SUSTAINABLE ALCOHOLYSIS OF SOYBEAN OIL IN SUPERCRITICAL CARBON DIOXIDE AND ANALYSIS OF THE STRUCTURED LIPIDS WITH NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

By

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ABSTRACT

Producing structured lipids (SLs) from oils that contain high levels of unsaturated fatty acids (UFAs) can be one of the keys to solving issues associated with *trans* and saturated fats in the food industry. Due to various nutritional and functional properties of SLs, they fulfill different applications in the food and pharmaceutical industries. This thesis has focused on production of two SLs - a high monoester mixture of soybean oil (HHMS) and a soybean oil-based DAG oil (SDO). HMMS can be used as an emulsifier mixture due to its rich composition of monoglycerides (MGs), diglycerides (DGs) and propylene glycol esters (PGEs). SDO can be used as a low caloric oil due to having a higher amount of 1,3-DGs compared to conventional oils. The main objective was producing and analyzing SLs in an environmentally-friendly manner. Enzymatic alcoholysis under supercritical CO_2 ($SCCO_2$) was chosen for production of both HMMS and SDO. Nuclear Magnetic Resonance (NMR) using ^{1}H, ^{13}C and ^{31}P spectroscopy was utilized for analysis of SLs. In the production of HMMS, enzyme and time significantly influenced the level of 1-MGs, 2-MGs and 1,2-DGs. The ^{31}P-NMR quantification of HMMS demonstrated that out of the total amount of MGs produced, they are composed of 21±2.9% of 2-MG and 4±0.3% of 1-MG at a pressure of 276 bar, at 70 °C, and an enzyme load of 10% (w/w) following 4h of enzymatic alcoholysis. Among the three methods, ^{31}P-NMR proved to be a practical methodology with high-reproducibility for the precise detection and quantification of partially esterified glycerols and free fatty acids in the SLs. After an 8h enzymatic alcoholysis in $SCCO_2$, the percentage change of total DGs in SDO increased dramatically (1700%) compared to the same reaction at atmospheric pressure. A total DG content of 44% (w/w), containing 60% of the healthier 1,3-DG isomer versus 40% 1,2-DG, was obtained at a pressure of 80 bar, at 60 °C, and an enzyme load of 10% (w/w) following 8h of enzymatic alcoholysis. Integration of

greener alternative processes of production and practical analysis of SLs contributes to the development of complex lipid mixtures that have functional and nutraceutical properties.

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DEDICATION

This writing is dedicated to

My grandmother, Bibi Zahra Hamzeie

My father, Gholamreza Vafaei

My mother, Mansoureh Emami

My brother, Dr. Ali Vafaei and his family, Dr. Tina Vafaei and Ryan Vafaei

My husband, Dr. Ehsan K. Esfahani and his parents, Ghasem K. Esfahani and Zahra Meisami, his sister, Dr. Elham k. Esfahani and his brother, Mohammad K. Esfahani

FOREWORD

This thesis is written in manuscript style and is composed of six chapters and four manuscripts. The first chapter includes “Overall Introduction” followed by manuscript 1, which will explain the current reaction routes and the effect of enzyme, reactants and reaction variables on production of monoglycerides and diglycerides. Research manuscripts, addressing specific objectives, will be presented in the following chapters 3 to 5. The final chapter discusses an “Overall Conclusions” of the thesis with concluding remarks of the work, its limitations, and proposed future directions of the research project.

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ABBREVIATIONS

AG	Acylglycerol
AM	Average molecular weight
ANOVA	Analysis of variance
CDCL3	Deuterated chloroform
CI	Chemical interesterification
CTDP	2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane
CVD	Cardio vascular disease
CY	Conversion yield
DAG	Diacylglycerol-enriched
DG	Diglycerides
DP	Diester of propylene glycol ester
EFSA	European food safety authority
FA	Fatty acid
FAEE	Fatty acid ethyl ester
FDA	Food and drugs administration-USA
FFA	Free fatty acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GE	Glycerol ester
GMO	Genetically modified organisms
GRAS	Generally recognized as safe
HPLC	High pressure liquid chromatography

LI	lipase-catalyzed interesterification
LN	Linolenic acid
LO	Linoleic acid
MDG	Mono- and di- glyceride
MG	Monoglyceride
MO	Monoolein
MP	Monoester of propylene glycol ester
MPSG	Manitoba pulse and soybean growers
MUFA	Monounsaturated fatty acid
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
OL	Oleic acid
PEG	Partially esterified glycerol
PGE	Propylene glycol ester
PUFA	Polyunsaturated fatty acid
$SCCO_2$	Supercritical CO_2
SDO	Soybean oil-based DAG oil
SEM	Standard of error mean
SFA	Saturated fatty acid
SI	Support information
SL	Structured lipid
STS	Stock solution
TG	Triglyceride

TLC	Thin layer chromatography
TMS	Tetramethylsilane
UFA	Unsaturated fatty acid
W/O	Water in oil

CHAPTER 1

OVERALL INTRODUCTION

1.1 INTRODUCTION

Producing structured lipids (SLs) from oils with high levels of unsaturated fatty acids (UFAs) which have emulsifying properties can be one of the keys to solve the main issues in the fat and oil industry. The current challenges include the abolition of partially hydrogenated fats, the reduction of saturated fats, replacing animal fats (hump, lard and tallow) with vegetable oils, and using oils which are produced in a sustainable way (Marangoni 2018). High monoester mixtures of oils and diacylglycerol-enriched oils have functional (Remonatto et al. 2015) and nutraceutical (Martin et al. 2014) applications in both the food (Zhang et al. 2017) and pharmaceutical (Feltes et al. 2013) industries.

SLs can be any type of acylglycerols (AGs) including monoglycerides (MGs), diglycerides (DGs) and triglycerides (TGs), all of which have different physical properties. AGs contain UFAs, such as linoleic acid (L) and oleic acid (O) and also saturated fatty acids, such as stearic acid (S). AGs with different fatty acids (FAs) have different melting points. For example, TGs with low (LOO and OOO) and high (SSS) melting point can be converted to structured TGs with intermediate melting points such as LOS, SOO and SOS (Jenab 2013). MGs and DGs are amphiphilic molecules, with free hydroxyl groups on the glycerol moiety (the hydrophilic part) and the fatty acid groups (the hydrophobic part) (Damstrup 2008). Both types of molecules have emulsifying properties (Feltes et al. 2013).

From the nutritional point of view, SLs have hypocaloric properties (Martin et al. 2014; Yanai et al. 2007). Different isomers of DGs and MGs have different health effects. For instance, during digestion, 1,3-DGs hydrolyze to 1-MGs instead of 2-MGs. 1-MGs versus 2-MGs are not suitable substrates for the acyltransferase enzymes of intestinal cells to re-synthesize the TGs. Therefore, 1-MGs increase fat oxidation and decrease the TG storage in adipose tissues. 2-monoacylglycerols (2-MGs) are essential molecules for lipid absorption and have a health protective effect when a high and rapid rate of absorption of UFAs is needed (Pfeffer et al. 2007). For example, transporting the polyunsaturated fatty acids (PUFAs) in a 2-MG form through the lymphatic system happens readily (Pfeffer et al. 2007). From the medicinal point of view, monounsaturated fatty acid (MUFA)-enriched MGs have antioxidant, antidiabetic, and antiatherogenic properties (Feltes et al. 2013; Martin et al. 2014).

From the functional point of view, SLs such as MGs, DGs and propylene glycol monoesters can be used with vegetable oils that contain UFA to produce shortenings and margarines without the need for hydrogenation of oils containing UFAs (Doucet 2005; Orthoefer 2008). Commercial diacylglycerol-enriched (DAG) oil, which contains 80% DG, has similar taste and colour to conventional vegetable oils. DAG oil can be used primarily in cooking and food product formulations (Chen et al. 2017) such as salad dressings, spreads, margarine, and confectionery fats (Katsuragi et al. 2008). Different types of FAs influence emulsifier properties. For example, MGs and DGs that contain UFAs provide softer texture and better emulsifying performance in fat based food products (Zhang et al. 2017).

SLs can be produced from various lipid reaction routes (Kim and Akoh 2015) with and without catalysts (He et al. 2016). Conventional chemical interesterification (CIE) suffers from a lack of FA selectivity (Farfán et al. 2015), the creation of undesired side products and disposing the toxic chemicals is needed. However, lipase-catalyzed interesterification (LIE) increases reaction selectivity so that there is control over the structure of the products by using enzymes with different specificities. LIE produces fewer side products than CIE, and using a biodegradable enzyme eliminates the need for removal of the alkaline catalysts and salt residue (Bart, Palmeri, and Cavallaro 2010). Conducting LIE under $SCCO_2$ pressure results in conducting the reaction at lower temperature and with lower solvent consumption compared to CIE. In addition, non-enzymatic reactions suffer from lack of FA selectivity (Willis, Lencki, and Marangoni 1998), low oxidative stability (Mensink et al. 2016) and the destruction of UFAs in the finished product (Solaesa et al. 2016). Therefore, using biocatalysts such as lipases has increased in use for the synthesis of SLs (Willis and Marangoni 2002). Various lipid reaction routes include esterification, hydrolysis, transesterification, acidolysis and alcoholysis (Venkata Rao, Jayaraman, and Lakshmanan 1993). Alcoholysis is one of the most common routes due to a high yield of MGs (Compton, Laszlo, and Evans 2013; Zhong et al. 2013) and DGs (Voll et al. 2011).

Sustainable technologies for the food industries are necessary in the next decade (Boye and Arcand 2013). Supercritical carbon dioxide ($SCCO_2$) is an eco-friendly technology which can be used in the food industry for conducting both enzymatic and non-enzymatic reactions (Loss et al. 2015). A $SCCO_2$ medium improves the reaction rates (Ramsey et al. 2009) and is easily separated from the reaction mixture by depressurization (Rezaei, Temelli, and Jenab 2007). $SCCO_2$ has valuable advantages for the production of SLs through enzymatic reactions (Jenab et al. 2014).

Glycerol, as a byproduct of biodiesel production, can be used to synthesize value-added products (Luo et al. 2016), and this is another eco-friendly option for production of SLs. For enzymatic glycerolysis at atmospheric pressure and in a solvent medium, a mixture of *tert*-butanol/isopropanol, has been used to attain a high yield of MGs (Zhong et al. 2009), and in tert-pentanol to obtain DG (Cai et al. 2016). Yet, how enzymatic alcoholysis in a supercritical carbon dioxide ($SCCO_2$) medium influences the levels of MGs, DGs and propylene glycol mono- and di-esters in a complex lipid mixture needs further studies. In addition, how enzymatic glycerolysis under atmospheric and $SCCO_2$ conditions influences the production of SLs containing different FA type at different positions of the AGs needs further studies.

Finding a fast, reliable and reproducible approach for accurate analyses of SLs is crucial for industries utilizing them. Nuclear magnetic resonance (NMR) spectroscopy is a rapid, reproducible and eco-friendly tool. Although NMR analysis of natural oils with high triglyceride (TG) levels has been conducted in many studies (Barison et al. 2010; Gao et al. 2009; Gouk et al. 2011; Lopes et al. 2016), there are few studies that have focused on NMR analysis of complex

lipid mixtures such as biodiesels (Anderson and Franz 2012) and other SLs (Magnusson et al. 2015). The short acquisition time of ^{1}H-NMR facilitates the analysis of lipid mixtures (Nieva-Echevarría et al. 2014). A single NMR experiment such as ^{31}P-NMR (Dais, Misiak, and Hatzakis 2015) can do a direct regio-isomeric analysis of a SL, such as an analysis of the 2-MG and 1-MG in the SLs. ^{13}C-NMR experiments also can analyse the position and type of fatty acids (FAs) directly without laborious sample preparation. In contrast, for determination of a FA type at the *sn*-2 position without NMR techniques, a combination of analytical techniques and reactions such as pancreatic lipase hydrolysis, thin layer chromatography (TLC), methanolysis, and gas chromatography (GC) is required (Yang et al. 2003).

In this thesis, a screening of 1D ^{1}H, ^{13}C and ^{31}P NMR spectroscopy to find the most feasible and easy NMR experiment for analysis of a high monoester mixture of soybean oil (HMMS), both quantitatively and qualitatively, was conducted. The results address the lack of research about NMR analysis of lipid mixture of mono- and diesters compared to NMR analysis of TGs. This study demonstrates how useful each nucleus is for determination of the conversion yield, the mono- and diester compositions, the FA composition, and the regio-isomeric distribution in the AGs of the complex lipid mixtures.

Additionally, it is expected that the changes in enzyme and reaction times alter the FA type, FA position and partially esterified glycerols (PEGs) profile of HMMS. The results will address shortfalls in understanding how levels of mono-and diesters, distribution of FAs, and their position within TGs are influenced by the presence or absence of a lipase during the different reaction times of an enzymatic alcoholysis in $SCCO_2$.

Moreover, it is anticipated that $SCCO_2$ develops the enzymatic alcoholysis. Therefore, two time-course studies of enzymatic glycerolysis under atmospheric and $SCCO_2$ conditions were conducted to produce soybean oil-based DAG oil (SDO). The effect of four independent variables, including pressure, reaction temperature, time, and enzyme load on the respective content of DG, MG, and FFA in the final products of SDO after enzymatic alcoholysis in $SCCO_2$ was examined.

1.2 RATIONALE

There is a lack of research on the role of NMR in quantitative and qualitative analysis of complex oils obtained through their lipase-catalyzed glycerolysis in a $SCCO_2$ medium. There has been no experimental effort to use eco-friendly methodologies in production and analysis of the products of lipase-catalyzed interesterification. Studying the effect of the presence or absence of enzyme on the alcoholysis of soybean oil and propylene glycol in $SCCO_2$ media to produce an emulsifier mixture with a high level of monoesters, is a neglected area of study. The specific aspect of utilizing green methodology to produce DAG oil is through use of surplus glycerol and enzyme. Testing different independent variables involving pressure, temperature, enzyme load and time on the composition of DAG oil merits additional study.

To support the hypothesis that NMR is a quick, reliable and eco-friendly method for analysis of complex oils, we examined the ^{1}H-, ^{13}C- and ^{31}P-NMR analysis of the lipid mixtures obtained through lipase-catalyzed glycerolysis to investigate the mono- and diester composition, and the FA composition of the reaction products. Moreover, to explore the hypothesis that lipase-catalyzed alcoholysis in a $SCCO_2$ medium influences the components of a complex lipid mixture

as a function of reaction time, I first studied if enzyme and time alter PEG composition, type and position of each FA in the TGs of HMMS. Secondly, I examined whether different variables of pressure, temperature, enzyme load, and time alter the components of SDO, to create a fitted mathematical model to maximize the DG level of SDO.

1.3 OBJECTIVES

The present research project has 4 specific objectives:

To screen ^{1}H, ^{13}C and ^{31}P spectroscopy to find the most feasible and easy NMR experiment for analysis of a complex lipid mixture, both quantitatively and qualitatively.

To explore how interesterification under the mild conditions of a $SCCO_2$ medium, with and without enzyme, influences the type and position of each FA in TGs and the PEG levels of HMMS as a function of reaction time.

To evaluate how enzymatic glycerolysis in $SCCO_2$ is effective for production of soybean oil-based DAG oil compared to enzymatic glycerolysis under atmospheric conditions.

To investigate the impact of four independent variables (pressure, temperature, enzyme load and time) on the maximizing the DG levels and minimizing the levels of MGs and free fatty acids.

1.4 HYPOTHESES

The hypotheses to be tested include:

1. NMR analysis is a versatile experiment for identifying and quantifying lipid mixture components.

2. ^{31}P-NMR is a reproducible and reliable test for the analysis of partially esterified glycerols and free fatty acids of high monoester mixture of soybean oil (HMMS) and soybean oil-based DAG oil (SDO).

3. Immobilized lipase from *Candida antarctica* (Novozym 435), also known as lipase B, is an effective catalyst for interesterification reactions under the mild conditions of $SCCO_2$ media.

4. Novozym 435 and reaction time affects the PEG contents and FA compositions of HMMS.

5. Reaction conditions such as reaction under $SCCO_2$ versus atmospheric conditions influences the production rate of DGs/ MGs and 1,3-DG/1,2-DG of SDO as a function of reaction time.

6. Variation of independent variables of reaction involving pressure, temperature, enzyme load and time alter the MGs, DGs and FFA levels of SDO.

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CHAPTER 2

MANUSCRIPT 1

PRODUCTION OF MONO AND DIACYLGLYCEROLS FROM FATS AND OILS: A MINI-REVIEW

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2.1 ABSTRACT

Emulsifiers can modulate health in humans and quality in food and pharmaceutical products. The main emulsifiers include the monoglycerides (MGs) and diglycerides (DGs), which are produced through different reaction routes such as hydrolysis, esterification, and interesterification. The reaction yield of MG and DG is influenced by the initial reactants, catalyst, and the reaction independent variables. The present review summarizes the knowledge of how reactants and catalysts interact to control the production yield of MGs and DGs. Recently, biocatalysts such as lipases have been increasingly used in MG and DG production due to their specificity, biodegradability and easy recovery. The reaction route, reactant types, pressure, temperature, reaction time, molar ratio, enzyme type, enzyme load, water content, and post-reaction techniques of enzymatic productions of MGs and DGs from various studies is reviewed. The information provided in this review helps to guide optimization of production conditions of the MGs and DGs. An outcome from this minireview is that favourable outcomes for the sustainable, safe, simple and economical production of MG and DG exist as a result of optimizing the reaction variables. Furthermore, diets that contain MG and DG can improve nutritional and quality benefits of food products they are incorporated in.

2.2 INTRODUCTION

Structured lipids (SLs) such as monoglycerides (MGs) and diglycerides (DGs) are products of interest in food (Remonatto et al. 2015; Zhang et al. 2017; Lee et al. 2018), pharmaceuticals (Damstrup et al. 2006; Pawongrat, Xu, and H-Kittikun 2007) and cosmetic industries due to their health benefits and their potential functionalities (Martin et al. 2014). One example is hard stock fat. The old way of hard stock production was partial hydrogenation of the vegetable oils which produces *trans* fatty acids (Klonoff 2007). Consumption of *trans* fatty acids is strongly correlated with cardiovascular diseases (CVD) (Wang and Hu 2017). SLs allow baking fats to have desirable physical properties without the need for addition of *trans* fatty acids (Akoh 2005). The health benefits of MGs and DGs are in large part ascribed to their different digestion and absorption pathways relative to the triglycerides (TG). MGs and DGs have emulsification properties. The functional properties of these emulsifiers are attributed to their amphiphilic properties of free hydroxyl groups on the glycerol moiety (the hydrophilic part) and their fatty acid groups (the hydrophobic part). MGs and DGs are approved as Generally Recognized As Safe (GRAS) by the U. S. Food and Drugs Administration (FDA) (Feltes et al. 2013).

MGs improve the functionality of bakery, confectionary (Pawongrat, Xu, and H-Kittikun 2007) (Damstrup et al. 2005), dairy, potato and pasta (Fregolente et al. 2007) products, margarines, peanut butter (Fregolente et al. 2010) and sauces (Fiametti et al. 2011) due to their emulsifying properties. Besides their emulsifying functionalities, MG have other excellent properties such as low odor, lack of taste, biodegradability, safety (Fureby, Adlercreutz, and Mattiasson 1996) and low cost (Fregolente et al. 2010) when used as ingredients in the food industry (Fiametti et al. 2011; Fregolente et al. 2010).

Besides their emulsifying functionality, DG have been recently clinically proven to assist in maintaining or reducing the fat mass (Kim et al. 2017), visceral fat area (Saito et al. 2017), and human body weight (Feltes et al. 2013) compared to TG; therefore, they are a beneficial partial substitute for TG (Fregolente et al. 2010). Nutraceutical and functional characteristics of DG have driven research interest globally (Pawongrat, Xu, and H-Kittikun 2007).

Production of mono and diacylglycerols has been performed through various routes according to the reaction type (Kim and Akoh 2015). Figure 2.1 shows various categories of lipid reaction routes include hydrolysis, esterification, and interesterification (Venkata Rao, Jayaraman, and Lakshmanan 1993).

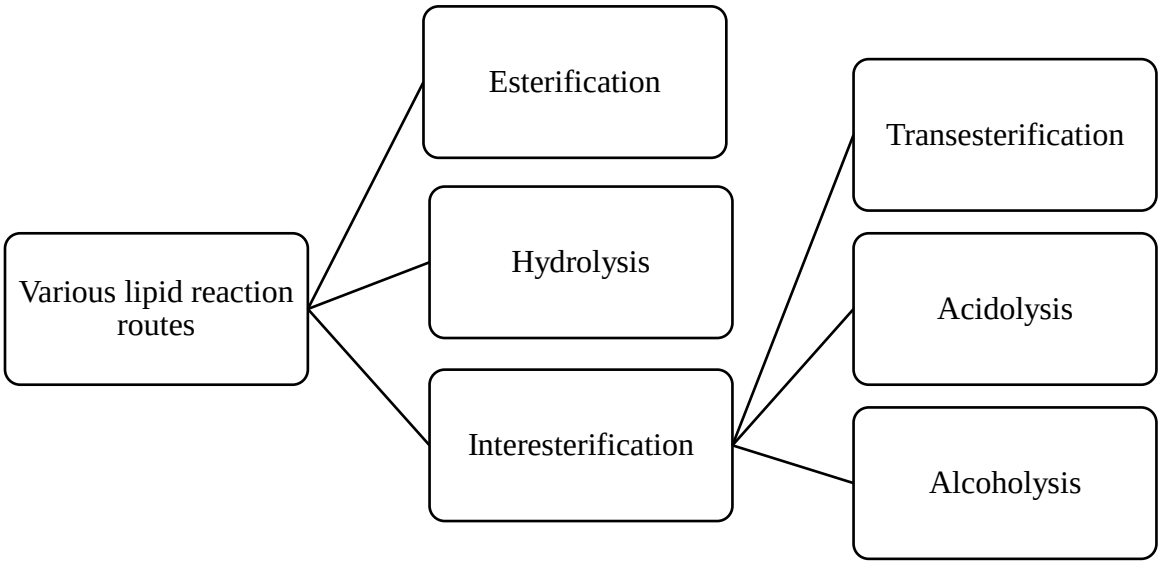

Figure 2. 1 Different possible reaction routes of lipids

All reactions can be conducted with a chemical or an enzymatic catalyst (He et al. 2016). The synthesis of MGs and DGs at large scale in industry is conducted via chemical glycerolysis at high temperature (Hasenhuettl 2008). Post-reaction techniques are used to obtain pure MGs or DGs (Damstrup et al. 2006; Hasenhuettl 2008b). However, a mixture of mono- and di-esters without purification steps has many applications and even can be attractive from ecological and economical point of views (Martin et al. 2014). For example, a mixture of MGs and DGs, which are synthesized from different sources of fats and oils, is very useful in ice cream production (Goff and Hartel 2013). A mixture of MGs and DGs develops the functionality of ice cream due to the amphiphilic property of the MGs and the dryness is improved due to the fat solubility of the DGs (Goff and Hartel 2013).

