ or $MgCl_2$ salt solution to prevent the enzyme configuration change due to the organic solvent in the environment, and retain the lipase activity (described in Chapter 1, Lu *et al.*, 2010); 3) increase the diameter of the microreactor to decrease the column back-pressure, and enable higher flow rate, thus realize lower run time; 4) decrease the

length of the microreactor and reduce the total residence time, without sacrificing the transesterification efficiency.

3.5 Conclusion

The lipase immobilized poly(GMA-*co*-EDMA) microreactor (PPMM) was validated for its use as a derivization device in fatty acid GC analysis. The transesterification efficiency for a commercial TAG standard mixture GLC 406 (Nuchek Prep. Inc.), and 3 vegetable oils (canola oil, camelina oil and RBD palm olein) were evaluated. These samples contain fatty acids varying from 12 to 21 carbon chain length, and different degrees of saturation. The four test samples were prepared in ethanol and hexane mixture (4:1, v/v) at a concentration of 0.42 mg/mL, passed through the PPMM microreactor at flow rate 0.3 µL/min at room temperature by a syringe drive. Products were analyzed by HPLC/ELSD and GC/FID.

The accuracy, intermediate precision and reusability of the PPMM microreactor were assessed according to a pre-established validation plan, with reference to a methylation process adopted in the 6th edition of Official Methods and Recommended Practices of the AOCS (AOCS, 2012). In summary, both the determination of total FA weight per mg sample and the measurement of individual FA weight percentage using PPMM derivatization microreactor shows equivalent overall accuracy when compared against measurements using the reference method. The measured amounts of FAEE derivatives per mg sample

obtained from 5 separate PPMM microreactors made on different days, showed high precision (0.70% RSD for the total FAEE content). Last but not least, each PPMM microreactor could be reused up to 4 times for a 1.5 h transesterification run with sodium phosphate buffer flush and ethanol flush after use, retaining its full derivatization efficiency.

Therefore, it can be concluded that the lipase immobilized porous polymer microreactor is a FA derivatization method for GC analysis that is equivalent to established methylation methods. It is advantageous because all that is required is a small amount of ethanol and hexane as the only reagents, and the procedure only requires these to flow through the microreactor. No further steps are required other than dilution prior to GC analysis. In contrast, other methylation schemes require the use of multiple reagents and sample manipulations. A further advantage is the potential for PPMM to be incorporated into the GC sample inlet robotics.

Chapter 4 Summary and Future Work

A flow-through microreactor for lipid derivatization prior to GC analysis was previously developed (Mugo et al., 2013; Mugo et al., 2014). This microreactor containing a monolithic structure of poly(GMA-co-EDMA) polymer within a fused silica capillary, was used as a substrate to immobilize lipase (PPMM). The PPMM can be used to perform small-scale transesterification of triglycerides (TAG) into fatty acid ethyl esters (FAEE), as a substitution for conventional acid or alkaline-catalyzed GC derivatization methods. The present thesis optimized the PPMM microreactor derivatization method on its total operation time (Chapter 2), and validated its use as a derivatization method in oil GC analysis (Chapter 3).

To determine fatty acid components in lipids by GC, non-volatile lipids are usually derivatizatized into fatty acid alkyl esters (FAAE) to increase their volatility in order to be detected. The conventional derivatization methods esterify or transesterify lipids with an alcohol, usually catalyzed by acid or alkaline reagent (i.e. BF_3, HCl, BCl_3, $NaOCH_3$, NaOH, etc.). Special precautions need to be taken when applying these methods, not only for operational safety, but also for the strict anhydrous environment required for alkaline-catalyzed reactions to avoid undesired side reactions, and the proper concentration of either an acid or an alkaline catalyst (Chapter 1). The esterification or transesterification time of the conventional methods vary from several minutes to several hours depending

on the type of catalyst. As well, because a batch system is used, harmful acids or alkalis need to be separated from the product solution before injecting a sample into the GC.