Molar ratio is a key reaction variable that plays an important role in the production of MGs and DGs (Hasenhuettl 2008; Yamane et al. 1994). Other reaction variables such as time, temperature, pressure, etc. need to be optimized based on the objective of the study. For example, if the purpose of the study is production of MGs with high unsaturated fatty acid levels, the goal of the optimization is to increase the MG yield with the temperature kept as low as possible. Low reaction temperatures minimize the problems of producing side reactions (Hasenhuettl 2008). Finding a safe, sustainable, and cost-efficient method, which provide SLs, such as a specific MG or DG that has a particular fatty acid at a specific position to produce a high-quality product, is crucial for manufacturers (Shahidi 2010; Srinivas and King 2010).

This paper reviews the knowledge of how the type of reactants (different sources of oil and fat) and catalysts (chemical or enzymatic catalysts) determine the yield of MG and DG via different reaction routes and compares the digestion and absorption pathways of TGs, DGs and MGs.

2.3 DIFFERENT HEALTH EFFECTS OF MG AND DG FROM TG DUE TO DIFFERENT DIGESTION AND ABSORPTION PATHWAYS

The importance of the nutritional and health effects of acylglycerols is due to their role as a carrier of the fatty acid (FA) and as an energy source for the human body. Figure 2.2 demonstrates that in the digestion of regular oils and fats, TG is hydrolyzed by pancreatic lipase to a T-shaped 2-MG molecule and the free fatty acids (FFA) at the intestinal lumen (Martin et al. 2014). The absorbed 2-MG in intestinal cells is quickly re-synthesized to a TG via re-esterification with FFA in the epithelial cells (Yanai et al. 2007) by monoacylglycerol

acyltransferases (MGAT) and diacylglycerol acyltransferases (DGAT) (Yanai et al. 2012). Then, TG are incorporated in chylomicrons, which are lipoproteins that transport lipids, and transferred to the intestinal lymph (Martin et al. 2014; Yanai et al. 2007).

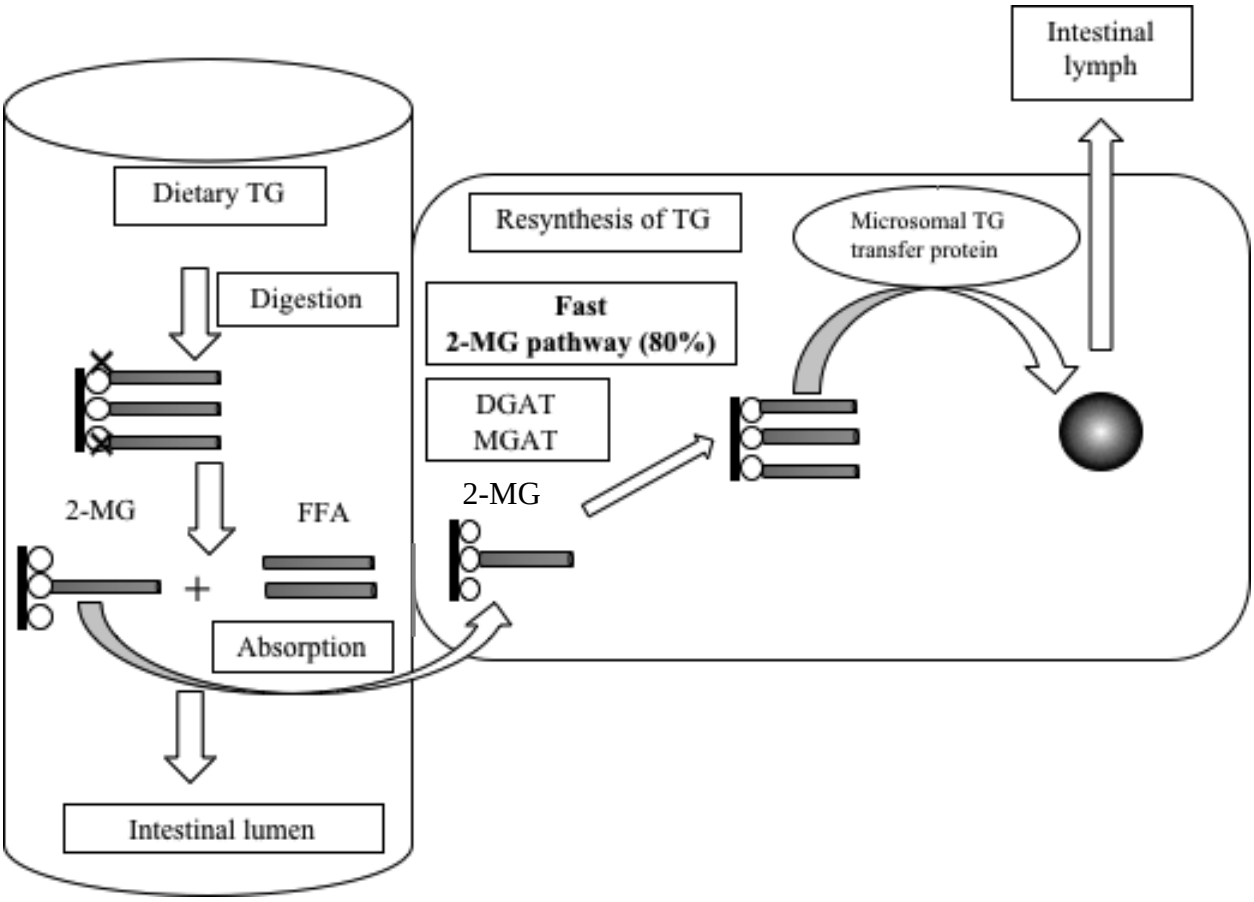

Figure 2. 2 Digestion and absorption pathway of triacylglycerols through the lumen and epithelial cells of the intestine. Modified from (Yanai et al. 2007)

Figure 2. 3 shows the dietary metabolic pathways for MG and DG and their difference from the TG metabolic pathway. During the digestion of DGs, 1,3-DG is hydrolyzed to an L-shaped 1-MG molecule and a FFA (Dhara, Dhar, and Ghosh 2013). The absorbed 1-MG in the intestinal cells, which can be obtained through either the digestion of dietary MG and DG, is not a suitable substrate for either acyltransferases (MGAT and DGAT) (Yanai et al. 2012) and is used less for synthesis of chylomicrons (Prabhavathi Devi et al. 2018). Therefore, less TG is re-synthesized through the 2-MG pathway. TG can be re-esterified, but through the glycerol-3-phosphate pathway which is a slow route (Martin et al. 2014; Yanai et al. 2007). Due to less amounts of 2-MG, more un-esterified fatty acids are re-directed via the vein to the liver where they undergo β-oxidation (Prabhavathi Devi et al. 2018). Increasing fat oxidation decreases lipid synthesis by enhancing the energy expenditure which may reduce the storage of TGs in the adipose tissues (Kristensen, Jørgensen, and Mu 2012).

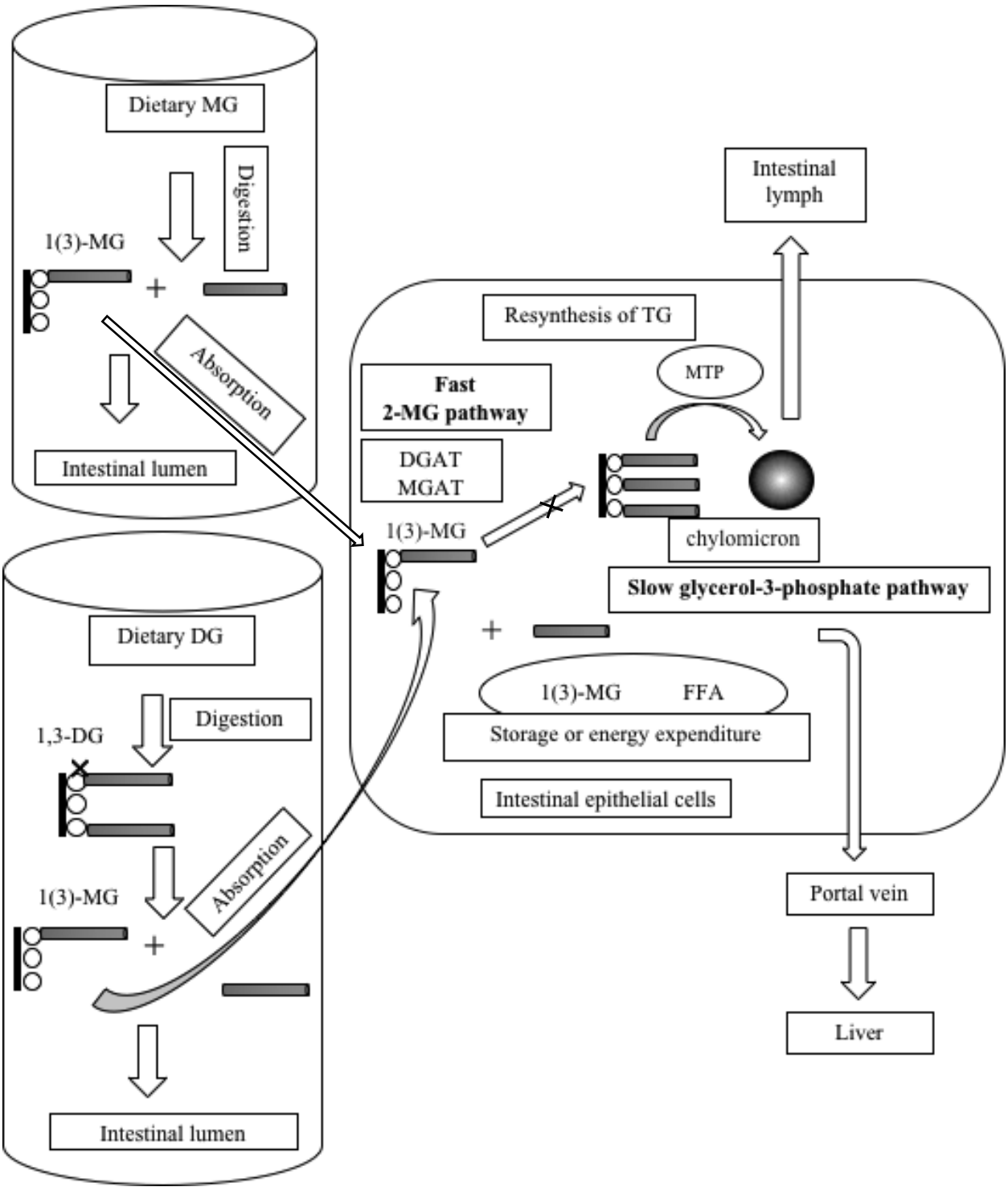

Figure 2. 3 Digestion and absorption pathway of diacylglycerols through the lumen and epithelial cells of the intestine. Modified from (Yanai et al. 2007)

2.4 LIPID REACTION ROUTES

Hydrolysis is a route to produce free fatty acid (FFA) from oils including TGs, DGs and MGs (equations 2-4). This reaction is usually performed before any lipid modification to produce FFA for interesterification in industry (Minami and Saka 2006). For example, in industry at a large scale a diacylglycerol-enriched (DAG) oil is produced using two steps of hydrolysis and esterification (Prabhavathi Devi et al. 2018). In the first step oils are hydrolyzed to produce FFAs then an esterification between glycerol and the obtained FFAs occurs (Prabhavathi Devi et al. 2018). Equation 2. 1 shows the general reaction of an ester ($(R_1OC(O)R_2$)with water. This reaction produces an FFA ($R_2C(O)OH$) after the loss of the alcohol moiety (R_1OH). Although hydrolysis accounts for FFA production, the stepwise nature of the hydrolysis reaction provides both MG and DG (equations 2. 2- 2. 4) (Moquin and Temelli 2008). $R,\ R_1, R_2, R_3$ and R_4 are different alkyl groups in equations (2. 1- 2. 9).

$$R_1OC(O)R_2 + H_2O \xrightarrow{Hydrolysis} R_2C(O)OH + R_1OH \quad (2.\ 1)$$

$$TG + H_2O \xrightarrow{Hydrolysis} DG + FFA \quad (2.\ 2)$$

$$DG + H_2O \xrightarrow{Hydrolysis} MG + FFA \quad (2.\ 3)$$

$$MG + H_2O \xrightarrow{Hydrolysis} G + FFA \quad (2.\ 4)$$

Esterification is a reaction between a carboxylic acid and an alcohol which produces an ester with subsequent loss of water (equation 2. 5).

$$R_1C(O)OH + R_2OH \xleftrightarrow{Esterification} R_2OC(O)R_1 + H_2O \quad (2.\ 5)$$

Interesterification, which is a fatty acyl ($RC(O)$) exchange between two substrates, includes transesterification, acidolysis, and alcoholysis (Jenab 2013). Transesterification is an exchange between fatty acids of two individual triglycerides or within a triglyceride (equation 2. 6). The application of transesterification is in the production of zero-*trans* products such as margarine and shortenings. For example, Jenab at al. (2014) used canola oil and fully hydrogenated canola oil in an enzymatic transesterification to modify the TG composition, melting and crystallization behaviours of oils to develop a base-stock for zero-*trans* margarines.

$$R_1OC(O)R_2 + R_4OC(O)R_3 \overset{Transesterification}{\Longleftrightarrow} R_1OC(O)R_3 + R_4OC(O)R_2 \qquad (2.\ 6)$$

Acidolysis is a fatty acyl exchange between an FFA ($R_3C(O)OH$) and ester ($R_1OC(O)R_2$). Equation 2. 7 shows an acyl exchange by acidolysis between an FFA and a TG. Instead of a FFA an ethyl ester also can react with the TG in the acidolysis reaction.

$$R_1OC(O)R_2 + R_3C(O)OH \overset{Acidolysis}{\Longleftrightarrow} R_1OC(O)R_3 + R_2C(O)OH \qquad (2.\ 7)$$

Acidolysis of conventional oils is predominantly used for the production of structured triacylglycerols, such as TGs containing MLM (medium: long: medium) fatty acids or human milk fat substitutes (Akoh 2005). For example, Bassan et al. (2019) reported an acidolysis reaction between a medium chain fatty acid, such as capric acid, and grape seed oil which is a source of PUFA and MUFA, to produce a MLM-type SL with high nutritional value.

Equation 2. 8 shows an alcoholysis reaction which is an esterification between an alcohol (R_3OH) such as ethanol and an ester ($R_1OC(O)R_2$). The products of this reaction are an ester ($R_3OC(O)R_2$) such as a fatty acid ethyl ester and an alcohol (R_1OH). The main application of alcoholysis is in ethanolysis of oils to produce biodiesel and in glycerolysis of fats and oils to produce MG and DG. Equation 2. 9 shows enzymatic glycerolysis to produce MG and DG (Marianne Linde Damstrup 2008; Willis and Marangoni 2002).

$$R_1OC(O)R_2 + R_3OH \overset{Alcoholysis}{\Longleftrightarrow\!\Longrightarrow} R_3OC(O)R_2 + R_1OH \qquad (2.\ 8)$$

$$\begin{matrix} O-C(O)R_1 \\ O-C(O)R_2 \\ O-C(O)R_3 \end{matrix} + \begin{matrix} OH \\ OH \\ OH \end{matrix} \overset{Glycerolysis}{\Longleftrightarrow\!\Longrightarrow} \begin{matrix} OH \\ O-C(O)R_2 \\ OH \end{matrix} + \begin{matrix} O-C(O)R_1 \\ OH \\ O-C(O)R_3 \end{matrix} \qquad (2.\ 9)$$

2.5 MONOGLYCERIDES

2.5.1 OVERVIEW

MGs are esters that are composed of a fatty acid esterified to the glycerol backbone in one of three positions (Figure 2. 4). The FA can be a short or long chain, saturated or unsaturated.

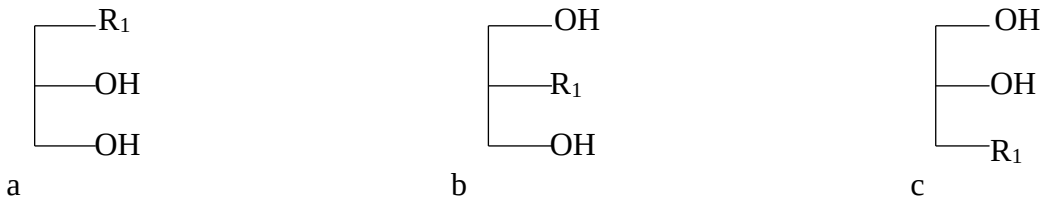

Figure 2. 4 Structures of stereoisomers of monoacyl-*sn*-glycerols. R represents a fatty acid residue. a) *sn*-1, b) *sn*-2 and c) *sn*-3.

2.5.2 MONOACYLGLYCEROL SYNTHESIS

Monoglycerides can be produced through all the above mentioned different lipid reaction routes, with or without enzyme.

2.5.2.1 NON-ENZYMATIC REACTIONS

Moquin and Temelli (2008) described the hydrolysis of canola oil without a catalyst in supercritical CO_2 ($SCCO_2$). They found a yield of 35% for MG after 2h reaction using a water to oil ratio of 3:1 at a temperature of 250 °C and a pressure of 100 bar. They also conducted a hydrolysis-glycerolysis reaction of canola oil to produce MG under $SCCO_2$ conditions (Moquin et al. 2006). A yield of more than 65% MG was obtained after 9 h reaction using a glycerol to oil ratio of 34:1 with 8 % initial water at a temperature of 250 °C and a pressure of 100 bar (Moquin et al. 2006). Although hydrolysis of TGs for MG production can be used as a one-step process in industry, non-enzymatic hydrolysis at high temperatures leads to a products with an unfavourable dark colour and a burnt taste (Voll et al. 2012).

A chemical esterification reaction was performed by Wee et al. (2013) between oleic acid and glycerol to produce a high yield of MG. The conversion yield of oleic acid to esters (MG and DG) was 40 %. The reaction was selective more for MG (98%) than DG. The reaction was conducted in a solvent of *tert*-butanol at 150 °C for 24h with a molar ratio of glycerol to oleic acid of 0.1. A catalyst of Sn–EOF was used, which is an elemental-organic framework (EOF) material obtained from element-carbon bonds between Sn^{4++} ions and 4,4'-Dibromooctafluorobiphenyl. Another chemical esterification between oleic acid and glycerol in a high-pressure tubular reactor at 35.5 bar and 240°C was conducted with a molar ratio of glycerol

to oleic acid of 6 (Herskowitz et al. 2015). After 2.4 h of reaction in a solvent of *tert*-butanol, a 74 % conversion rate with 95% selectivity for monoolein was achieved (Herskowitz et al. 2015).

Go et al. (2015) conducted an interesterification (alcoholysis) reaction of glycerol and sunflower oil in acetone under a high pressure of nitrogen. They reported the optimal reaction condition to obtain 43% MG was for 2 h of reaction at a pressure of 180 bar, a temperature of 250 °C, and a glycerol to oil molar ratio of 5. An alcoholysis reaction of soybean oil and glycerol with sodium methoxide and sodium hydroxide catalysts was conducted by (Echeverri, Cardeño, and Rios 2011). They found that 43% MG could be obtained after 1.5h at 200 °C, with a glycerol to oil molar ratio of 2.5 and with 10% water.

These examples show that most of the non-enzymatic reactions, no matter which route type, need a reaction temperature above 150°C. The reaction time varied between 1.5 h to 24 h depending on pressure and catalysts in the reaction system.

2.5.2.2 ENZYMATIC REACTIONS

Lipase-catalyzed reactions are an alternative method to conduct all types of reaction routes under milder conditions. Table 2. 1 shows that MG production with enzyme eliminates the need for high-reaction temperature (above 150 °C) in contrast with non-enzymatic reactions (section 2.5.2.1).

Table 2. 1 Summary of literature about lipase-catalyzed reactions for production of MGs in the last decade

Study	Reaction type	Reactant	Pressure (bar)	Temp. (°C)	Time (h)	Molar ratio	Enzyme type	Enzyme load	Water addition	Post-reaction	Yield
Tai and Brunner 2011	Esterification	Palmitic acid	CO_2-expanded acetone, 85	50	5	G/O= 3	N. 435	25% of fatty acid weight	-	-	59%
Tai and Brunner 2011	Hydrolysis	Triolein	Atm. 1	50	1.6	W/O=5	L. RM IM	10% of oil weight	-	-	21%
Zhang et al. 2018	Ethanolysis	Tuna oil	Atm. 1	Room temp.	3	E/O= 3	L. 435	8% of total reactants	-	Liquid-liquid extraction and crystallization by acetonitrile and hexane	90%
Compton, Laszlo, and Evans 2013	Ethanolysis	Soybean	Atm. 1	20-40	4	E/O= 31	N. 435	50% of oil weight	-	Flash chromatography purification with acetone and hexane	60%
Abreu Silveira et al. 2017	Ethanolysis in hexane	High oleic sunflower	Atm. 1	40	4	E/O= 8	TLL	9% of oil weight	-	-	33%
Fregolente et al. 2010	Glycerolysis	Soybean	Atm. 1	70	24	G/O= 8	N. 435	2% of oil weight	3.5% of glycerol weight	Short path distillation	80%
Solaesa et al. 2016	Glycerolysis in *tert*-pentanol	Sardine	Atm. 1	50	2	G/O= 3	L. 435	5% of total substrates	-	Short path distillation	67%
Voll et al. 2011	Glycerolysis in *tert*-butanol	Olive	Amt. 1	50	12	G/O= 6	N. 435	10% of total substrates	-	Solvent evaporation at 70 °C, 0.5 bar for 1 h in a vacuum oven	60%
Zhong et al. 2013	Glycerolysis in *tert*-butanol	Soybean	Atm. 1	50	4	G/O= 5	N. 435	10% of oil weight	-	-	80%
Fiametti et al. 2011	Glycerolysis under the influence of ultrasound irradiation	Olive	Atm. 1	65	2	G/O= 2	N. 435	7.5% of total substrates	-	-	39%

Temp.: Temperature, Atm.: atmosphere, G/O: glycerol/oil, E/O: ethanol/oil. N.435: Novozym 435 (lipase B from *Candida antarctica*, immobilized on macroporous polyacrylate resin beads), L. 435: Lipozyme 435 (lipase B from *Candida antarctica*, immobilized on a Lewatit VP OC 1600), L. RM IM; Lipozyme RM IM (a commercial immobilized 1,3-specific lipase from *Rhizomucor miehei*, immobilized on macroporous anion exchange resins), TLL; lipase from *Thermomyces lanuginosus* adsorbed on hydrophobic supports.