The disadvantages mentioned above for conventional acid or alkaline-catalyzed GC derivatization could all be prevented in an enzymatic-catalyzed reaction performed on a lipase immobilized PPMM microreactor. Instead of reacting as a mixture in a batch system, the TAG oil in an ethanol/hexane mixture (4:1, v/v) passed through the PPMM become fully derivatized into FAEE due to the exposure of the oil to the enzyme immobilized on the internal surface of the reactor. In addition to the FAEE products dissolved in hexane and excess ethanol, the collected product solution only contains a very small amount of glycerol as a byproduct, which did not affect the fatty acid determination by GC. Thus, the PPMM products can be directly injected into the GC without any wash or separation. Additionally, the PPMM microreactor is used at a micro-scale both for oils and organic solvents, compatible with the amounts required for GC analysis. Both of these attributes make the PPMM transesterification microreactor method a “green” GC derivatization method. More importantly, the microreactor has potential to be coupled with GC as an automated fatty acid determination system, integrating sample preparation (derivatization) and analysis on a single platform.

The total time required for the PPMM microreactor procedure, including passing the reactant oil through the column for reaction and collecting enough FAEE derivatives for GC analysis, was reduced from 5 h to 1.5 h. It has been

demonstrated that the decreased collection time did not compromise the full conversion from TAG to FAEE as shown in the HPLC/ELSD and GC/FID results (Chapter 2). It is highly desirable to have a fast derivatization method to allow higher throughput and to minimize any immobilized lipase activity loss. The collection time used in this work (1.5 h) makes the speed of the microreactor method comparable with conventional methods using alkaline catalysts which can complete transesterification of TAGs within less than 1 hour. At the same time, the shortened infusion and collection time enables greater reusability of the PPMM microreactor.

The PPMM microreactor fabrication procedure reported previously was reproduced in Chapter 2. The results from scanning electron microscopy (SEM), porosity test and lipase loading test revealed that the PPMM microreactor had large surface area for lipase to bind, and the amount of lipase immobilized onto the reactor was in agreement with that reported previously (Mugo et al., 2014). The transesterification of a pure TAG standard dissolved in ethanol and hexane mixture (4:1, v/v) was successfully completed on the PPMM microreactor, indicating the derivatization efficiency of the microreactor method.

However, the application of PPMM microreactor on commercial oil samples would be a different case, since they contain much more complicated fatty acid constituents, and the degree of unsaturation and the carbon chain length will both affect the fatty acid determination on GC. Therefore, it is important to validate the derivatization efficiency of the PPMM microreactor method on

commercial oil products, in order to confirm that it can be an equivalent but advantageous substitution for the conventional acid or alkaline-catalyzed batch system GC derivatization methods.

One commercial TAG standard mixture (GLC 406 from NuChek) and three vegetable oils (canola oil, camelina oil and refined-bleached-deodorized RBD palm olein) were used as test samples for the PPMM microreactor. HPLC/ELSD analyses indicated that all of the TAG from the initial samples were consumed, while GC/FID results indicated that they were quantitatively converted to FAEEs (Chapter 3). The GC/FID results for fatty acid determination using PPMM microreactor methods were compared to the AOCS official methylation method *Ce 1k-09* to demonstrate equivalent accuracy (AOCS, 2012). Not only was the microreactor method accurate, it also gave precise FA determination results over different batches, with the relative standard deviation of 5 individual microreactor measurements of total FAEE amount being 0.70%. Furthermore, the microreactor could be reused 4 times without any loss in efficiency, and this might be a result of the reduced reaction time optimized in earlier research.

A future study that could be carried out is the optimization of the length and diameter of the PPMM microreactor. The current study already demonstrated that the transesterification reaction was completed during the passage of oil through the microreactor. The point at which all of the TAG is converted to FAEE can be used to in the future to investigate the shortest microreactor length enabling full conversion, and thus the shortest flow-through time for the lipid

sample. Also, a larger microreactor diameter can decrease the flow-through time by enabling a higher reactant flow rate, since larger diameter means less backpressure impeding the flow. Therefore, by optimizing the length and diameter of the PPMM microreactor, a shorter total procedure time can be achieved.

In addition, the validation of the lipase immobilized poly(GMA-*co*-EDMA) microreactor in this study was limited to the transesterification of plant oils, which contain mainly triglycerides. However, lipid GC analysis also involves transesterification of other fatty acid containing compounds such as phospholipids and cholesterol esters, and the esterification of free fatty acids. Thus, the future validation of the microreactor method could focus on the transesterification or esterification of other lipids. For example, if the transesterification of phospholipids can be successfully achieved using the microreactor, it could be employed in clinical diagnosis such as blood lipid profiling.

After the above tests have been done, future development of the PPMM microreactor may involve the design, assembly and testing of an automated GC-derivatization-analysis system. This fully automated analysis system will realize the lipid derivatization, and GC analysis directly from a vial of sample solution. In this way, a fast and direct GC analysis for fats and oil can be achieved.

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Functional Motions of *Candida antarctica* Lipase B: A Survey through Open-Close Conformations

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Candida antarctica lipase B (CALB) belongs to psychrophilic lipases which hydrolyze carboxyl ester bonds at low temperatures. There have been some features reported about cold-activity of the enzyme through experimental methods, whereas there is no detailed information on its mechanism of action at molecular level. Herein, a comparative molecular dynamics simulation and essential dynamics analysis have been carried out at three temperatures (5, 35 and 50°C) to trace the dominant factors in the psychrophilic properties of CALB under cold condition. The results clearly describe the effect of temperature on CALB

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Appendix 2

Figure A2-1 The Flow-through GC Derivatization Microreactor Device

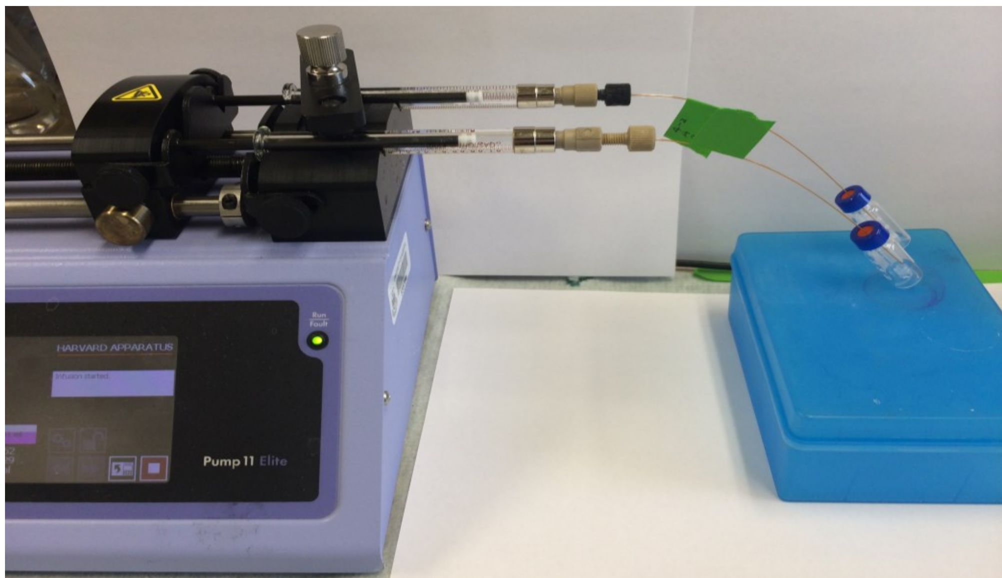

Table A2-1 Microreactor Weight with or without Monolith and Immobilized Lipase

	weight/ g
15 cm of empty capillary	0.02485
	0.02489
	0.02512
15cm of monolith in capillary	0.02699
	0.02721
	0.02783
15 cm of empty capillary with water	0.03093
	0.02982
	0.02997
15cm of monolith in capillary with water	0.03171
	0.03140
	0.03165
Porosity	0.7763
	0.8499
	0.7876
Porosity Average	0.81
Porosity %RSD	3.24%