However, considering ‘low temperature’ in enzymatic reactions results in challenges to obtain a high yield, and so there is a need for some post-reaction techniques to increase the MG yield. For example, short path distillation, liquid-liquid extraction and other purification techniques have been used to increase MG yields above 60% (Table 2. 1). Only the reaction in supercritical fluid showed a high yield (59%) without a purification step (Table 2. 1). Therefore, to achieve a yield with a high percentage of MG, the contributions of alternative solutions such as reaction under supercritical CO_2 pressure (Tai and Brunner 2011), the use of assistance solvents such as *tert*-butanol (Zhong et al. 2013) or *tert* -pentanol (Solaesa et al. 2016), and increasing the reaction time (Fregolente et al. 2010; Voll et al. 2011) are helpful.

2.6 DIGLYCERIDES

2.6.1 OVERVIEW

DGs are esters that are constituted of two fatty acids that are esterified at two of three positions to the glycerol backbone (Figure 2. 5). The FA can be a short or long chain, saturated or unsaturated. The saturation degree of FAs can control their positional distribution. For example, saturated FAs usually prefer to be located at the *sn*-1,3 positions (Karupaiah and Sundram 2007; Vlahov 2009).

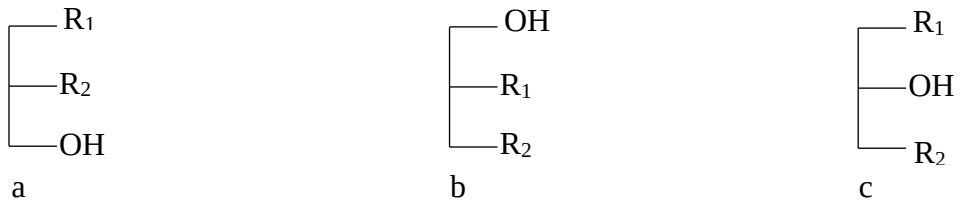

Figure 2. 5 Structures of stereoisomers of diacyl-*sn*-glycerols. R represents a fatty acid residue. a) *sn*-1,2, b) *sn*-2,3 and c) *sn*-1,3.

2.6.2 DIACYLGLYCEROL SYNTHESIS

As for monoglycerides, diglycerides can be produced through different lipid reaction routes, but mostly through glycerolysis with and without enzyme (Jenab 2013).

2.6.2.1 NON-ENZYMATIC REACTIONS

The non-enzymatic process for DG production requires high temperature or a chemical catalyst. Glycerolysis of sunflower oil in acetone was conducted by Go et al. (2015). They verified that a reaction system with a high pressure of nitrogen at 180 bar, 250 °C and a glycerol to oil molar ratio of 5 (with 10% added water) produced 34% DG after 2h. Moquin and Temelli (2008) conducted a non-enzymatic hydrolysis under supercritical carbon dioxide conditions. After 2 hours of reaction 0.045 mole of DG per 100g of canola oil was obtained using a molar ratio of water to oil of 3:1 (w/w) at 100 bar and 250 °C. High-temperature reactions lead to increased energetic cost (Voll et al. 2012). Additionally, unsaturated fatty acids will be destroyed at high temperatures (Naik, Naik, and Mohanty 2014). Moreover, during the DAG oil production process, contaminants such as glycidol fatty acid esters (GEs) can form at a temperature above 150 °C (Zhong et al. 2014). GEs, as probably carcinogenic compounds, are the major problem in

commercializing DAG oil. Utilizing low temperatures for the production and refining processes can decrease GE formation (Zhong et al. 2014).

A non-enzymatic glycerolysis reaction was conducted at 200 °C and a pressure of 70 bar of nitrogen gas using a flow rate of 100 ml/min (Yang et al. 2004). Dry butter fat was mixed with dry glycerol and NaOH as a catalyst (16% and 0.1 % (w/w), respectively). Reaction was ended after 2h and products containing 40-45 % DG were obtained. After cooling the product to 80°C it was washed with water and dried under vacuum. The product was purified by short path distillation. To do an isomerization, the purified sample was heated to 80°C and stirred at 300 rpm for 29 h. The DG content of the final product after isomerization reached 77% (Yang et al. 2004).

Not all the chemical lipid reactions for production of DG are conducted at high temperatures (above 150 °C). For example, Zhong et al. (2014) utilized KOH-MgO catalyst to synthesize DG from soybean oil through the glycerolysis route. The optimal conditions for obtaining a yield of 42% DG were a temperature of 80 °C, an acetone to oil molar ratio of 3, and 12 h of reaction. In another study, Zhong et al. (2010) used ultrasonic irradiation with a NaOH catalyst at 50 °C, and they could achieve 52% DG after 1h reaction.

2.6.2.2 ENZYMATIC REACTIONS

Enzymatic reactions to produce DG not only decrease the required temperature for the reaction system, but they also eliminate the need for chemical production within a solvent (Zhong et al. 2014). Thus, they can provide an economical and quality advantage for the food (Voll et al.

2012) and pharmaceutical industries (Obitte et al. 2018). Pure diacylglycerols can be added to food or pharmaceutical products as emulsifiers (Obitte et al. 2018). Diacylglycerol-enriched oil can also be used as a food product instead of conventional cooking oil which has a high level of triacylglycerols (Lo et al. 2008). Enzymatic production of DG is a sustainable method due to less production of by-products and results in less solvent consumption for separation of the unwanted products (Monte Blanco et al. 2015). High-quality of DG, easy separation of DG, recovery and reutilization of the biocatalysts are the advantages of the enzymatic reactions regardless of the reaction route type (Monte Blanco et al. 2015).

Table 2. 2 shows the studies that have increased for the DG yield with the same post-reaction techniques (Eom et al. 2010; Fregolente et al. 2010; Saberi et al. 2011). To obtain a reaction that favours DG rather than MG when Novozym 435 is used as a catalyst, a lower molar ratio of glycerol to oil gives greater yield without the need for post-reaction techniques (Tables 2. 1 and 2. 2). For example, for DG production by glycerolysis when Novozym 435 is used as a catalyst, the molar ratio of glycerol to oil is ≤ 1 (Kahveci et al. 2010; Miranda et al. 2013; Voll et al. 2011) while this value for MG production is ≥ 2 (Fiametti et al. 2011; Fregolente et al. 2010; Voll et al. 2011; Zhong et al. 2013). The reason can be explained by the stoichiometric molar ratio of glycerol to TG which is 0.5 for the complete conversion of TG to DG (Yamane et al. 1994). Results from Tables 2.1 and 2.2 show Lipase B from *Candida antarctica* is a promising lipase for most of the reaction routes for DG and MG production. In enzymatic alcoholysis with Novozym 435, the alcohol to oil molar ratio for MG production is greater than the same molar ratio needed for DG production.

Table 2. 2 Summary of the literature about lipase-catalyzed reaction processes for production of DGs in the last decade

Study	Reaction type	Reactant	Pressure (bar)	Temp. (°C)	Time (h)	Molar ratio	Enzyme type	Enzyme load	Water addition	Post-reaction	Yield (wt%)
Hares Júnior et al. 2018	Hydrolysis	Triolein	Atm. 1	60	2	W/O = 5	L. RM IM	10% of oil weight	-	-	40%
Voll et al. 2012	Hydrolysis	Palm	Atm. 1	55	72	W/O = 0.2	L. RM IM	3% of oil weight	2%	-	36%
Kahveci et al. 2010	Glycerolysis in binary ionic liquid system	Triolein	Atm. 1	55	24	G/O = 0.9	N. 435	15% of oil weight	-	-	70%
Liu et al. 2012	Glycerolysis	Soybean	Atm. 1	55	12	G/O = 10	Lecitase	5% of oil weight	5%	-	54%
Saberi et al. 2011	Glycerolysis	Palm	Atm.1	65	7	G/O = 1	N. 435	10% of oil weight	5%	short path distillation	90%
Eom et al. 2010	Glycerolysis	Tuna	Atm. 1	40	24	G/O = 3	L. RM IM	10 % of substrates weight	10 %	Short path distillation	70%
(Voll et al. 2011)	Glycerolysis in tert-butanol solvent	Olive	Atm. 1	55	12	G/O = 0.33	N. 435	-	-	-	50%
Miranda et al. 2013	Glycerolysis	Fish	Atm. 1	60	24	G/O = 0.5	N. 435	15% of dry weight basis	-	-	50%
Fregolente et al. 2010	Glycerolysis	Soybean	Atm. 1	70	24	G/O = 8	N. 435	2% of oil weight	3.5% of glycerol weight	Short path distillation	53%
Lubary, Hofland, and Horst 2011	Ethanolysis	Milk fat	SC. 100	42	24	E/O = 0.5	N. 435	2% of fat weight	-	$SCCO_2$ extraction	32%

Temp.: Temperature, Atm.: atmosphere, Sc.: supercritical CO_2 G/O: glycerol/oil, W/O: water/oil, E/O: ethanol/oil
N.435: Novozym 435 (lipase B from *Candida antarctica*, immobilized on macroporous polyacrylate resin beads), RM IM; Lipozyme RM IM (a commercial immobilized 1,3-specific lipase from *Rhizomucor miehei*, immobilized on macroporous anion exchange resin

Using alcoholysis in the production of MG and DG is developing. Recently, studies that have focused on a sustainable way of production have been developed to use the by-products of biofuel production such as glycerol (Remón et al. 2018) and ethanol (He et al. 2016). Although ethanolysis and glycerolysis are helpful for eco-friendly production of MG and DG, a large amount of organic solvent is required to increase the miscibility of reactants (Naik, Naik, and Mohanty 2014) or to improve the yield of the reaction (Zhang et al. 2018) if the reaction is conducted at atmospheric pressure. Therefore, using other technologies to reduce the consumption of solvent, such as conducting the reactions under $SCCO_2$ conditions, can be more eco-friendly compared to conducting the reactions under atmospheric conditions (Nautiyal 2016).

2.7 CONCLUSION

Alcoholysis reactions, especially glycerolysis, are the most common lipid reaction routes used to produce MGs and DGs. Alcoholysis is a sustainable route, and based on choosing a proper molar ratio, provides better yields of DG or MG compared to other routes. The production of MGs and DGs are highly influenced by the reaction conditions and their yield varies based on the various initial reactants and the catalyst types. Enzyme and reaction type alter the yields of the product of interest. Enzymatic reactions are preferred by most of the recent studies because the milder reaction conditions produce less potential side reactions relative to the non-enzymatic reactions.

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CHAPTER 3

MANUSCRIPT 2

HIGH-RESOLUTION NMR SPECTROSCOPY- A VERSATILE TOOL FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF AN EMULSIFIER MIXTURE OF SOYBEAN OIL

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3.1 ABSTRACT

Emulsifiers are multifunctional additives for food and beverages, pharmaceuticals, personal care products, cosmetics and agrochemicals. Interest in these end-use products has increased due to their highly functional properties. Therefore, finding a fast, reliable and reproducible approach for an accurate analysis of these complex lipid mixtures is crucial for industries utilizing them. In the current study, a comprehensive qualitative and quantitative nuclear magnetic resonance (NMR) analysis of a high monoester mixture of soybean oil (HMMS) was conducted using one-dimensional (1D) ^{1}H, ^{13}C and ^{31}P spectroscopy. For ^{31}P-NMR analysis samples were derivatized with a phosphorous reagent. The HMMS was produced by enzymatic alcoholysis of soybean oil and 1.2-propanediol in a supercritical CO_2 system. The NMR method was validated by using model pure compounds and literature data. Quantification of the glycerol region with ^{13}C-NMR analysis indicated that the conversion yields of triglycerides (TGs) to monoesters and diesters of propylene glycol were 43% and 11%, respectively. Compositional distribution analysis, quantified by aliphatic carbons with ^{13}C-NMR, showed that HMMS is composed of more unsaturated fatty acids (UFA) (82%) than saturated fatty acids (SFA) (18%). The ^{31}P-NMR quantification of HMMS demonstrated that the monoglycerides (MGs) are composed of 21% of 2-MG and 4% of 1-MG. Among the three nuclei, ^{31}P-NMR spectroscopy is a practical methodology with high-reproducibility for the precise detection and quantification of partially esterified glycerols and free fatty acids.

3.2 INTRODUCTION

Reducing the caloric content of convenience foods has resulted in a significant growth in the market for food emulsifiers, multifunctional additives that are widely used in food and beverage applications (Pazdur et al. 2015). Emulsifiers are useful for reducing or eliminating partially hydrogenated fats such as confectionary fats (Skeaff 2009). Specifically, low saturated, high *cis*-unsaturated and *trans*-free fat emulsifiers are healthier than those with saturated fatty acids (SFA) or partially hydrogenated fats(Skeaff 2009).

High mono- and di- ester mixture oils such as propylene glycol (1,2-propanediol) esters (PGEs) and a mixture of mono- and di-glycerides (MDGs) have been known as functional emulsifiers and their market is developing in Europe and North America. PGEs and MDGs are both effective non-ionic surfactants and are used as water-in-oil (W/O) emulsifiers (Szeląg and Sadecka 2009) in both the pharmaceutical (St. John's University report, 2017) and food industries (Ahmad et al. 2014; Goff and Hartel 2013; Liu and Shaw 1995). For example, in the dairy industry, only 0.3% of propylene glycol monoester is sufficient to inhibit recrystallization of ice in ice cream (Goff and Hartel 2013). Mixtures of PGEs and MDGs have also been used to make high-quality bakery products (Shaw and Lo 1994). For instance, they confer good rheological behaviour to cake batters so that they produce excellent structure and volume in the final product (Shaw and Lo 1994).

Reports on the nutritional and functional benefits of PGEs and MDGs have strengthened the demand for analytical methods that provide detailed qualification and quantification of esters of glycerol and propylene glycol, tocopherols, plant sterols and fatty acid (FA) composition.

High-resolution NMR was first applied for the analysis of food lipids around 50 years ago (Paduano 2009). Recent advances in instrumentation and data processing techniques have greatly improved the application of this method (Dais, Misiak, and Hatzakis 2015). NMR spectroscopy has been used previously as a useful and easy technique for the analysis of lipid mixtures such as diacylglycerol oil (Hatzakis et al. 2011) and biodiesel (Nagy et al. 2009). Accordingly, analysis of the fats and lipids in complex matrices, such as food, nutraceutical and pharmaceutical products, has largely been achieved with NMR spectroscopy (Dais, Misiak, and Hatzakis 2015; Paduano 2009).

In contrast to gas chromatography-mass spectrometry (GC-MS) methods, NMR requires no calibration with standards nor the construction of calibration curves. For example, for the GC-MS analysis of standard diglycerides (DGs), which are a mixture of 1,2-DG and 1,3-DG, at least 40 runs (4 standards × 5 concentrations × 2 replications) are needed (Zhu et al. 2013). However, the NMR analytical technique can determine several minor components such as DGs, MGs, monoesters of propylene glycol (MPs) and diesters of propylene glycol (DPs) in a single replicated experiment. In contrast to gas chromatography (GC), NMR can distinguish between FAs from different groups such as TGs and MGs (Beyer, Diehl, and Holzgrabe 2010).

NMR is also a rapid technique, due to less need for sample pretreatments such as methyl esterification and fractionation (Merkle et al. 2017), and due to relatively short instrument time. For example, an optimized ^{1}H-NMR methodology has been able to detect the FA types of sunflower, olive, and linseed oils in less than a minute (Castejon et al. 2014). Further advantages of NMR are the small amount of sample (10-100 mg) required and less exposure of the sample to air in the long and thin NMR tubes, which results in lower solvent evaporation (Dais, Misiak, and Hatzakis 2015). Unlike in high-performance liquid chromatography (HPLC), there is no need to measure the response factor (Dais, Misiak, and Hatzakis 2015). In a single experiment, without any sample pre-reaction, the ^{13}C-NMR and ^{1}H-NMR spectroscopies provide extensive information on FA composition directly from the sample, mainly on the different types of the unsaturated fatty acids (UFA) present (Barison et al. 2010). In addition, ^{31}P-NMR has been used as a highly accurate and reproducible method to identify partially esterified glycerols (PEGs), alcohols and the FAs of biodiesel, (Nagy et al. 2009) and free glycerol in olive oil (Hatzakis, Agiomyrgianaki, and Dais 2010).

An enzymatic alcoholysis in a supercritical CO_2 system was used to convert soybean oil into a complex mixture containing a high level of monoesters. The initial soybean oil and the HMMS were analyzed for glycerol esters (GEs) (1-MG, 2-MG, 1,3-DG, 1,2-DG and TG) and PGEs (1-monoester of propylene glycol (1-MP), 2-monoester of propylene glycol (2-MP) and 1,2-diester of propylene glycol (1,2-DP)), tocopherols and FA composition.

Therefore, the main contribution of this study was screening 1D ^{1}H, ^{13}C and ^{31}P spectroscopy techniques to find the most feasible and easy NMR assay for analysis of as many compounds as

possible in a un-purified complex lipid mixture in a single experiment, both quantitatively and qualitatively.

3.3 MATERIALS AND METHODS

3.3.1 MATERIALS

Refined soybean oil was donated by Manitoba Pulse and Soybean Growers (MPSG). 1,2-propanediol, the enzyme Novozym 435 (lipase B, from *Candida antarctica*), phosphorus reagent (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane) (CTDP), 4-Å molecular sieves, chromium (III) acetylacetonate Cr $(acac)_3$, pyridine, cyclohexanol (99%), deuterated chloroform ($CDCl_3$), and 1-mono-, 1,3-di- and triolein standards were purchased from Sigma-Aldrich (Canada). EMD Millipore Steritop™ sterile vacuum bottle-top filters were purchased from Fisher Scientific (Canada).

3.3.2 ALCOHOLYSIS REACTION OF SOYBEAN OIL

The substrates utilized for enzymatic synthesis of HMMS consisted of soybean oil and 1,2-propanediol. The volume ratio of 1,2-propanediol to soybean oil was selected to be 0.75 to obtain the highest conversion of soybean oil (Jackson and King 1997). Enzyme and water (10% and 0.2% of the oil, respectively) were then added to the mixture. The reactor vessel was sealed and pressurized by a gas booster pump at a flow rate of 100 g/min to reach 276 bar after 15 minutes, heated to 70°C, and maintained within ±5°C by a controller. After 4 hours (h), the transesterification was ended by depressurizing the system and removing the enzyme from the reaction materials by vacuum filtration. The upper layer of each sample was collected by pipet and transferred into 15 mL graduated centrifuge tubes. In order to separate excess alcohol, the samples were centrifuged for 15 min at 2900 g (Kristensen, Xu, and Mu 2005). The composition of the oil layer was then analyzed by NMR.

3.3.3 NMR ANALYSIS SAMPLE PREPARATION

20 mg of each oil sample (soybean oil or HMMS) was weighed into a 1.5 ml microcentrifuge tube, to which 500 µl of $CDCl_3$ was added, and after vortexing, it was inserted directly into the 5mm NMR tube for obtaining one- and two-dimensional ^{1}H- and ^{13}C-NMR spectra. For quantitative ^{13}C-NMR analysis, the procedure was repeated with a 100 mg oil sample. For ^{31}P-NMR analysis, 50 ml of a stock solution (STS) consisting of a 1.6:1 volume ratio of pyridine and $CDCl_3$ with 30 mg of $Cr(acac)_3$ relaxation reagent was prepared. To this STS, 67.5 mg of cyclohexanol, the internal quantification standard was then added. The STS was tightly stoppered and protected from moisture with a 4-Å molecular sieve. For each ^{31}P analysis, 100 mg of oil sample was weighed into a 1.5 ml tube to which 0.4 ml of STS and 50 µl of CTDP reagent were added; this was left to allow reaction of the CTDP with free hydroxyl groups of the oil sample. The ^{31}P spectrum was recorded after a reaction time of 15 min. Two replicate analyses for ^{1}H- and ^{13}C-NMR and three for ^{31}P-NMR were performed.

3.3.4 NMR SPECTROSCOPY PROCEDURE

All the NMR analysis were conducted at 25°C on a Bruker Avance III 500 spectrometer (Bruker Corporation, Billerica, MA, USA) and the probe temperature was controlled at 25°C at all times. The temperature calibration also was checked by standard methods. All the spectra were processed by the control software Topspin (Bruker Biospin, Germany) and evaluation software SpinWorks 4.2.0 (Copyright © 2015, Kirk Marat, University of Manitoba).

3.3.4.1 ^{1}H-NMR

One dimensional proton-NMR spectra were obtained with 32 scans, in the time domain and processed sizes of 64k points, and a spectral width of 20 ppm. Chemical shifts were reported in

ppm from tetramethylsilane (TMS) using the residual $CDCl_3$ peak ($\delta = 7.26$) as a secondary reference.

3.3.4.2 ^{13}C-NMR

One dimensional ^{13}C-NMR spectra were recorded with proton decoupling. The ^{13}C spectra were recorded with 400 scans, a time domain size of 64k points, a processed size of 128k points, and a spectral width of 237 ppm. Chemical shifts were reported in ppm from TMS using the $CDCl_3$ peak at 77.0 ppm for dilute samples and at 77.07 ppm for concentrated samples as secondary references.

3.3.4.3 QUANTITATIVE ^{13}C-NMR

For quantitative ^{13}C-NMR, Nuclear Overhauser Effect (NOE) was suppressed by using inverse-gated decoupling, and full relaxation was ensured with a 10 sec relaxation delay and a 30^{o} C flip angle. At least 64 scans were recorded, while other parameters were identical to those of the conventional ^{13}C spectra.