Table A2-2 Amount of Lipase Immobilized onto the PPMM Microreactor Using UV Spectrometer (average of 3 microreactors)

	Absorbance (AU)	Concentration (mg/mL)	Amount of Lipase (mg)
Initial lipase solution (10 times diluted)	0.0979	0. 7678	7.678
Collected lipase solution (10 times diluted)	0.0715	0.5542	5.542
Loaded lipase	-	-	2.136

Table A2-3 GC-FID Results for Quantifying Ethyl Oleate Concentration after Transesterification of Triolein on PPMM Microreactor

0.5mg/ml TO + 30uL 0.1mg/ml C19:1ME IS after 1.5hr reaction					
	C18:1	C19:1	dilution factor	Real Conc.	conversion rate
		RUN 1			
Area	27481.48	6430.24	0.41	0.5105	97.07%
Conc. After GC dilution	0.2117	0.0585			
		RUN 2			
Area	27790.03	6467.32	0.41	0.5210	99.07%
Conc. After GC dilution	0.2142	0.0589			
		RUN 3			
Area	27280.39	6507.54	0.41	0.5170	98.30%
Conc. After GC dilution	0.2105	0.0593			
Average %FAEE				0.5162	98.15%
RSD of each component	0.89%			1.03%	
theoratical value				0.5259	

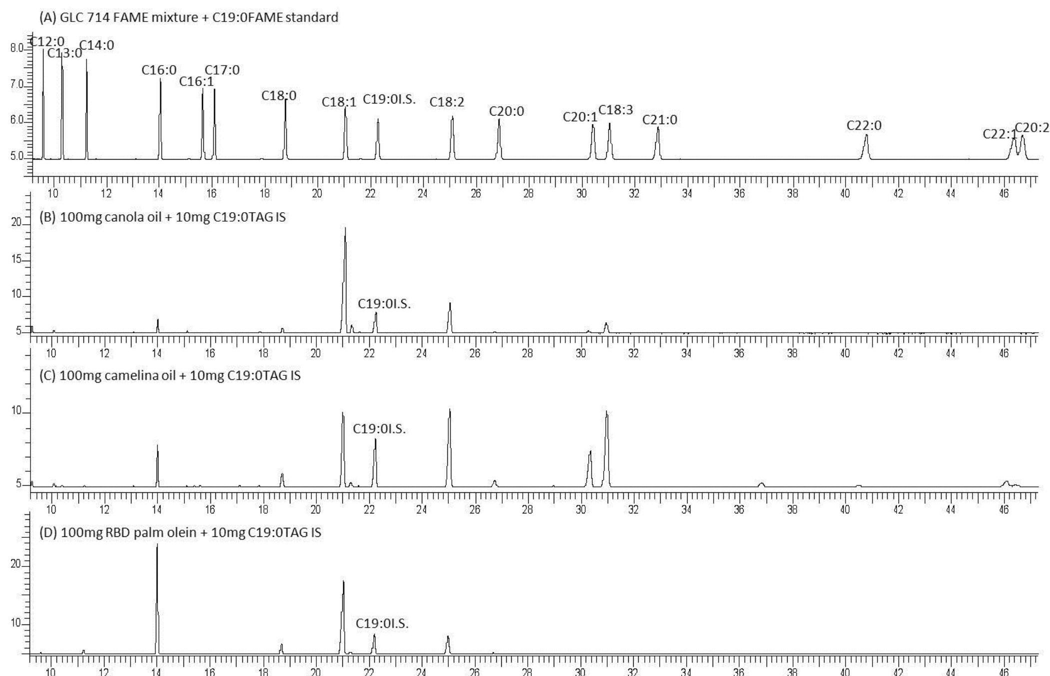

Figure A3-1 AOCS Methylation GC/FID Traces Using Isothermal Condition (a) GLC 714 FAME standard mixture with C19:0 fame internal standard; (b) canola oil methylation products with C19:0 TAG as internal standard; (c) camelina oil methylation products with C19:0 TAG as internal standard; (d) RBD palm olein methylation products with C19:0 TAG as internal standard