3.3.4.4 ^{31}P-NMR

Proton-decoupled ^{31}P spectra were recorded without NOE, with a 10 sec relaxation delay, and a spectral width of 83 ppm. For each spectrum, 32 scans were recorded with 32k acquisition data points that were zero-filled to 64k points.^{31}P chemical shifts were reported relative to the hydrolysis product of CTDP at 131.18 ppm as a secondary reference (Hatzakis et al. 2011).

3.4 RESULTS AND DISCUSSION

NMR analysis relies on correct chemical shift assignment, and so this has a key role in identification and quantification of oil samples. In this research, ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra of standard linoleic acid, monoolein, diolein, and triolein were used as model pure compounds. More detailed tables can be found in the Appendix (Tables A. 1. 1 and A. 1. 2) and literature data (Hatzakis et al. 2011; Hasenhuettl and Hartel 2008; Jin et al. 2007; Vlahov, Giuliani, and Del Re 2010). These data were used as the basis for analysis of the high monoester mixture of soybean oil (HMMS). Since the ^{31}P-NMR analysis relies on derivatization of the free hydroxyl groups of the HMMS with the CTDP reagent, compounds with no free hydroxyl groups, such as triglycerides (TGs), cannot be seen.

3.4.1 IDENTIFICATION OF THE SOYBEAN OIL AND HMMS COMPONENTS WITH ^{1}H-NMR

Figure 3. 1 shows the expanded ^{1}H-NMR of the glyceridic protons of soybean oil and HMMS. Compared to the spectra of soybean oil, a signal is observed at 4.10 ppm, clearly showing the production of 1-MG in HMMS. ^{1}H-NMR analysis was used to identify the composition of HMMS including its GEs and PGEs, their positional isomers, FA composition and plant sterols.

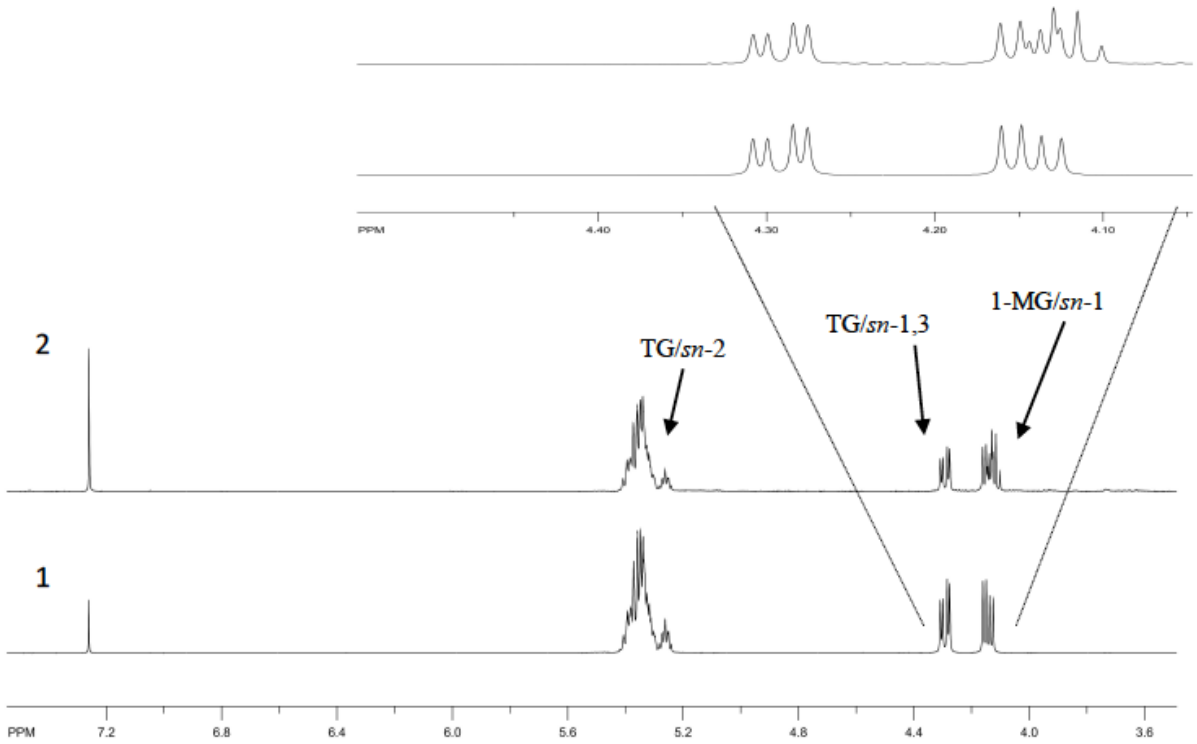

Figure 3. 1 ^{1}H-NMR spectra of glyceridic protons of a high monoester mixture of soybean oil (HMMS). (1) Soybean oil (time = 0h of enzymatic alcoholysis), (2) HMMS (time = 4 h of enzymatic alcoholysis). Two spectrograms are referenced to the chloroform peak at 7.26 ppm

Table A. 1. 5 in Appendix summarizes the proton NMR chemical shifts of HMMS after 4 h of enzymatic alcoholysis. Assignment of the signals was based on data reported for model pure compounds and previous literature (Andreis, Meić, and Veksli 1983; Castejon et al. 2014; Dais, Misiak, and Hatzakis 2015; Jin et al. 2007). Because of peak overlap between the linoleic and linolenic acid chains, or between the acyl chains of MG and the same chains in 1,3-DG, the ^{1}H NMR data were used only for identification of the HMMS components, not for their quantification. The chemical shifts of HMMS are consistent with the data of model pure compounds and previous literature.

3.4.2 ANALYSIS OF SOYBEAN OIL AND HMMS WITH ^{13}C-NMR

To quantify the mono- and di-esters, along with the positional distribution of the FAs, ^{13}C-NMR analysis of the HMMS was carried out. The greater spectral dispersion of the carbon spectra relative to that of the proton spectra allowed for better characterization of both the TGs and the mono- and di-esters. Tables A. 1. 2 and A. 1. 3 in the appendix show the ^{13}C-NMR chemical shifts of model pure compounds, soybean oil and HMMS, respectively. All the chemical shifts are specific for HMMS and their repeatability was checked for at least three replications. There are four distinct regions in the ^{13}C-NMR spectrum of acyl glycerides: *i*) carbonyl, *ii*) olefinic, *iii*) glycerol, and *iv*) aliphatic carbons (Brescia and Sacco 2006).

3.4.2.1 CARBONYL CARBONS

The carbonyl carbons of soybean oil and HMMS lie between 172.8-173.3 ppm. The group of signals at 172.8 ppm is related to carbonyl signals of low frequency sets (*sn*-2), while the group at 173.3 ppm is related to the high (*sn*-1,3) frequency sets (Hatzakis et al. 2011). These data provide information on FA composition of HMMS and the different positions of the esterified chains.

The ^{13}C-NMR spectrum of the carbonyl carbons of different oils was employed to identify the acyl positional distribution (*sn*-1,3 *vs sn*-2) of the SFA and UFA groups (Gouk et al. 2011), and also to provide information on the length and unsaturation pattern of the FAs (Vlahov, Giuliani, and Del Re 2010).

Table 3.1 compares the chemical shifts of soybean oil with those of HMMS. The chains esterified at the *sn*-2 position are seen to be 0.4 ppm lower in chemical shift compared to those esterified at the *sn*-1,3 positions, in both soybean and HMMS oils, a finding that is in agreement with other studies conducted on DAG oil and olive oil (Hatzakis et al. 2011; Vlahov, Giuliani, and Del Re 2010). In each position, the highest shift belongs to SFA while the lower frequency is related to the LO and LN chains (Table 3.1). LO and LN chains usually overlap (Hatzakis et al. 2011). The order of the frequencies of the chains (from high to low) is SFA > OL > LO in TGs. Therefore, this order confirms that the signal moves to a lower chemical shift as a result of the higher number of double bonds in the acyl chains (Vlahov, Giuliani, and Del Re 2010). The TG of HMMS at the *sn*-1,3 positions, comprises three resonances at 173.20, 173.21 and 173.24 ppm, attributed to LO and LN, OL and SFA chains, respectively. The majority of the soybean oil and HMMS at the *sn*-2 position is comprised of LO and LN chains at 22% and 24.5%, respectively.

Table 3. 1 ^{13}C-NMR chemical shifts and fatty acid composition of the carbonyl group of soybean oil and HMMS

Acylglycerol	**Carbon position of C1**	**Fatty acid**	**Soybean oil 0 h**		**HMMS 4 h**	
			Chemical shift (ppm)	Fatty acid composition (%)	Chemical shift (ppm)	Fatty acid composition (%)
TG	*sn*-2	LO+LN	172.79	22.0±0.9	172.79	24.5±1.1
		OL	172.82	10.0±2.3	172.80	8.2±0.4
		SFA	-	0	172.87	1.2±0.1
	sn-1,3	LO+LN	173.19	37.6±3.3	173.20	36.1±0.5
		OL	173.20	26.3±1.7	173.21	15.9±0.5
		SFA	173.23	4.2±0.8	173.24	14.2±1.0

Although there are some changes in the percentage of each FA after enzymatic alcoholysis, the distribution of SFA and UFA between *sn*-2 and *sn*-1,3 positions were unchanged (Table 3. 1). The UFA, which have the highest concentration amongst the FAs in soybean oil and in the TGs of HMMS, were distributed equally at the *sn*-2 and *sn*-1,3 positions. However, SFA prefer *sn*-1,3 positions to the *sn*-2 position. This is in agreement with the study by Vlahov (2009) where it was concluded that the chain concentration and positional specificity determine the FA distribution in acylglycerols.

3.4.2.2 OLEFINIC CARBONS

The ^{13}C-NMR spectrum of the olefinic carbons (127.1 to 131.9 ppm) was used to determine the acyl distribution and acyl positional distribution of the UFA groups in the HMMS. Although the total amount of LN and OL chains increases slightly after the enzymatic reaction, the LO chain decreases dramatically (Table 3. 2). The olefinic region provides better quantification of UFA composition compared to the carbonyl region, owing to less peak overlap in the LO and LN chains (Wollenberg 1990).

Table 3. 2 ^{13}C-NMR chemical shifts and fatty acid composition of the olefinic group of soybean oil and HMMS

Component Name	Carbon	Soybean oil 0 h		HMMS 4 h	
		Chemical shift (ppm)	Fatty acid composition (%)	Chemical shift (ppm)	Fatty acid composition (%)
LN	C-15	127.14	3.0±0.1	127.11	2.8±0.1
LN	C-10	127.77	3.6±0.1	127.75	3.1±0.3
LO	C-12	127.92	17.5±0.7	127.90	18.0±0.4
LO	C-10	128.09	17.7±0.3	128.06	17.0±0.8
LN	C-13	128.24	3.2±0.2	128.22	2.5±0.2
LN	C-12	128.29	2.9±0.4	128.26	2.4±0.3
OL	C-9	129.71	7.4±0.5	129.68	5.8±0.2
LO	C-9	129.97	1.8±0.3	129.96	7.4±0.3
OL	C-10	129.99	24.5±0.3	129.98	19.0±2.4
LO	C-13	130.20	15.7±1.2	130.17	9.8±0.2
LN	C-9	130.23	5.2±1.0	130.18	9.9±0.2
LN	C-16	131.93	2.5±0.1	131.90	2.5±0.1
	Total LN		20.4±1.2		23.2±0.5
	Total LO		52.7±0.6		51.8±1.5
	Total OL		32.0±0.7		25.0±1.9

3.4.2.3 GLYCEROL CARBONS

The glycerol region of the ^{13}C spectrum (62.1 to 71.8 ppm) provides information on the conversion yield of the TGs to monoesters. Figure 3. 2 shows that the glycerol region also provides information on the position of the glycerol α (*sn*-1) and β carbons (*sn*-2) of propylene glycol mono- and diesters (Jin et al. 2007; Ng 2000).

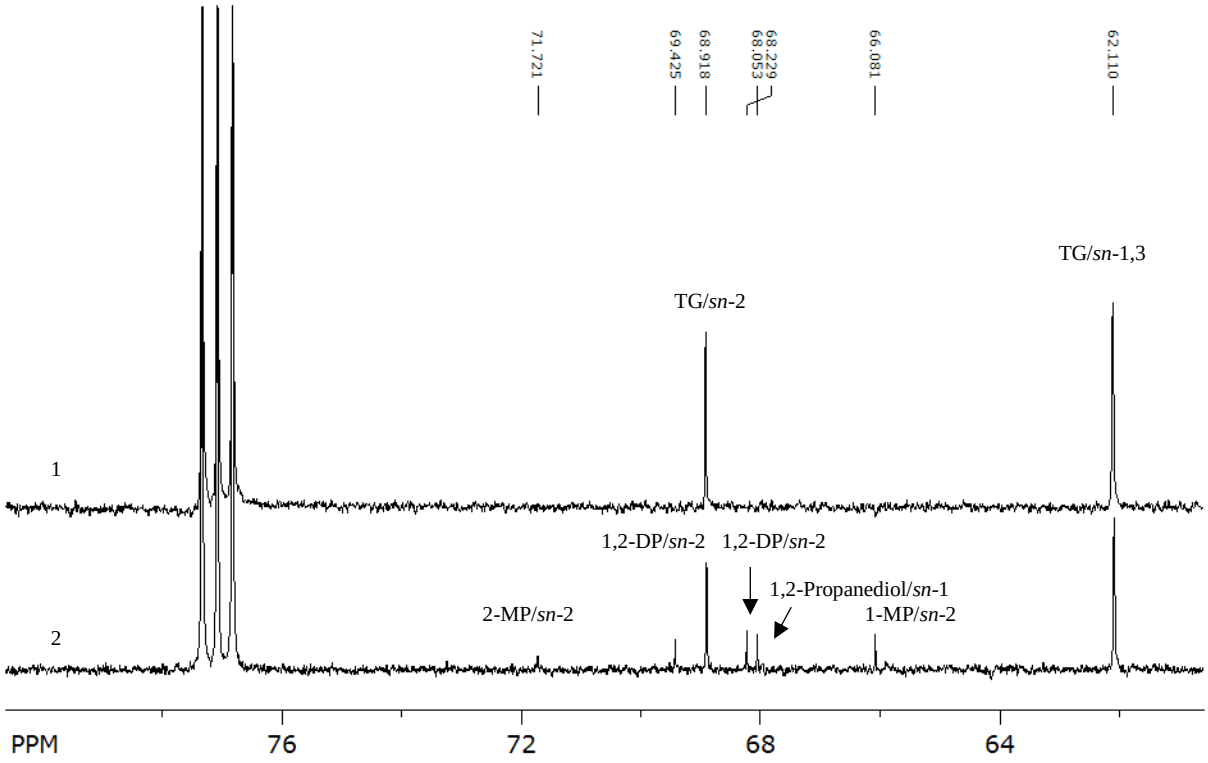

Figure 3. 2 ^{13}C-NMR spectra of glycerol carbons of a high monoester mixture of soybean oil (HMMS). (1) Soybean oil (time = 0h of enzymatic alcoholysis), (2) HMMS (time = 4 h of enzymatic alcoholysis). Two spectrograms are referenced to chloroform peak at 77.07 ppm

The proportion of each PGE in HMMS was determined from the total integrated intensity relative to the integrated intensity of the *sn*-2 peak of the TG at 68.9 ppm. The 1-MP, 2-MP, and

1,2-DP components were quantified from the *sn*-2 peak integrations at 66.1, 71.7, and 68.0 ppm, respectively. The conversion yield (CY) of TGs to monoesters was calculated from ^{13}C-NMR peak integrations using Eq. (3. 1). The % proportion of each type of component, P_{MPs} and $P_{1,2\text{-}DPs}$ was calculated from the division of the integration amount of each 1-MP, 2-MP and 1,2-DP, peak, respectively, by the sum of all peaks multiplied by one hundred:

$$CY(\%) = \left[\frac{(P_{Mps}/AM_{Mps})}{(P_{TGs}/M_{Soy})+(P_{Mps}/AM_{Mps})+(P_{Dps}/AM_{Dps})}\right] \times 100 \qquad (3.\ 1)$$

Where M_{soy} represents the average molecular weight (AM) of the soybean oil. AM_{MPs} and $AM_{1,2\text{-}DPs}$ denote the average molecular weight of MPs and 1,2-DPs, respectively (equation 3. 1).
From the NMR analysis of the enzymatic alcoholysis, the CY of TG to monoesters (1-MP and 2-MP) was 43±2.8%, and to di-esters (1, 2-DPs) was 11± 4.5%.

3.4.2.4 ALIPHATIC CARBONS

The ^{13}C-NMR spectrum of the aliphatic carbons starts at 14.05 ppm for C-18 of the OL chains and extends to 34.23 ppm for C-2 of the OL chains. From this wide range, the region of 31.51-31.91 ppm for C-16 was selected for confirmation of the FA composition of all glyceride species that were measured *via* the olefinic carbons. In Table 3. 3, the information corresponding to C-16 indicates that the major part (60%) of the FAs of all GEs and PGEs of HMMS is composed of LO chains.

Table 3. 3 ^{13}C-NMR chemical shifts and fatty acid composition of C-16 of all TGs and mono- and diesters of soybean oil and HMMS after a 4-hour enzymatic alcoholysis

Component name	Carbon of all acylglycerols	Soybean oil 0 h		HMMS 4 h	
		Chemical shift (ppm)	Fatty acid composition (%)	Chemical shift (ppm)	Fatty acid composition (%)
LO	*sn*-1,3/*sn*-2/C16	31.55	53.8±0.3	31.53	59.7±1.1
OL	*sn*-1,3/*sn*-2/C16	31.94	26.3±1.1	31.91	21.9±0.8
SFA	*sn*-1,3/*sn*-2/C16	31.96	19.9±1.2	31.93	18.4±0.9

The ^{13}C-NMR spectrum of carbonyl carbons provides information about chain positional and compositional distribution of the SFA and UFA esterifying at the *sn*-1,3 and *sn*-2 positions of glycerol and propylene glycol esters of soybean oil and HMMS. Considering both the *sn*-1,3 and the *sn*-2 positions of the TGs of HMMS, 25% of the *sn*-2 position is occupied by LO and LN chains, while 1% of the *sn*-2 position is composed of SFA. These numbers change to 36% and 16%, respectively, at the *sn*-1,3 positions.

3.4.3 QUALIFICATION AND QUANTIFICATION OF PEGS, FREE FA AND TOCOPHEROLS OF SOYBEAN OIL AND HMMS WITH ^{31}P-NMR

Derivatization with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane followed by ^{31}P-NMR provided a practical, rapid, and precise technique (Hatzakis et al. 2011) for both identifying and quantifying each component in the lipid mixtures. ^{31}P-NMR provides sharp signals in pyridine/$CDCl_3$ at specific chemical shifts without overlap, making the peak integration and data processing easy. The location of residual acylglycerols, FFA and tocopherol are well established, starting with the elegant work of Spyros and Dias for analysis of olive oil (Spyros and Dais

2000) and continued by (Hatzakis et al. 2011) for DAG oil. Integration of tocopherols at 144.9 ppm and the residual acylglycerols from 146.4-148.2 ppm, in combination with the internal standard cyclohexanol at 145.2 ppm, made for an easy and absolute quantification of each component in soybean oil and HMMS. Table 3. 4 summarizes the signal assignments and absolute weight percentage for each component of HMMS obtained by the enzymatic alcoholysis reaction. For example, 2-MG appears at 148.00 ppm and has a weight percentage of 21%, while the total amount of MGs and DGs was 45%.

Table 3. 4 ^{31}P-NMR chemical shifts and weight percentage of each component of soybean oil and HMMS after a 4 h enzymatic alcoholysis

Component Name	**Chemical shift (PPM)**	**Soybean oil (%) 0 h**	**HMMS (%) 4 h**
Free FA	134.8	-	3.08±0.3
Tocopherol	144.9	0.9±0.1	0.9±0.0
1-MG	146.4	0.1±0.0	4.2±0.3
1,3-DG	146.7	1.3±0.0	4.1±0.3
2-MG	148.0	-	20.7±2.9
1,2-DG	148.2	0.7±0.0	16.2±0.5
Total DG		2.0±0.1	20.6±0.4
Total MG		0.2±0.0	24.7±3.0
Total MG+DG		2.1±0.1	45.3±3.4
Ratio of MG/DG		0.1±0.1	1.2±0.1

3.4.4 THE COMPARISON OF ANALYTICAL CAPACITY OF ^{1}H-, ^{13}C- AND ^{31}P-NMR FOR DTECTION OF ACYLGLYCEROLS

Table 3. 5 shows that among all three nuclei, only ^{31}P-NMR analysis of HMMS detects the 2-MG component. Furthermore, ^{31}P-NMR provides evidence about the existence of PEGs in soybean oil (Table 3. 4).

Table 3. 5 The discrimination capacity of each nucleus for analysis of a complex mixture of acylglycerols with 1D-NMR

	Component Name	^{1}H-NMR	^{13}C-NMR	^{31}P-NMR
Soybean oil	1-MG/*sn*-1,3	×	×	✓
	1-MG/*sn*-2	×	×	✓
	1,3-DG	×	×	✓
	1,2-DG	×	×	✓
	TG/*sn*-2	✓	✓	×
	TG/*sn*-1,3	✓	✓	×
HMMS	1-MG/*sn*-1,3	✓	×	✓
	1-MG/*sn*-2	✓	×	✓
	2-MG	×	×	✓
	1,3-DG	✓	×	✓
	1,2-DG	✓	×	✓
	TG/*sn*-2	✓	✓	×
	TG/*sn*-1,3	✓	✓	×

An efficient analytical methodology of multinuclear NMR spectroscopy provides a powerful platform to analyze complex emulsifier mixtures such as HMMS quantitatively and qualitatively while only using a tiny amount of solvent. The same sample in a small amount of $CDCl_3$ can be

used for both ^{1}H-NMR and ^{13}C-NMR. ^{1}H-NMR is quick, simple and informative, but for a complex oil such as HMMS, it does not provide enough spectral dispersion for accurate quantification. ^{13}C-NMR, on the other hand, provides better dispersion, but suffers from lower sensitivity, and care is required to ensure complete relaxation and suppression of the NOE. The FA composition, GEs and PGEs of HMMS are determined by ^{1}H-NMR and ^{13}C-NMR spectroscopy.

The ^{31}P-NMR spectroscopy is able to detect and determine the absolute amounts of 1-MG, 2-MG, 1,2-DG 1,3-DG, but is unable to quantify TG. However, ^{13}C-NMR is able to provide this measurement. Esterification of oil with alcohol to synthesize a high monoester mixture produces both 1-MGs and 2-MGs. Most of the current studies, using GC (Simsek, Degirmenci, and Murtezaoglu 2016) and HPLC (Remonatto et al. 2018) as analysis techniques, consider only 1-MGs, and they are taken to be representative for all MGs content while MGs include both 1-MGs and 2-MGs. As a result, the high level of 2-MGs in the mixture after an esterification reaction is ignored. ^{31}P-NMR spectroscopy, with more sensitivity than ^{13}C-NMR, demonstrates a significant signal for 2-MG, which was not detectable with carbon NMR.

3.5 CONCLUSION

This research contributed to development of ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR methodologies to facilitate the analysis of HMMS which is a mixture of emulsifiers such as monoglycerides, diglycerides, propylene glycol monoesters and propylene glycol diesters. In addition, the results verified that ^{31}P-NMR spectroscopy is a practical methodology with high reproducibility for the precise detection and quantification of PEGs and other minor compounds such as tocopherol and free FAs in a lipid mixture that are obtained directly after enzymatic reaction without any purification.

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CHAPTER 4

MANUSCRIPT 3

TIME COURSE STUDY OF INTERESTERIFICATION OF SOYBEAN (*GLYCINE MAX*) OIL WITH PROPYLENE GLYCOL IN SUPERCRITICAL CO_2 AND ANALYSIS BY NMR SPECTROSCOPY

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4.1 ABSTRACT

The time course study of high monoester mixtures from soybean oil (HMMS) synthesis, as healthier alternatives to *trans* food products, in a supercritical CO_2 ($SCCO_2$) medium with and without enzyme was investigated. Phosphorous nuclear magnetic resonance (^{31}P-NMR) was used to quantify the absolute amount of partially esterified acylglycerols (PEGs). Carbon NMR was utilized to determine type and position of the fatty acids (FAs) of HMMS. Enzyme and time significantly influenced the synthesis of 1-monoglycerides (1-MGs), 2-MGs and 1,2-diglycerides (1,2-DGs) in this alcoholysis of soybean oil with 1,2-propanediol, based on high catalytic activity and operational stability of Novozym 435 in $SCCO_2$ during a short reaction time. Results suggest that 4 h is a suitable reaction time for this lipase-catalyzed interesterification (LIE) system for the synthesis of 2-MGs with a yield of 20%. The highest polyunsaturated fatty acid (PUFA) (65%) in the triglyceride (TG) of HMMS was produced after 4 h of reaction. After 6 h of reaction, a high level (20%) of saturated fatty acids (SFAs) was found in the TGs of HMMS, which were distributed between the *sn*-2 (5%) and *sn*-1, 3 (15%) positions.

Keywords: monoglycerides, diglycerides, fatty acid distribution, lipase-catalyzed interesterification, supercritical CO_2, Novozym 435, NMR, soybean (*Glycine max*) oil

4.2 INTRODUCTION

The type of dietary fatty acids (FAs) and the serum concentration of triglycerides (TGs) are linked with noncommunicable diseases such as cardiovascular disease (CVD) (Wang and Hu 2017) and cancer (VHM & PP Study Group et al. 2009). For example, *trans* fatty acids are strongly correlated with CVD, and replacing saturated fatty acids (SFAs) with polyunsaturated fatty acids (PUFAs) such as α-linoleic, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) helps lower blood triglyceride levels (Handelsman and Shapiro 2017) and reduces the CVD risk significantly (Wang and Hu 2017).

Structured lipids (SLs) are synthetic novel fats which are produced through interesterification reactions of natural oils and fats. SLs can be a structured TG, a monoester and a diester, or a mixture of all of them. Interesterification changes the type and the positions of FAs in TGs and partially esterified glycerols (PEGs), resulting in an alteration in physical and nutraceutical properties of TGs and PEGs after reaction (Kadhum and Shamma 2017). SLs are attracting attention recently due to both healthy and functional benefits. The position and the type of FAs within the acylglycerol molecules of the SLs have important roles in both the health and the functional properties of the SLs.

From the nutritional point of view, SLs can help in reducing the amount of undesired FAs such as *trans* and saturated FA obtained from food products. Mono- and diglycerides are interesting products possessing various potential nutraceutical properties simultaneously, including high bioactivity and hypocaloric effects (Martin et al. 2014). Antioxidant, antidiabetic, and antiatherogenic properties of monoglycerides (MGs) containing oleic acid have also been identified (Feltes et al. 2013; Martin et al. 2014).

Additionally, SLs with a specific FA at a particular position are products of interest for many pharmaceutical companies, and the products are targeted at infants and people with specific diseases or disorders. It is essential to know the position and type of FAs in TGs because their hydrolyzation occurs regiospecifically in the mouth, stomach and intestinal mucosa (Pfeffer et al. 2007). For instance, the pancreatic lipase cleaves FAs at the *sn*-1,3 positions and leads to formation of 2-MGs in the intestine. TGs can be absorbed in greater amounts when there is a PUFA at the *sn*-2 position compared with TGs containing PUFA at the *sn*-1,3 positions (Zhang et al. 2018).

From the functional point of view, synthesizing SLs containing a higher amount of mono- or diester, or a mixture of them, will reduce *trans* fat consumption (List 2014). For example, partially esterified glycerols and liquid unsaturated oil can be used instead of partially hydrogenated fats to produce low *trans*/saturated baking fats (Klonoff 2007; Orthoefer 2008). Doucet (2005) has produced a shortening system that contains an emulsifier composition consisting of three various type of emulsifiers, including MGs and DGs, an emulsifier such as mono- and diesters of propylene glycol, and very little of an ionic emulsifier (e.g., lecithin). Therefore, partially hydrogenated fats, that may have 15-35% *trans* isomers of fatty acids, can be

replaced by a shortening system which is produced by blending of this emulsifier composition with an unsaturated oil such as soybean oil (Doucet 2005).

Mono- and diglycerides, approved as generally recognized as safe (GRAS) ingredients by the Food and Drugs Administration-USA (FDA) (Feltes et al. 2013), are effective non-ionic surfactants and are used as water-in-oil (W/O) emulsifiers (Feltes et al. 2013; Szeląg and Sadecka 2009) in both the pharmaceutical (Damstrup et al. 2006; Martin et al. 2014; Pawongrat, Xu, and H-Kittikun 2007) and food industries (Lee et al. 2018; Remonatto et al. 2015; Zhang et al. 2017). MGs, as amphiphilic molecules, can form different types of colloidal structures, such as micelles, which are thermodynamically stable in aqueous solutions at a reasonable concentration (Damstrup 2008). MGs can have other roles in different industries. For instance, MGs are emulsifiers and stabilizers in spreads or ice creams and clarifiers in beverages (Feltes et al. 2013).

The type of FA has an important role in the functionality of the SLs. Some food companies prefer emulsifiers with higher levels of unsaturated fatty acids (UFAs) because of their better health benefits and emulsibility (Li et al. 2017). Highly unsaturated emulsifiers provide softer texture and better emulsifying performance (Zhang et al. 2017) and can improve the functionalization of fat products (Acevedo, Block, and Marangoni 2012). Saturated diglycerides are effective emulsifiers for use in some baking products (Skogerson and Boutté 2010). Utilizing 1,3-DGs and 1-MGs with specific FA provides double benefits to the food industry due to their emulsifying and bioactivity properties (Doucet 2005; Martin et al. 2014).

The disadvantages of conventional chemical interesterification (CIE) approaches include the fact that they are both energy- and solvent- intensive and require the addition and removal of solvents. Further drawbacks of CIE include a low yield of the interesterification products (Fureby, Adlercreutz, and Mattiasson 1996), especially in the production of PEGs (Pfeffer et al. 2007), high oil loss (Mensink et al. 2016), high post-purification costs (Fiametti et al. 2011; Pfeffer et al. 2007), lack of FA selectivity (Willis, Lencki, and Marangoni 1998), low oxidative stability (Mensink et al. 2016) and the destruction of highly unsaturated FAs in the finished product (Solaesa et al. 2016). CIE methods also suffer from unwanted side products (Jackson 1998), and produce final products with a dark colour (Jackson and King 1997) and burnt flavour, due to the use of catalysts such as NaOH, KOH, $Ca(OH)_2$ at high temperatures (Liu and Shaw 1995; Temelli, King, and List 1996; Solaesa et al. 2016). Toxic chemicals such as these sodium alkylates (Akoh 2017) should be used less in food products (Zhang et al. 2018).

An alternative to the CIE method for producing a high monoester mixture is lipase-catalyzed interesterification (LIE) involving the use of enzymes, alcohol, and oils (Shaw and Lo 1994; Fureby, Adlercreutz, and Mattiasson 1996). LIE is a promising method for obtaining SLs with very particular compositions that have improved nutritional and medicinal applications (Willis, Lencki, and Marangoni 1998). In addition to the advantages of LIE from an environmental point of view, due to using a biodegradable protein and not having to dispose of toxic chemicals like in CIE reactions (Ibrahim and Xu 2008), the post-reaction cleaning is a simple separation (Srivastava, Madras, and Modak 2003). Enzyme separation facilitates the recovery of products (Zhang et al. 2018), decreases solvent residues, and reduces the cost of treating the residue (Feltes et al. 2013). Low-temperature LIE under pressure reduces undesired polymerization (Remonatto et al. 2015),

inhibits oxidation, and prevents toxic substance formation, outcomes that very important when the initial reactant is an oil containing a high level of polyunsaturated fatty acids (PUFAs) (Feltes et al. 2013). PUFAs are more susceptible to being oxidized at higher temperatures compared to monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) (Vieira, McClements, and Decker 2015).

Various lipases have different catalytic activities (Zhang et al. 2018) and enantioselectivity (Feltes et al. 2013). Lipases have different position-, stereo- and FA- specificity or they can be non-specific (Remonatto et al. 2015; Feltes et al. 2013; Willis, Lencki, and Marangoni 1998). Immobilized lipase from *Candida antarctica* (Novozym 435), also known as lipase B, is efficient in the production of a high content of MGs (Solaesa et al. 2016), especially 2-MGs (Zhang et al. 2018). Novozym 435 has been usually considered as a non-specific enzyme (Zhang et al. 2018). However, the solvent medium can affect lipase activity, specificity, and positional selectivity (Zhang et al. 2018) and can turn Novozym 435 into a 1,3-specific lipase (Pfeffer et al. 2007). The reason for specificity alteration of Novozym 435 by the solvent may be due to the low flexibility of the tertiary structure of the enzyme in the presence of excess solvent. Excess polar solvent leads to a delay in access of the catalytic binding pocket to the acyl group at the *sn*-2 position of the TG (Pfeffer et al. 2007) because the tertiary structure of the enzyme is fixed by polar solvents (Irimescu, Iwasaki, and Hou 2002). Novozym 435 has a high enantio-selectivity towards secondary alcohols because this enzyme prefers the R-enantiomer of secondary alcohols (Pfeffer et al. 2007). This preference is due to the tertiary structure of the protein and extremely complex stereo-interactions (Anderson, Larsson, and Kirk 1998). Therefore Novozym 435's high enantio-selectivity against secondary alcohols (Pfeffer et al. 2007), specifically high relative activity

toward 1,2-propanediol (90.6%) (King 2004), has been explained by its protein structure (Anderson, Larsson, and Kirk 1998). In addition, the tolerance of Novozym 435 to high pressures in $SCCO_2$ media has been noted (Tai and Brunner 2011; Jackson 1998; King 2005). Lipases with different positional and FA specificity can alter some nutritional properties of the final lipid products after LIE relative to CIE or non-enzymatic interesterification (Willis, Lencki, and Marangoni 1998).

Few studies have reported the differences of interesterification under the mild conditions of a $SCCO_2$ medium with and without enzyme to produce high monoester mixtures from soybean oil (HMMS). In this study, the effect of enzyme and reaction times on FA type, FA position and the profile of partially esterified glycerols (PEGs) in HMMS including 1-monoglycerides (1-MGs), 2-monoglycerides (2-MGs), 1,3-diglycerides (1,3-DGs), and 1,2-diglycerides (1,2-DGs) will be analyzed quantitatively and qualitatively. This knowledge would create a good foundation for the production of functional and nutraceutical SLs which are synthesized under $SCCO_2$ pressure and low temperature without any purification step. Furthermore, NMR analysis of the products will result in better understanding of how levels of production of PEGs and the positional distribution of FA are influenced by the activity of the enzyme and reaction times during the interesterification of soybean oil. Thus, the objective of this study was to explore the effects of the presence or absence of the enzyme and reaction times on the synthesis of HMMS with subsequent analysis of the PEGs in HMMS qualitatively and quantitatively using ^{31}P-NMR and assessing the distribution and type of FAs in the HMMS using ^{13}C-NMR spectroscopy.

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

Refined soybean oil was donated by the Manitoba Pulse and Soybean Growers (MPSG). 1,2-propanediol and Novozym 435 (lipase B, from *Candida antarctica*) were purchased from Sigma-Aldrich (Canada). EMD Millipore Steritop™ sterile vacuum bottle-top filters were purchased from Fisher Scientific (Canada). Phosphorus reagent (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane) (CTDP), 4-Å molecular sieves, chromium (III) acetylacetonate (Cr $(acac)_3$), pyridine, cyclohexanol (99%) and deuterated chloroform ($CDCl_3$) were purchased from Sigma-Aldrich (Canada).

4.3.2 INTERESTERIFICATION OF SOYBEAN OIL

Both enzymatic and non-enzymatic reactions were conducted under a $SCCO_2$ pressure of 276 bar using a 1,2-propanediol/oil mixture at a molar ratio of 10:1. For the enzymatic reaction, Novozym 435, an immobilized lipase preparation on acrylic resin, and water (10% and 1% of the oil weight, respectively) were then added to the mixture. The reactor vessel was sealed and pressurized by a gas booster pump at a flow rate of 100 g/min to reach 276 bar after 15 min, heated to 70°C, and maintained within ±5°C by a controller. LIE was ended after 2, 4, or 6 hours by depressurizing the system and removing the enzyme from the reaction materials by vacuum filtration. For the non-enzymatic reaction, the reactor vessel containing the 1,2-propanediol/oil mixture was sealed and pressurized by a gas booster pump at a flow rate of 100 g/min to reach 276 bar after 15 min, heated to 70°C, and maintained within ±5°C by a controller. Depressurizing occurred after 2, 4, or 6 hours to end the non-enzymatic reaction. The upper layer of each sample from both enzymatic and non-enzymatic reactions was collected by pipet and transferred into 15-mL centrifuge tubes. To separate the excess alcohol, samples were

centrifuged for 15 min at 2900 g. NMR was used to analyze the oil separated from the excess alcohol. Fig. 4. 1 shows the reaction products of enzymatic alcoholysis of soybean oil and 1,2-propanediol.

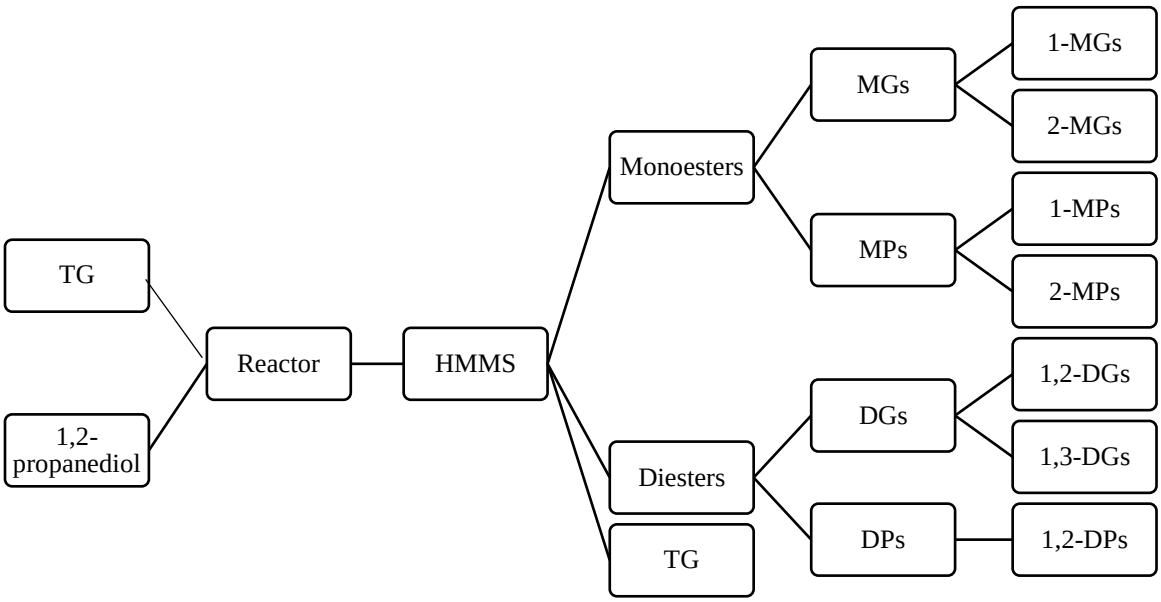


Figure 4. 1 The products of enzymatic alcoholysis of TG (soybean oil). 1-MGs (1-monoglycerides), 2-MGs (2-monoglycerides), 1-MPs (1-monoester of propylene glycol), 2-MPs (2-monoester of propylene glycol), 1,2-DGs (1,2-diglycerides), 1,3-DGs (1,3-diglycerides), 1,2-DPs (1,2-diesters of propylene glycol)

4.3.3 NMR EXPERIMENTS

A Bruker Avance III 500 spectrometer (Bruker Corporation, Billerica, MA, USA) was used to conduct the NMR analysis. The probe temperature was controlled at 25°C at all times. Temperature calibration was checked by standard methods.

50 ml of a stock solution consisting of a 1.6:1 volume ratio of pyridine and $CDCl_3$ with 30 mg of Cr $(acac)_3$ relaxation reagent was prepared. To this stock solution, 67.5 mg of cyclohexanol, the internal quantification standard was then added. The container of stock solution was tightly stoppered and protected from moisture with a 4-Å molecular sieve. For each ^{31}P analysis, 100 mg of oil sample (soybean oil or HMMS) was weighed into a 1.5 ml tube to which 0.4 ml of stock solution and 50 µl of CTDP reagent were added; this was left to allow reaction of the CTDP with free hydroxyl groups of the oil sample. The ^{31}P spectrum was recorded after a reaction time of 15 min.

For ^{13}C-NMR analysis, 20 mg of each sample was weighed into microcentrifuge tubes, to which 500 µl of $CDCl_3$ was added, and after vortexing, it was inserted directly into the 5mm NMR tube for obtaining the ^{13}C-NMR spectra. Two replicate analyses for ^{13}C-NMR and three for ^{31}P-NMR were performed.

4.3.4 STATISTICAL ANALYSIS

All the data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). The goal was to study significant differences between components of HMMS at $P < 0.05$ during reactions of both enzymatic and non-enzymatic interesterification. The enzymatic and non-enzymatic experiments were performed in triplicate and duplicate, respectively. The data presented are average values with the standard error of the mean.

4.4 RESULTS AND DISCUSSION

4.4.1 ^{31}P-NMR ANALYSIS TO QUANTIFY THE PARTIALLY ESTERIFIED GLYCEROLS OF HMMS

^{31}P-NMR provides sharp signals in pyridine/$CDCl_3$ at specific chemical shifts without overlaps, making the peak integration and data processing easy. ^{31}P-NMR spectroscopy, with better sensitivity than ^{13}C-NMR, demonstrates a significant signal for 2-MG, which was not detectable with carbon NMR in this study. Therefore, ^{31}P-NMR was selected to quantify the exact amount of MGs, including 1-MGs and 2-MGs, and DGs (1,2-DGs and 1,3-DGs) in the HMMS.

As shown in Table 4. 1, enzyme and time were both highly significant determining factors for production outcomes for the MG, and the effect of the enzyme was more significant ($P<0.00001$) than the time variable ($P=0.0002$).

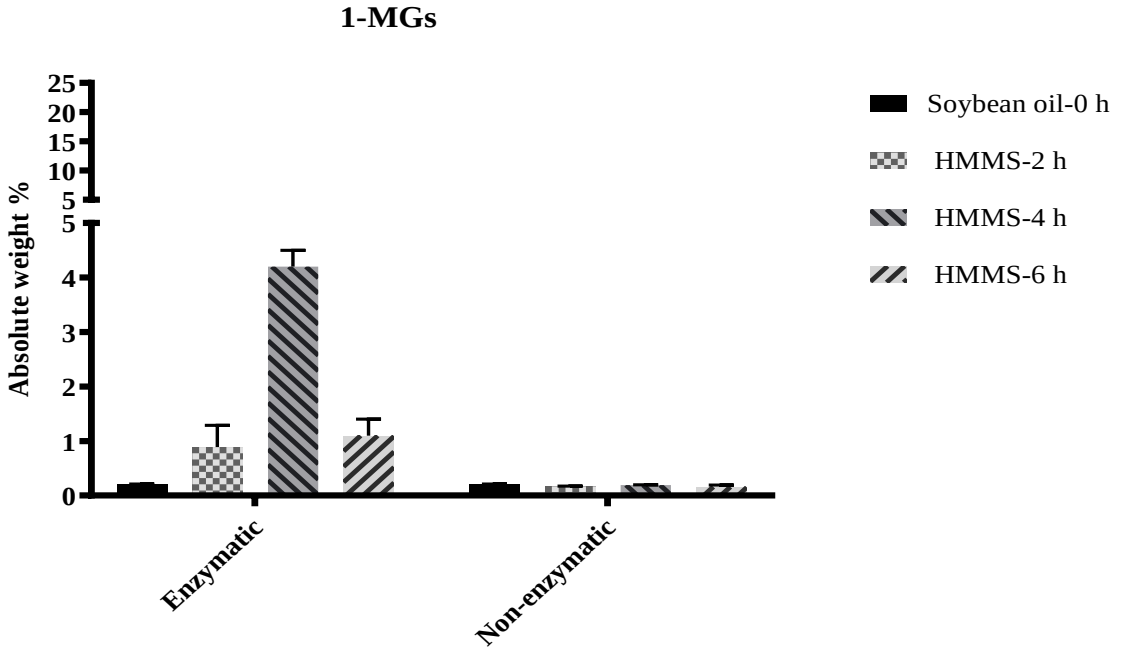
Table 4. 1 Effect of time and enzyme on production of MGs and DGs

Two-way ANOVA for PEGs*	% of total variation	SS (Type III)	DF	MS	F value (DFn, DFd)	P value
Source of Variation for 1-MGs						
Enzyme	26.5	9.9	1	9.9	F (1, 14) 33.9	<0.0001****
Time (h)	31.4	11.7	3	3.9	F (3, 14) 13.4	0.0002***
Interaction	31.2	11.6	3	3.9	F (3, 14) 13.3	0.0002***
Residual		4.1	14	0.3		
Source of Variation for 2-MGs						
Enzyme	29.6	354	1	354.4	F (1, 14) 39.7	<0.0001****
Time (h)	30.0	358	3	119.5	F (3, 14) 13.4	0.0002***
Interaction	30.0	358	3	119.5	F (3, 14) 13.4	0.0002***
Residual		125	14	8.9		
Source of Variation for 1,2-DGs						
Enzyme	38	305	1	305	F (1, 14) 54	<0.0001****
Time (h)	27	217	3	72	F (3, 14) 13	0.0003***
Interaction	25	203	3	68	F (3, 14) 12	0.0004***
Residual		79	14	6.0		
Source of Variation for 1,3-DGs						
Enzyme	43	54	1	54	F (1, 14) 25	0.0002***
Time (h)	10	13	3	4.4	F (3, 14) 2.0	0.1584^{ns}
Interaction	22	28	3	9.4	F (3, 14) 4.3	0.0237*
Residual		30	14	2.2		

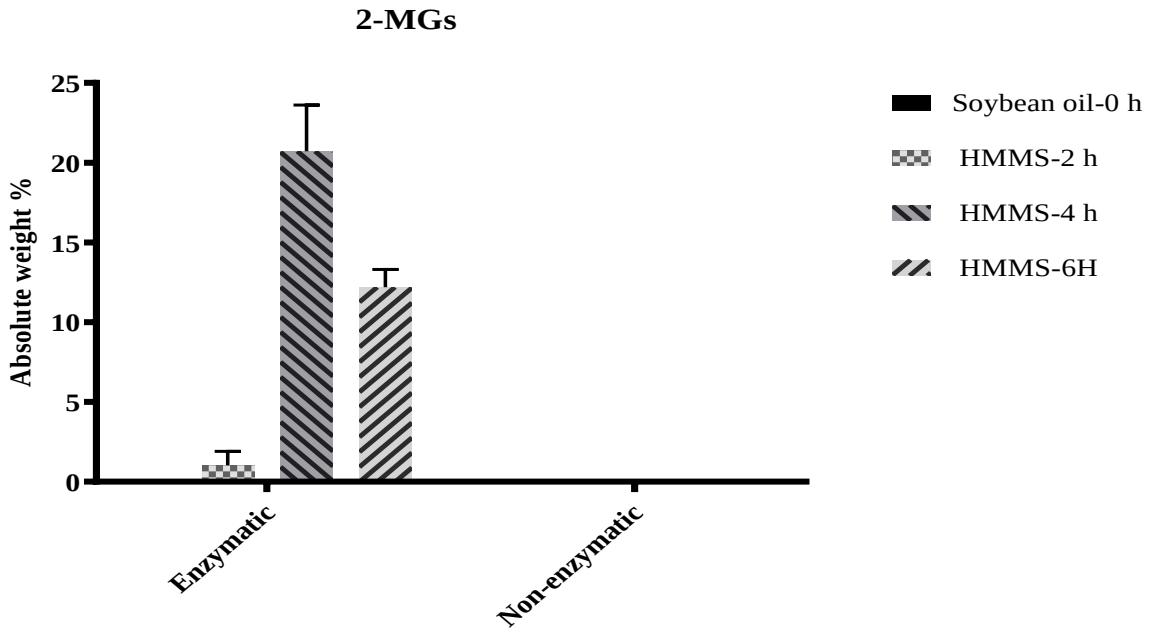
*Alpha level is 0.05

SS: sum of squares, DF: degree of freedom, MS: mean sum of squares.

Fig. 4. 2 demonstrates that 2-MGs were not detected in the alcoholysis products at any reaction time if the reaction had no enzyme. The highest contents of 2-MGs (20%) in the HMMS resulted from enzymatic alcoholysis after 4h of reaction. Higher level of 2-MG compared to other partially esterified glycerols in HMMS occurred only when Novozym 435 (lipase B) was used in the reaction. This is in agreement with other studies where lipase B and A of *Candida antarctica* has been shown to produce a higher level of 2-MG in products of an alcoholysis reaction (Abreu Silveira et al. 2017; Gunstone 1999). Most lipases are not able to convert TG that contains PUFA at all positions (the *sn*-1,3 and *sn*-2) to the 2-MG (Pfeffer et al. 2007). The molecular reason is that these types of TGs are sterically demanding for the substrate binding sites of these type of lipases (Pfeffer et al. 2007). However, Novozym 435 is able to convert TGs that contain any type of FA to the 2-MG (Pfeffer et al. 2007) because this enzyme can change the steric effects of TG containing PUFA to interact with the substrate binding sites (Pfeffer et al. 2007).



(a)



(b)

Figure 4. 2 Production of MGs after 2, 4 and 6 hours of enzymatic and non-enzymatic alcoholysis

Fig. 4.2 shows there are 0% 2-MGs and 0.2% 1-MG in soybean oil, but there are 21% 2-MG and 4% 1-MG in the HMMS produced under enzymatic conditions after 4 h. By considering the total MGs (including 1-MGs and 2-MGs) equal to 100, it can be concluded that in a short reaction time (4 h), Novozym 435 has altered the amounts of 1-MGs from 100% in soybean oil to 20% in the HMMS and the amounts of 2-MGs from 0% in soybean oil to 80% in the HMMS. In commercial preparations of MGs, which are produced by CIE methods, MGs are an equilibrium mixture of 90% 1-MG and 10% 2-MG (Gunstone 1999). The reason for greater amount of 1-MG compared to 2-MG can be related to the thermodynamic stability of acylglycerols with fatty acids in positions 1 and 3 compared to *sn*-2 (Feltes et al. 2013), which results in easy acyl migration from the center toward the *sn*-1,3 positions. Although the amount of both 2-MG and 1-MG were predominant at 4 h of enzymatic reaction (Fig. 4. 2), the ratio of 2-MG/1-MG increased from 5 to 12 when the reaction time increased from 4 to 6 h.

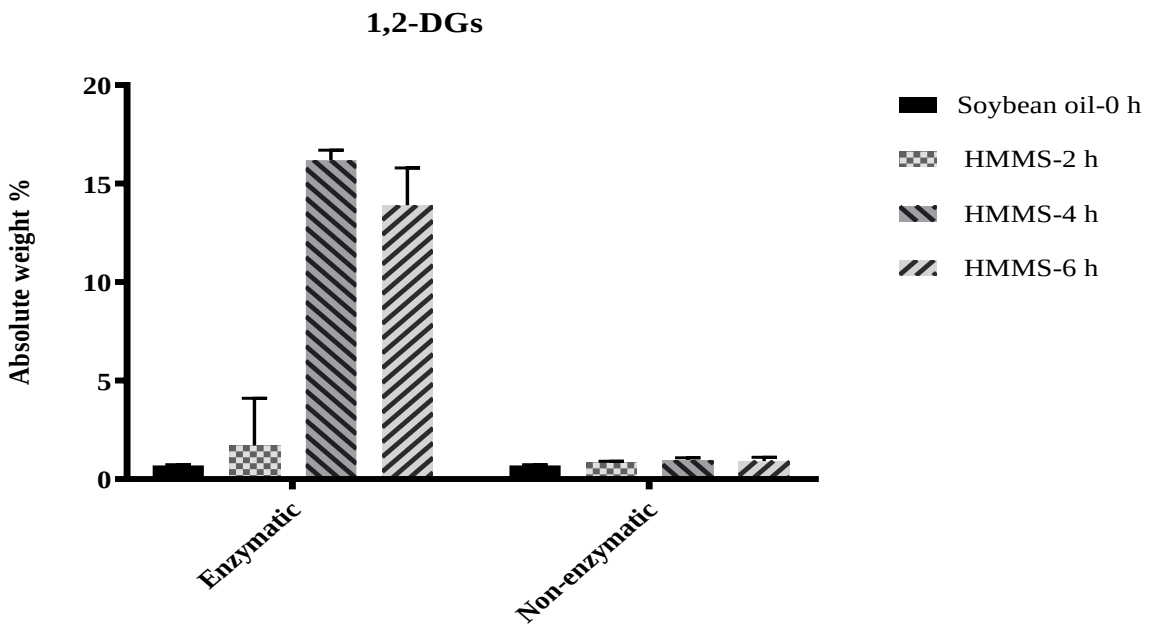
2-MGs, as the results of digestion of TGs, are easily absorbable components in the intestine. Thus, they have a health protective effect by transporting PUFAs through the lymphatic system and acting as a high-energy lipid source (Pfeffer et al. 2007). In biosynthetic pathways, 2-MGs are used in the production of phosphatidylcholine (Zhang et al. 2018). 2-MGs with specific FA can be useful components for infants and patients with exocrine pancreatic insufficiency (Martin et al. 2014).

MGs can be digested entirely and their consumption is nutritionally as safe as natural vegetable oils (Feltes et al. 2013). The two isomers of MGs have different absorption rates. Indeed, 2-MGs can be more easily absorbed than 1-MGs in the small intestine (Willis, Lencki, and Marangoni

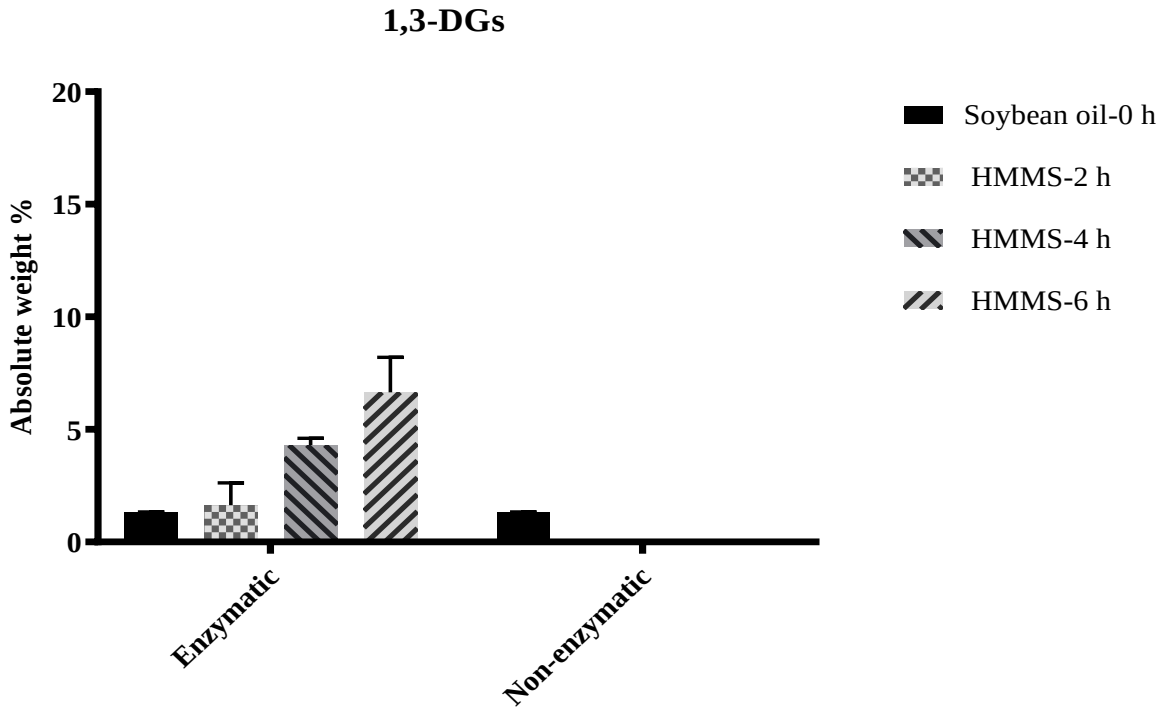
1998). However, both isomers of MGs can inhibit rises in postprandial blood triglyceride and insulin levels and keep TG and insulin levels under control in the blood. Therefore, MGs are able to prevent obesity and diabetes (Feltes et al. 2013; Martin et al. 2014). For instance, Shimotoyodome et al. (2012) reported that the consumption of dietary 1-monoolein by rodents decreased transport of both glucose and FA.

DGs are the other products of interesterification of soybean oil in the production of HMMS. The interaction of time and enzyme was effective in DG production. However, synthesis of the 1,3-DG isomer of DGs did not significantly change over time (Table 4. 1).

Fig. 4. 3a. shows that 1,2-DG reached the highest level in HMMS after 4 h of alcoholysis in the enzymatic reactions. The only product, obtained at all the time periods of the non-enzymatic alcoholysis, was 1,2-DG (Fig. 4. 3a.).



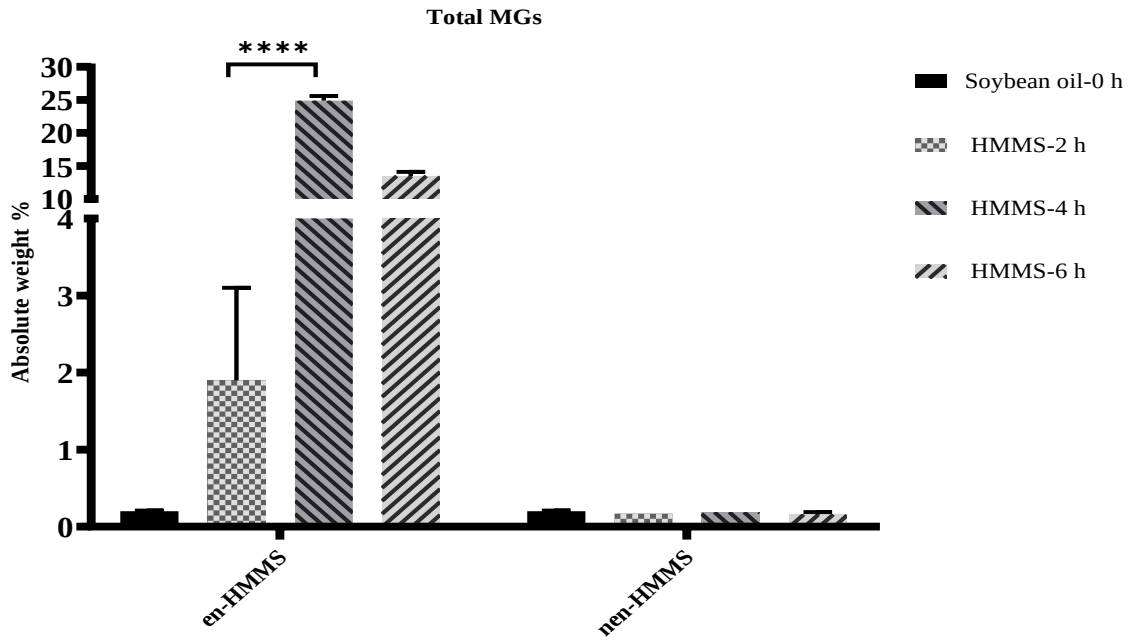
(a)



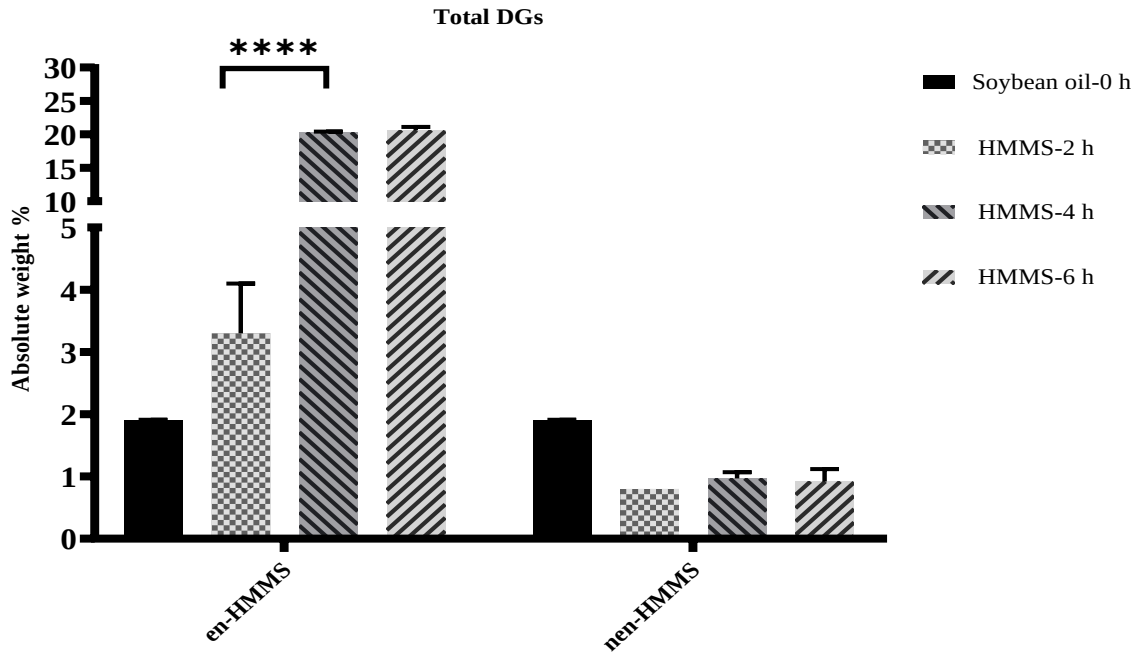
(b)

Figure 4. 3 Production of DGs after 2,4 and 6 hours enzymatic and non-enzymatic alcoholysis

The alcoholysis in $SCCO_2$ carried out with Novozym 435 resulted in a good yield of MGs and DGs (45%) after 4 h of reaction. For the non-enzymatic reaction conducted under the same conditions the yield of MGs and DGs was 1.2%. MG content was significantly ($P<0.0001$) maximized to 25% after 4 h of enzymatic alcoholysis. In contrast, the MG content remained at 0.2% even after 6 h of non-enzymatic alcoholysis. (Fig. 4. 4). Lubary et al (2011) conducted an enzymatic alcoholysis of milk fat in $SCCO_2$ with *Pseudomonas fluorescens* lipase and removed the fatty acid ethyl ester, which is a by-product of the ethanolysis, using a $SCCO_2$ extraction procedure to produce 32% DG and 6% MG.



(a)



(b)

Figure 4. 4 Total MGs and total DGs after 2, 4 and 6 hours of enzymatic and non-enzymatic alcoholysis

4.4.2 FATTY ACID COMPOSITION AND ANALYSIS OF HMMS BY ^{13}C-NMR

^{13}C-NMR is an effective methodology in analysis of FA composition and the positional distribution of FAs. The position of each FA is distinguished when the carbonyl carbons of each FA resonates at a specific chemical shift. Gouk et al. (2011) used ^{13}C-NMR to analyse the carbonyl carbons of different oils, which are mostly composed of TGs, to identify the acyl positional distribution (*sn*-1,3 *vs sn*-2) of SFA and UFA.

In this study, ^{13}C-NMR was used to analyse the carbonyl carbons of HMMS, which is a mixture of TGs and mono- and di-esters, to identify the positional distribution of the different FA types at different reaction times. Table 4. 2 shows two positions of FAs within the TGs of HMMS at different reaction times compared with that of soybean oil and also with each other. For example, soybean oil is compared with HMMS after 4h or HMMS after 2h with HMMS after 6 h. Therefore, all the changes of linoleic (LO), linolenic (LN), oleic acid (OL) and saturated fatty acids (SFAs) contained at two positions (*sn*-1,3 and *sn*-2) in the TGs of soybean oil and HMMS after 2, 4 and 6 h of reaction were compared, but only the values that are significant are reported in Table 4.2. The results showed that the percent of MUFA (OL) at the *sn*-2 position in TGs of soybean oil compared to that of HMMS after 4 h reaction decreased significantly (P= 0.0009) (Table 4.2). Most of the significant differences between fatty acid composition of soybean oil and HMMS at different reaction times happened at the *sn*-2 position for UFAs (LO, LN and OL) (Table 4.2). Only OL varied significantly at both the *sn*-2 and *sn*-1,3 positions, and only then after 6 h of reaction. SFAs of soybean oil and HMMS had a significant increase only at the *sn*-1,3 position. Therefore, Table 4 shows Novozym 435 had a substantial effect in altering the UFA at the s*n*-2 and the SFAs at the s*n*-1,3 in HMMS at different reaction times.

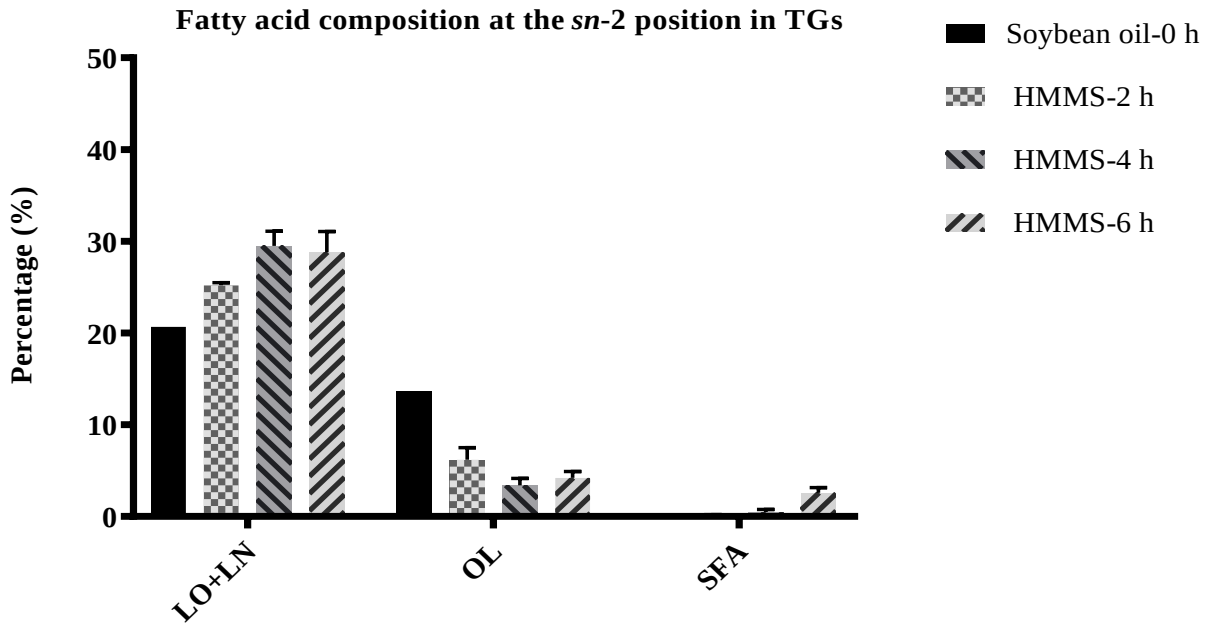
Table 4. 2 Tukey's multiple comparison test of fatty acids in TGs of soybean oil and HMMS after 2, 4 and 6 h of enzymatic reaction

	***sn*-2**				***sn*-1,3**			
Fatty acid type in Soybean oil and HMMS	**Mean* diff.**	**95% CI of diff.**	**SE of diff.**	**P value**	**Mean diff.**	**95% CI of diff.**	**SE of diff.**	**P value**
LO+LN								
Soy vs. HMMS-4h	-8.8	-15.1 to -2.5	2.2	0.004**	2.1	-6.3 to 10.5	3.1	0.89^{ns}
Soy vs. HMMS-6h	-8.1	-14.6 to -1.6	2.3	0.01*	-1.6	-10.2 to 7.0	3.0	0.95 ^{ns}
HMMS-2h vs. HMMS-6h	-4.3	-8.6 to -0.02	1.6	0.04*	-4	-10.1 to 2.1	3.1	0.29 ^{ns}
OL								
Soy vs. HMMS-2h	7.4	0.9 to 13.9	2.3	0.02*	7.1	-1.5 to 15.7	3.1	0.13 ^{ns}
Soy vs. HMMS-4h	10.2	3.9 to 16.5	2.2	0.0009***	6.9	-1.5 to 15.3	3.0	0.13 ^{ns}
Soy vs. HMMS-6h	9.4	2.9 to 15.9	2.3	0.003**	12.5	3.9 to 21.1	3.1	0.003**
SFA								
Soy vs. HMMS-2h	-0.1	-6.6 to 6.4	2.3	>0.99 ^{ns}	-12.1	-20.7 to -3.5	3.1	0.004**
Soy vs. HMMS-4h	-0.5	-6.8 to 5.8	2.2	0.99 ^{ns}	-9.7	-18.1 to -1.3	3.0	0.02*
Soy vs. HMMS-6h	-2.6	-9.1 to 3.9	2.3	0.68 ^{ns}	-9.4	-18.0 to - 0.8	3.1	0.03*

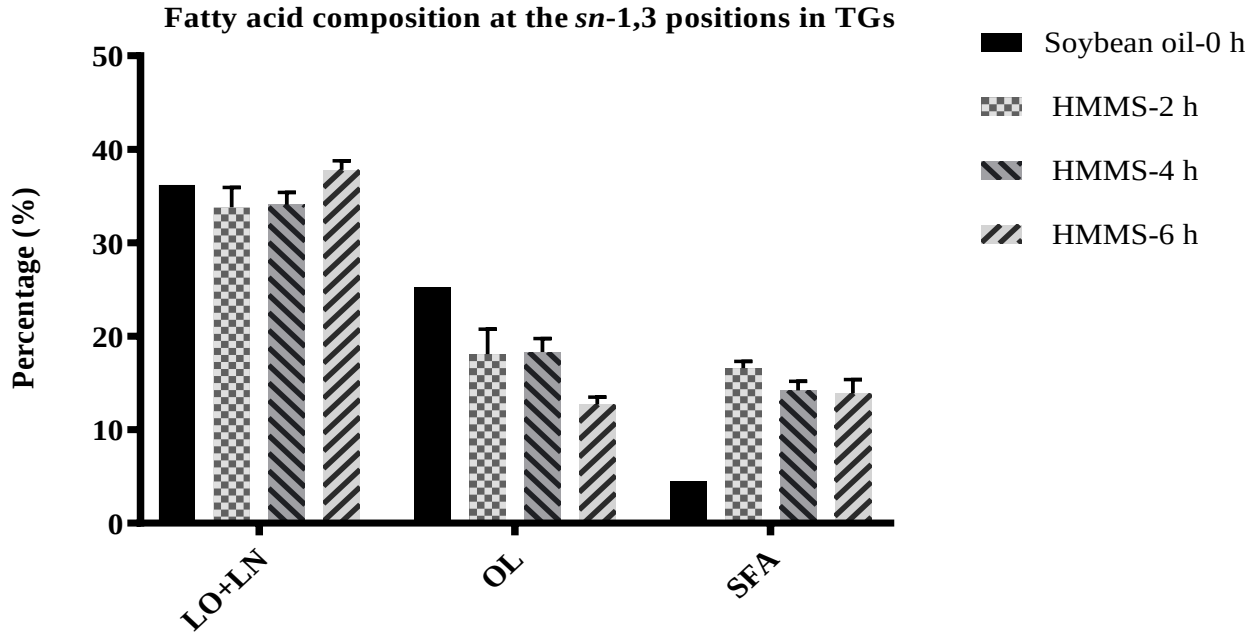
*Degrees of freedom (DF) for all is 21.

LO: linoleic acid, LN: linolenic acid, OL: oleic acid and SFA: saturated fatty acid.

Fig. 4. 5 shows that the content of PUFAs (LO and LN) at the *sn*-2 position of TGs increased significantly (P=0.004) and reached 30% after 4 h. It is hard to retain PUFAs at the *sn*-2 position because of a spontaneous shift of FAs from the center to the peripheral positions of glycerol (Compton et al. 2012). However, the conditions of this alcoholysis with Novozym 435 stabilized the PUFAs at the *sn*-2 position of TGs at all time periods of the enzymatic reaction (Fig. 4. 5a). PUFAs are safer at position 2 with respect to protection against oxidation because of steric reasons. For example, TGs with PUFAs at *sn*-3 and SFAs at *sn*-1,2 can be oxidized easier than 1,3-disaturated-2-PUFA molecules (Pfeffer et al. 2007).



(a)



(b)

Figure 4. 5 Fatty acid composition at two positions in the TG of soybean oil and HMMS after 2, 4 and 6 hours of enzymatic reaction

The level of SFAs at the *sn*-1,3 of TGs increased significantly after the enzymatic reaction (Table 4. 2). Fig. 4. 5 demonstrates that the highest level (17%) of SFAs at the *sn*-1,3 was obtained after 2 h of reaction (P=0.004). The UFAs, which have the highest concentration amongst the FAs in soybean oil and the TGs of HMMS, are distributed equally at both the *sn*-2 and *sn*-1,3 positions. However, SFAs preferred *sn*-1,3 positions to *sn*-2 position at all time periods of the enzymatic reaction (Fig. 4. 5). This was in agreement with a study by Vlahov (2009). Enzymes have a crucial role in the distribution of FAs. For example, in an acidolysis reaction with *Mucor miehei* enzyme, PUFAs were limited to the *sn*-1,3 positions, whereas in a reaction with an immobilized lipase from *Candida antarctica*, PUFAs sat at both *sn*-1,3 and *sn*-2 positions (Gunstone 1999). After 4 h of enzymatic alcoholysis, the contents of PUFAs and MUFAs at the *sn*-1,3 position of TGs were 34% and 18%, respectively, compared with 30% and 6% at the *sn*-2 position.

4.5 CONCLUSION

The present study provided valuable data for the partially esterified acylglycerol contents of a high monoester mixture of soybean oil based on a time course study of an alcoholysis reaction, with 1,2-propanediol in $SCCO_2$, with and without enzyme. Furthermore, an analysis of FAs within the TGs was conducted to show the positions of PUFA, MUFA and SFA. Based on high catalytic performance of Novozym 435 and its operational stability in $SCCO_2$ our results suggest that this enzymatic alcoholysis is a suitable type of LIE for the preparation of greater amounts of 2-MGs than1-MGs in HMMS in a short reaction time. Higher levels of PUFA (30%) at the *sn*-2 position in the TGs of HMMS compared to PUFA in the soybean oil at the same position was obtained after 4 h of enzymatic alcoholysis. Therefore, LIE of soybean oil with 1,2-propanediol in $SCCO_2$ can be used by food manufacturers to produce more eco-friendly and safer shortenings with reduced *trans* fats contents, compared to CIE methods.

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CHAPTER 5

MANUSCRIPT 4

OPTIMIZATION AND ANALYSIS OF AN ENZYMATIC GLYCEROLYSIS OF SOYBEAN OIL TO PRODUCE DIACYLGLYCEROL-ENRICHED OIL IN A SUPERCRITICAL CARBON DIOXIDE SYSTEM

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5.1 ABSTRACT

This research addresses an alternative green methodology for the synthesis of diacylglycerol-enriched (DAG) oil by enzymatic glycerolysis of soybean oil in a supercritical carbon dioxide ($SCCO_2$) medium. DAG oil has been recently highlighted as a healthy oil. Phosphorous nuclear magnetic resonance (^{31}P-NMR), an eco-friendly, fast, and precise analytical technique, was used for analysis of the different isomers of partially esterified glycerols (PEGs), free fatty acids (FFAs), and tocopherols of the soybean oil-based DAG oil (SDO) mixture taken from the reaction. After 8 hours (h) reaction, enzymatic glycerolysis in $SCCO_2$ increased dramatically (1700%) the percentage change of total diglyceride (DG) in SDO compared to the same reaction at atmospheric pressure. A total DG content of 44% (w/w), containing 60% of the healthier 1,3-DG isomer versus 40% 1,2-DG, was obtained at a pressure of 80 bar, temperature of 60 °C, and an enzyme load of 10% (w/w) following 8 h of enzymatic glycerolysis. A mathematical model was developed to find the relationship between the total DG production and the independent variables (*i.e.*, pressure, time, enzyme load and temperature). To validate the efficacy of the model, the Least Square technique was employed and the value of R^2 was 0.94 which shows that the model is well fitted to the experimental results.

5.2 INTRODUCTION

A transformational path to sustainable practices is urgent for today's global industries. Biodiesel production processes are challenged ecologically and economically due to the production of surplus glycerol which forms about 10% of total output products (Fiametti et al. 2011; Remón et al. 2018). Using glycerol as a primary reactant to create novel functional foods, such as diacylglycerol-enriched (DAG) oil, can make bio-fuel production more eco-friendly and economically efficient (Ferretti, Spotti, and Di Cosimo 2018).

DAG oil has been marketed as a healthy oil (Zhang et al. 2017) since diglycerides (DGs) have been clinically proven to aid in the maintenance or loss of weight and to reduce body (Kim et al. 2017) and visceral (Saito et al. 2017) fat. DAG oil also lowers blood cholesterol levels (Dhara, Dhar, and Ghosh 2013) and helps to maintain healthy blood triacylglycerol levels (Song et al. 2012; Yamanaka et al. 2016). DG, particularly 1,3-DG, is digested and absorbed in the small intestine mucosa, and without converting into a triglyceride, is consumed as a source of energy (Ferretti, Spotti, and Di Cosimo 2018). As a result, DAG oil reduces the level of blood triglyceride more than triacylglycerol (TAG) oils which are the conventional edible oils.

DGs occur naturally in some vegetable oils but they are present in small amounts (Liu et al. 2016). DGs have emulsifying functionality (Feltes et al. 2013) and can be used with unsaturated oils to produce a shortening system (Doucet 2005) which can replace partially hydrogenated fats, thereby reducing the consumption of undesired *trans* and saturated FAs of many food products (Orthoefer 2008). DGs are used as emulsifiers in the food, pharmaceutical, and cosmetic industries due to having both hydrophilic and hydrophobic components in their molecular structures (Cai et al. 2016; Feltes et al. 2013; Hartel and Hasenhuettl 2013).

Commercial DAG oil is produced from esterification of glycerol with unsaturated fatty acids that are hydrolyzed from soybean and canola oils (Katsuragi et al. 2004). Since DAG oil has similar taste and colour to conventional vegetable oils, it can be used in a variety of food products, increasing the health benefits of each product significantly (Katsuragi et al. 2008). It can be used primarily in cooking and food product formulations (Chen et al. 2017) such as in mayonnaise, salad dressings, spreads, margarine, nutritional bars, and confectionery fats (Katsuragi et al. 2008).

DAG oil can be created from various routes such as esterification (Watanabe et al. 2003), hydrolysis (Voll et al. 2012), acidolysis (Xu et al. 1999), ethanolysis (Blasi et al. 2007), and glycerolysis (Ferreira and Tonetto 2017; F. Voll et al. 2011). Hydrolysis produces small amounts of DG after a long reaction time (Voll et al. 2012). In the ethanolysis reaction, separation of fatty acid ethyl esters (FAEEs) is required to increase the DG yield (Lubary 2011). Esterification of free fatty acids (FFAs) with glycerol produces water (Watanabe et al. 2003), and the drawback is the separation of water. Using a water adsorbent may cause the removal of monoglycerides (MGs) and DGs (Ferreira and Tonetto 2017). Glycerolysis produces a higher yield of DG (Yamane et al. 1994) and compared to esterification does not need separation of the water directly nor hydrolysis of oil to obtain the mixture of FFA (Ferretti, Spotti, and Di Cosimo 2018).

The chemical glycerolysis of oils to produce DAG oil has high energy consumption and solvent cost. Besides, chemical glycerolysis methods are time-consuming and laborious due to adding and removing metal catalysts such as NaOH, KOH, $Ca(OH)_2$ (M. Liu et al. 2016). Some chemical glycerolysis reactions need to be conducted at high temperature (150-200 °C). High reaction temperature leads to degradation of heat-sensitive unsaturated fatty acids (Zhong et al. 2010). This produces final products that are dark in colour and burnt in flavour (Temelli, King, and List 1996).

As an alternative to chemical glycerolysis for DAG oil production, glycerolysis can be conducted with an enzyme (Liu et al. 2012). Enzymes are non-toxic catalysts for use in production of food-related products, and are more eco-friendly, especially when they can be reused (Ferreira and Tonetto 2017). Enzymatic interesterification also eliminates excessively expensive protection/de-protection steps that are necessary for chemical interesterification (Laszlo, Compton, and Vermillion 2008). However, enzymatic glycerolysis still requires long reaction times (Kahveci et al. 2010; Miranda et al. 2013) because of low mass transfer rates, the high density of glycerol, and adsorption of the glycerol to the hydrophilic carrier of the immobilized enzyme (Liu et al. 2016). To solve this problem many studies have conducted enzymatic glycerolysis in a solvent medium such as a *tert*-butyl alcohol. For example, Naik et al. (2014) used different co-solvents in the enzymatic glycerolysis of sunflower oil and reached a maximum conversion rate after 5 h. However, enzymatic glycerolysis in solvent does not meet all criteria of a green chemistry approach since there is extra energy consumption for solvent separation from the products and there is the need for disposal of high amounts of the solvent.

Supercritical carbon dioxide ($SCCO_2$) has many benefits as a solvent because it is safe, non-flammable and it also works as an inexpensive and green catalyst that enhances the reaction rate. Also, it does not leave a residue in the food product (Loss et al. 2015). Conducting reactions in $SCCO_2$ media has developed because the mass transfer rate is increased by $SCCO_2$ due to its high diffusivity and low viscosity (Ramsey et al. 2009). Temperature and pressure can be manipulated to efficiently control the solvent power and the $SCCO_2$ media reaction performance (Ramsey et al. 2009). Moreover, one of the most critical advantages of $SCCO_2$ is its easy separation from the reaction medium by depressurization (Rezaei, Temelli, and Jenab 2007).

Recently, supercritical fluid media have been developed to conduct enzymatic reactions, and this shows valuable advantages due to the elimination of solvents in the manufacturing process (Jenab et al. 2014).

Elimination of a process which only works based on solvents reduces the cost of production. Recovery and reuse of the enzyme result in further cost reduction. Selective separation of desired products such as DGs is enabled by merely adjusting the pressure of the $SCCO_2$ of the system and no solvent residue remains in the final products (Lubary 2011). Lubary et al. (2011) reported a synthesis of a DAG milk fat with an immobilized lipase (*P. fluorescens*) in a $SCCO_2$ medium through an alcoholysis reaction, yielding 33mol% DG. However, a purification step for separation of FAEE was required to increase the DG yield up to 51mol%, and another step of enzymatic isomerization was required to increase the content of the 1,3-DG isomer from 34mol% to 64mol% by promoting acyl migration (Lubary, Hofland, and ter Horst 2011).

^{31}P-NMR, as a novel quantitative analytical tool, has been used in the analysis of a complex oil such as biodiesel, which is a mixture of alcohols, FFA, MG, and DG. This rapid and accurate methodology was used in the entire analysis of conversion of triglycerides to biodiesels by calculating the yield at each step and analyzing the composition of the substrate mixture, and the product during a commercial production process (Nagy, Foston, and Ragauskas 2010).

The enzymatic glycerolysis of soybean oil to produce specifically a soybean oil-based DAG oil (SDO) in $SCCO_2$ and the analysis of this lipid mixture with a quick, reliable ^{31}P-NMR have not been reported previously. SDO is a lipid mixture of MG, DG, TG, FFA and free glycerol. Glycerolysis in $SCCO_2$ to obtain a high level of DGs in SDO without any purification or isomerization steps is an outcome of this study. In addition, an in-depth analysis of the effect of the different reaction conditions on DG content is investigated to find the optimum conditions defined through a fitted model.

Therefore, the first objective of this study is to compare the content of all components of SDO after the enzymatic glycerolysis of soybean oil under atmospheric and $SCCO_2$ conditions to show how effective $SCCO_2$ is in the production of SDO with a high content of DG containing the healthier isomer, 1,3-DG. The second objective is to better understand the effect of the independent variables, including pressure, reaction temperature, time, and enzyme load on the respective content of DG, MG, and FFA in the final products of SDO after the enzyme-catalyzed reaction. The second objective results in establishment of an efficient method for enzymatic glycerolysis of SDO from soybean oil in supercritical system by deriving a mathematical model for the maximum content of DG in SDO.

5.3 THE ROLE OF MOLAR RATIO AND WATER CONTENT IN DG PRODUCTION

Scheme 5.1 illustrates the complete conversion of TG to DG when the molar ratio of glycerol: TG is 0.5. The study of Yamane et al. (1994) revealed that when the stoichiometric molar ratio of glycerol to oil (TG) is 0.5, the best DG yield was obtained; increasing the ratio from 0.5 to 1 led to a significant decrease in DG production. Other studies showed that the optimum molar ratio is

0.5 for glycerol to TG (Miranda et al. 2013) and with this molar ratio the maximum yield of DG was obtained. Zhong et al. (2013) reported that a molar ratio of 5 (glycerol: TG) increased MG production. Besides, a large amount of glycerol remains after glycerolysis at glycerol: TG molar ratios between 3.5 and 4.0:1, and this has to be removed (Yamane et al. 1994). The excess glycerol in the reaction medium limits the catalytic performance and activity of the enzyme because it adheres to the enzyme support (Weber and Mukherjee 2004).

$$2\,TG + Glycerol \overset{Enz}{\longleftrightarrow} 3\,DG$$

Scheme 5.1. Synthesis of DG via enzymatic glycerolysis

Most glycerolysis studies, aiming at producing DG, are conducted with a small amount of water (Voll et al. 2011). Some studies used the water that is naturally associated with glycerol, which is between 3% (Voll et al. 2011) and 3.5% (Kristensen, Xu, and Mu 2005), for the glycerolysis reaction. Adding extra water increases the amount of FFA produced and the MG content, thereby lowering the production of DG (Weber and Mukherjee 2004).

5.4 MATERIALS AND METHODS

5.4.1 MATERIALS

Refined soybean oil was donated by Manitoba Pulse and Soybean Growers (MPSG). Glycerol, Novozym 435 (lipase B, from Candida antarctica), phosphorus reagent (2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane) (CTDP), 4-Å molecular sieves, Chromium (III) acetylacetonate $Cr(acac)_3$, pyridine, cyclohexanol (99%) and deuterated solvent (chloroform) were purchased from Sigma-Aldrich (Canada). EMD Millipore Steritop™ sterile vacuum bottle-top filters were purchased from Fisher Scientific (Canada).

5.4.2 GLYCEROLYSIS REACTION

The substrates utilized for the enzymatic reaction of SDO consisted of soybean oil and glycerol. The molar ratio of glycerol to soybean oil was selected to be 0.5 to obtain the highest DG content (Yamane et al. 1994). Water was not added in the reactor vessel as glycerol contains around 3% moisture (Voll et al. 2011).

To conduct the enzymatic reactions for the first objective, 3% of total substrate weight of immobilized Novozym 435 was added to the mixture of soybean oil and glycerol. The atmospheric reaction was started in a screw-capped Erlenmeyer flask in an oven at atmospheric pressure at 60°C for different times - 1, 3, 4 and 8 h. Glycerolysis was ended by removing the enzyme from the reaction materials using a filter connected to a vacuum pump. For reaction under $SCCO_2$ conditions, the reactor vessel was sealed and pressurized by a gas booster pump at a flow rate of 100 g/min to reach 80 bar. The flow rate was then reduced to 20g/min and was kept at this rate for the rest of the reaction. The vessel was heated to 60°C and maintained within ± 5 °C by a controller for the different times required for the study. Glycerolysis was ended by

depressurizing the system and removing the enzyme from the reaction materials utilizing vacuum filtration. The upper layer of the reaction products from each glycerolysis reaction was collected by pipet and transferred into 15 mL centrifuge tubes. In order to separate excess glycerol, the samples were centrifuged for 15 min at 2900 g (Kristensen, Xu, and Mu 2005). The oil layer was then analyzed by ^{31}P-NMR.

For the second objective, several experiments were conducted to examine the effect of four independent variables including operating pressure (80-450 bar), operating temperature (40-70 °C), time (1-12 h), and enzyme concentration (3-10%) on the production of SDO components (*i.e.*, DG, MG, FFA). Table 5. 1 shows different experiments based on various reaction conditions. As illustrated in Table 5. 1, fifteen experiments were carried out to consider a wide range of changes in each independent variable. In order to obtain an optimum value for each independent variable, a specific variable was varied over a defined range while the other three variables were kept unchanged. For instance, the value of pressure changed from 80 bar to 450 bar in the first five experiments, while the values of time, temperature and enzyme load remained constant at 4 h, 60 °C and 3%, respectively. The optimum value was then chosen considering the DG content of SDO and industrial demands such as the process costs (Haar et al. 2015). For example, if the DG content at 100 bar was a little higher than the DG content at 80 bar, the 80 bar was selected as the optimum pressure to reduce energy usage and production cost.

Table 5. 1 Experiments based on the different independent variables

Run.	Pressure (bar)	Temperature (°C)	Time (h)	Enzyme load (%)
1	80	60	4	3
2	100	60	4	3
3	150	60	4	3
4	300	60	4	3
5	450	60	4	3
6	80	40	4	3
7	80	70	4	3
8	80	60	1	3
9	80	60	2	3
10	80	60	3	3
11	80	60	5	3
12	80	60	8	3
13	80	60	12	3
14	80	60	4	6.5
15	80	60	4	10

5.4.3 SAMPLE PREPARATION FOR NMR ANALYSIS

To conduct the analysis, a Bruker Avance III 500 spectrometer (Bruker Corporation, Billerica, MA, USA) was used at 25°C. 50 ml of a stock solution (STS) consisting of a 1.6:1 volume ratio of pyridine and $CDCl_3$ was prepared where 30 mg of $Cr(acac)_3$ relaxation reagent was added to STS. 67.5 mg of cyclohexanol, the internal quantification standard, was then added to the STS. To protect the STS solution from moisture, 4-Å molecular sieves were added and the container was tightly sealed. 100 mg of oil sample (soybean oil or SDO) was weighed into a 1.5 ml tube to which 0.4 ml of STS and 50 µl of CTDP reagent were added; this was left 15 minutes to allow

reaction of the CTDP with the free hydroxyl groups of the oil sample. ^{31}P NMR was performed in three replications for analysis of SDO. ^{31}P chemical shifts were reported in ppm relative to a sharp signal in pyridine/$CDCl_3$ of STS at 132.18 ppm. The reaction between CTDP reagent and the moisture in the oil samples produces this sharp signal. Table 5. 2 shows the ^{31}P-NMR chemical shift assignment of partial acylglycerols, FFA and tocopherols. The absolute quantification of each component of the soybean oil and the SDO was obtained by integration of peaks for FFAs at 134.7 ppm, tocopherols at 144.9 ppm and MGs and DGs from 146.4-148.2 ppm where the internal standard cyclohexanol gives a peak at 145.2 ppm (Table 5. 2).

Table 5. 2 ^{31}P-NMR chemical shifts of minor components of oils

Name of compound	FFA	Tocopherols	Cyclohexanol	Free glycerol secondary	1-MG secondary	1,3-DG	Free glycerol primary	1-MG primary	2-MG	1,2-DG
Chemical shift (ppm)	134.7	144.9	145.2	146.3	146.4	146.6	147.4	147.7	148.0	148.2

5.4.4 STATISTICAL ANALYSIS AND MATHEMATICAL MODEL DERIVATION

Data of the first and second objectives were analyzed by two-way and one-way analysis of variance (ANOVA), respectively. The analyses were followed by Tukey's multiple comparisons test using GraphPad Prism Software (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com).

The goal was to investigate significant differences between components of SDO at $P < 0.05$ during the enzymatic reaction. The enzymatic experiments were performed at least in duplicate, and the data are reported as average values with the corresponding standard error of the mean

(SEM). A mathematical model for the DG content as a function of four independent variables was derived based on the experimental data. Details about the steps followed to establish the model are provided in Appendix A. 2.

5.5 RESULTS AND DISCUSSION

5.5.1 ENZYMATIC GLYCEROLYSIS UNDER ATMOSPHERIC AND SUPERCRITICAL CO_2 PRESSURES

For the first objective, two time course studies of enzymatic glycerolysis under both atmospheric and $SCCO_2$ conditions were conducted and five components (1-MG, 2-MG, 1,2-DG, 1,3-DG and tocopherol) of SO and SDO were obtained after 1, 2, 3 and 8 h of reaction times (see Figure A. 2. 2). The results showed that $SCCO_2$ is an effective reaction medium for production of SDO with high content of DG, which is in line with the results of a study carried out by Jenab et al. (2013) on the application of $SCCO_2$ in the enzymatic interesterification of fats and oils. Figure 5. 1 shows ^{31}P-NMR spectra of minor components and 1,3-DG at 146.6 ppm for typical samples of soybean oil and SDO after 4 and 8 hours of reaction under $SCCO_2$ conditions.

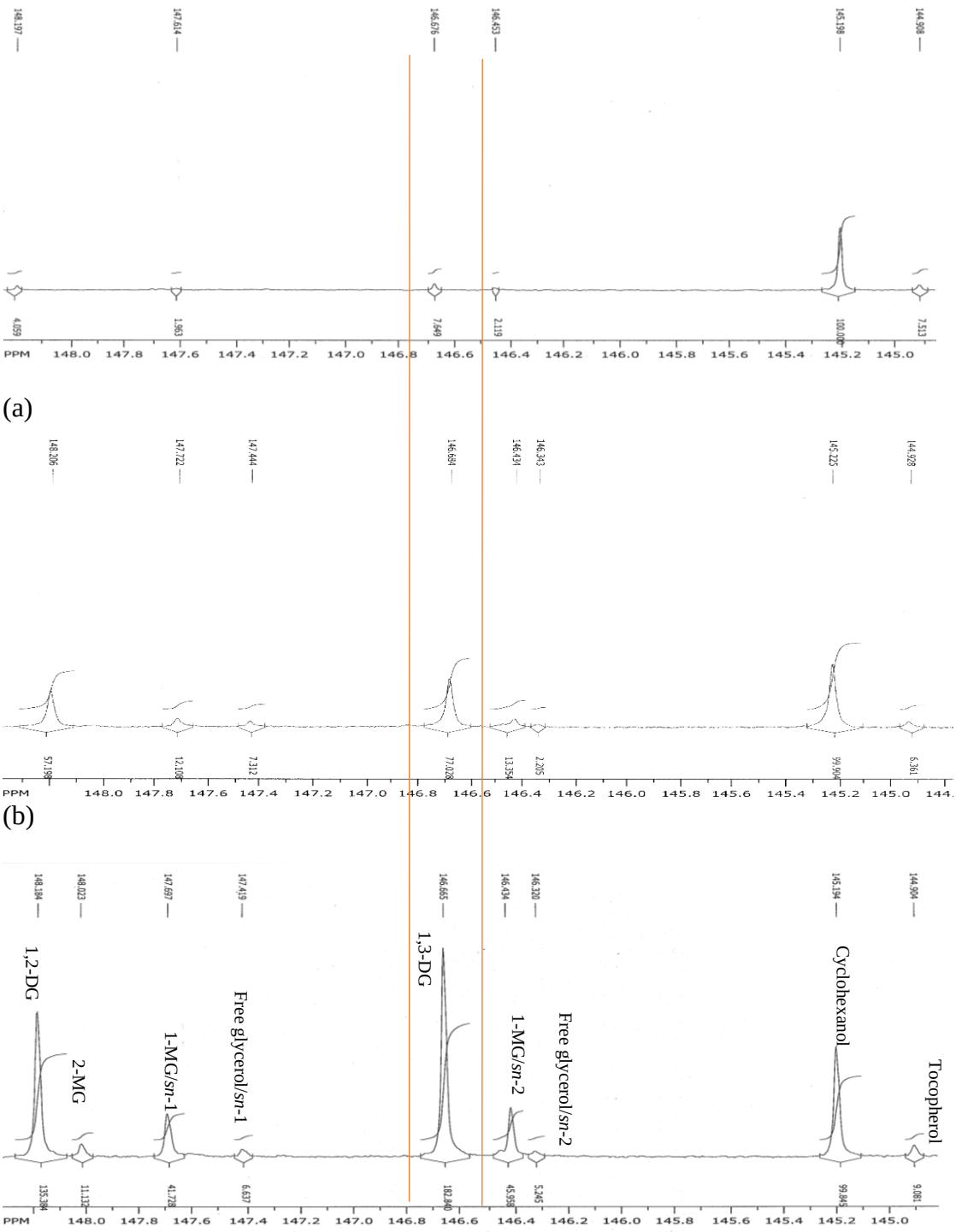


Figure 5. 1 ^{31}P-NMR spectrum of minor components and 1,3-DG at 146.6 ppm for typical samples of soybean oil. (a) SDO-4h, (b) and SDO-8h (c). SDO was obtained under $SCCO_2$ conditions at a pressure of 80 bar and a temperature of 60°C with 3% of Novozym 435

1,3-DG was selected to show a comparison of the time course of the reaction under both conditions in Figure 5. 1 due to its nutritional benefits in DAG oil (Lubary, Hofland, and ter Horst 2011). 1,3-DG content was maximized at 4±0.7% for atmospheric conditions while this value was reached after 1 h of reaction under $SCCO_2$ conditions. After 8 h of reaction the 1,3-DG content of SDO under $SCCO_2$ conditions was 27±2.9% (Figure 5. 2).

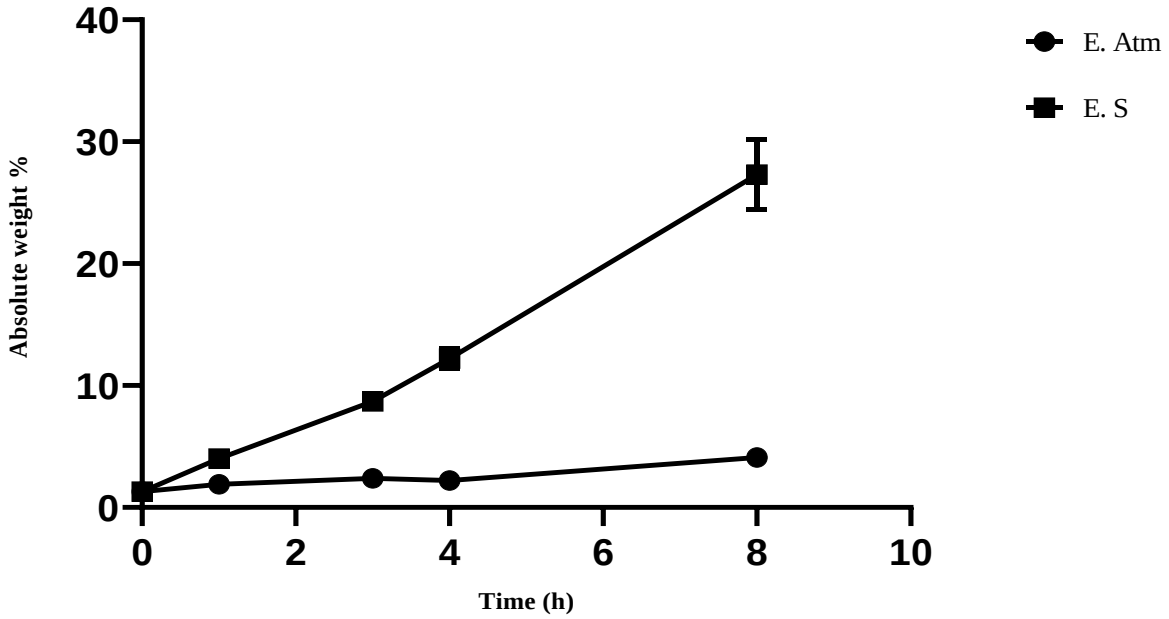


Figure 5. 2 The content of 1,3-DG in SDO obtained from enzymatic glycerolysis reaction under atmospheric (E. Atm.) and $SCCO_2$ (E. S.) conditions

Table 5. 3 summarizes the mean values of the weight percentage of each SDO component along with their SEM values. To find the level of significance, the mean value of SDO components, obtained at different reaction times, was compared with the corresponding amount of soybean oil components. Moreover, the highest amount of total DG was found to be 42±4.6% after 8 hours of enzymatic glycerolysis in contact with $SCCO_2$ while this value was only 6±0.6 % under atmospheric conditions.

Table 5. 3 Mean value of weight percentage of each component within soybean oil and SDO after enzymatic glycerolysis under atmospheric and $SCCO_2$ conditions

Enzymatic glycerolysis at 1 atm.					
Component	**Soybean Oil**	**SDO-1h**	**SDO-3h**	**SDO-4h**	**SDO-8h**
Tocopherols	0.9±0.0	1.1±0.0 ^{ns}	1.2±0.1^{ns}	1.1±0.1^{ns}	1.2±0.0^{ns}
1,3-DG	1.3±0.0	1.9±0.1^{ns}	2.4±0.1^{ns}	2.2±0.2^{ns}	4.1±0.7^{ns}
Total MG	0.2±0.0	0.2±0.0 ^{ns}	0.2±0.0 ^{ns}	0.2±0.0 ^{ns}	0.4±0.1^{ns}
Total DG	1.9±0.1	3.0±0.2 ^{ns}	3.8±0.1 ^{ns}	3.5±0.1 ^{ns}	6.2±0.6 ^{ns}
Ratio of 1,3-DG/1,2-DG	1.9±0.0	1.9±0.1 ^{ns}	1.7±0.1^{ns}	1.8±0.0^{ns}	1.9±0.3^{ns}
Ratio of DG/MG	2.1±0.1	13.7±1.1 ^{ns}	18.5±3.6^{ns}	16.6±1.3^{ns}	15.6±1.4^{ns}
Enzymatic glycerolysis under $SCCO_2$ pressure					
Tocopherols	0.9±0.0	1.1±0.02^{ns}	1.1±0.04 ^{ns}	1.0±0.04 ^{ns}	1.1±0.03 ^{ns}
1,3-DG	1.3±0.0	4.0±0.3 ^{ns}	8.7±0.47 ^{ns}	12.2±0.8 ^{ns}	**27.3±2.9******
Total MG	0.2±0.0	0.6±0.2^{ns}	1.5±0.2^{ns}	1.7±0.1^{ns}	**6.2±1.5****
Total DG	1.9±0.1	7.6±0.4 ^{ns}	15.8±1.0^{ns}	21.8±1.1^{ns}	**41.5±4.6******
Ratio of 1,3-DG/1,2-DG	1.9±0.0	1.1±0.1 ^{ns}	1.2±0.0 ^{ns}	1.3±0.1 ^{ns}	1.4±0.1 ^{ns}
Ratio of DG/MG	2.1±0.1	**16.9±4.5***	10.7±0.8^{ns}	13.3±1.2^{ns}	8.1±1.2^{ns}

The significance levels are shown by "****" for P<0.0001(0.01%), "***" for P<0.001 (0.1%), "**" for P<0.01(1%), "*" for P<0.05 (5%) and "ns" which represents non-significant. Means of each column are compared to means of the next column

The percentage change of DG content in SDO (DG_{SDO_i}) with respect to the level of DG in soybean oil (DG_{soy}) was derived using Eq. (5. 2):

$$\delta_{DG_i} = \left(\frac{DG_{SDO_i} - DG_{soy}}{DG_{soy}}\right) \times 100 \qquad (5.\ 2)$$

where δ_{DG_i} is the percentage change and DG_i stands for DG of SDO in the hour i.

$SCCO_2$ dramatically increased (1700%) the percentage change of total DG content in SDO relative to the DG content in soybean oil after 8 hours of reaction (Figure 5. 3).

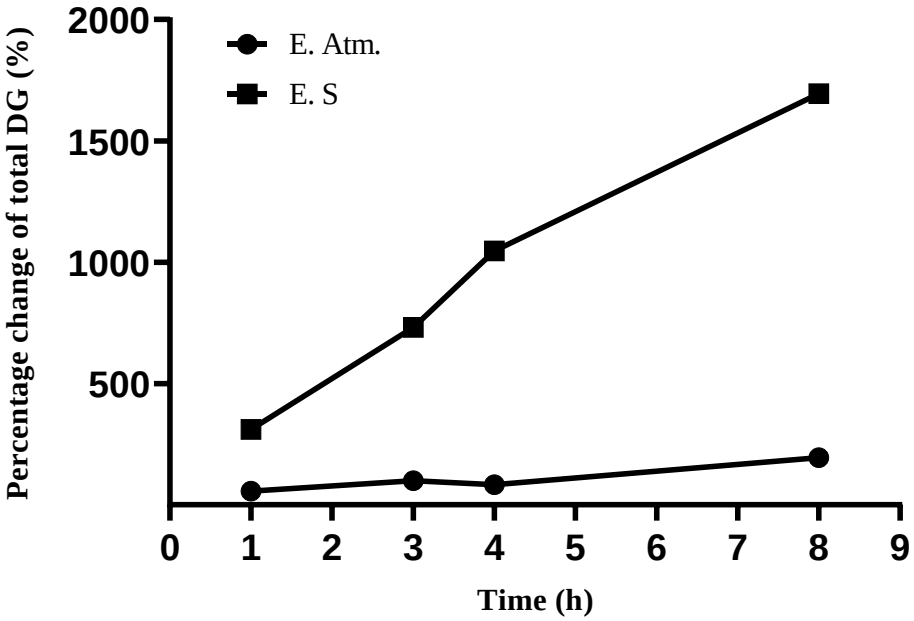


Figure 5. 3 Percentage change of total DG level in SDO relative to that of soybean oil with time of enzymatic glycerolysis under atmospheric (E. Atm.) and $SCCO_2$ (E. S) pressures

5.5.2 EFFECT OF PROCESS PARAMETERS ON ENZYMATIC GLYCEROLYSIS IN $SCCO_2$

In order to maximize the production of total DG and 1,3-DG levels and minimize the MG and FFA levels, the interesterification between soybean oil and glycerol in the presence of immobilized Novozym 435 lipase in contact with $SCCO_2$ needed to be optimized. Therefore, the effect of four independent variables, namely pressure, reaction temperature, time, and enzyme load on the contents of DG, MG, and FFA in the SDO after the enzyme-catalyzed reaction was investigated (Table 5. 4).

Table 5. 4 Effect of time, enzyme load, temperature and pressure on the contents of DG, MG, and FFA after enzymatic glycerolysis in $SCCO_2$

Effect of pressure (bar) at 60°C after 4 h of reaction with 3% enzyme							
Component	**80**	**100**	**150**	**300**	**450**		
1,3-DG	**12.2±0.8******	11.7±0.6^{ns}	8.7±0.4 ^{ns}	10.6±0.2^{ns}	12.7±1.3^{ns}		
Total MG	1.7±0.1^{ns}	**2.6±0.2*****	**1.5±0.1*****	1.6±0.1^{ns}	**2.6±0.0** ***		
Total DG	**21.8±1.1******	20.3±0.6^{ns}	**14.3±0.6***	17.6±0.4^{ns}	21.5±2.4^{ns}		
FFA	**4.1±0.6*****	**6.6±0.1***	**2.5±0.1*****	2.9±0.3^{ns}	4.7±0.6^{ns}		
Effect of temperature (°C) at 80 bar after 4 h of reaction with 3% enzyme							
Component	**40**	**60**	**70**				
1,3-DG	**6.4±0.3****	**12.2±0.8*****	11.7±0.2^{ns}				
Total MG	**1.4±0.1****	1.7±0.1 ^{ns}	**2.2±0.8*****				
Total DG	**12.3±0.2*****	**21.8±1.1*****	20.6±0.5 ^{ns}				
FFA	**3.4±0.1****	4.1±0.6^{ns}	4.6±0.2 ^{ns}				
Effect of time (h) at 60°C and 80 bar with 3% enzyme							
Component	**1**	**2**	**3**	**4**	**5**	**8**	**12**
1,3-DG	4.0±0.3^{ns}	5.7±0.1^{ns}	**8.7±0.5***	**12.2±0.8****	11.2±0.5^{ns}	**15.6±0.5****	17.8±0.3^{ns}
Total MG	**0.6±0.2*****	0.8±0.1^{ns}	**1.5±0***	1.7±0.1 ^{ns}	**1.1±0.0***	**6.2±0.1******	**1.8±0.0***
Total DG	**7.6±0.4****	11.0±0.8^{ns}	**15.8±1.0***	**21.8±1.1****	19.2±1.0^{ns}	**27.6±0.9*****	30.4±0.6^{ns}
FFA	**2.5±0.4***	1.7±0.6^{ns}	2.7±0.1^{ns}	4.1±0.6^{ns}	3.1±0.1^{ns}	**5.9±0.0****	5.4±0.0^{ns}
Effect of enzyme load (%) at 60°C and 80 bar after 4h of reaction							
Component	**3**	**6.5**	**10**				
1,3-DG	**12.2±0.8*****	**16.6±0.4***	21.1±1.0^{ns}				
Total MG	**1.7±0.1*****	**2.4±0.2***	**3.7±0.2****				
Total DG	**21.8±1.1******	**28.6±0.7***	**36.2±1.9***				
FFA	**4.1±0.6***	**10.8±0.0*****	**14.7±0.9***				

Means of each column are compared to means of the next column. Means of the first column are compared to the control (column of the soybean oil in Table 5.3)

5.5.2.1 EFFECT OF PRESSURE

The effect of pressure on the lipase-catalyzed esterification of glycerol and soybean oil at a molar ratio of 0.5 at 60 °C and enzyme load of 3% (w/w of initial substrates) after 4 h of reaction was investigated and the results are shown in Table 5. 4. The results indicate that pressure had a significant effect on the synthesis of total DG in the pressure ranges from 0 to 80 bar and 100 to 150 bar. However, the total DG did not change significantly from 80 to 100 bar and from 150 bar to 450 bar. Furthermore, the total DG level at a pressure of 80 bar (22±1.1%) is very close to total DG level at 450 bar (21.5±2.4%). However, it decreases with an increase in pressure from 100 to 300 bar. The reduction in total DG can be explained by the effect of pressure on the phase behaviour of the reaction mixture, reaction rate constant, and the reactants' solubility (Fornari and Stateva 2014; Jenab 2013). With a pressure increase the density of the oil increases and this results in a reduction of mass transfer in the supercritical phase at a constant temperature (Jenab 2013). Furthermore, the increase of solubility of CO_2 in the lipid phase results in dilution of the reactants in the lipid phase (Jenab 2013).

The results shown in Figure 5. 4 reveal that the 1,3-DG level is maximized at 450 bar (12.7±1.3%) but its level is close to the 1,3-DG amount at 80 bar (12.2±0.8%). The increased level of total DG and 1,3-DG that occurs from 300 to 450 bar may be explained by the enhanced solubility of $SCCO_2$ (Jenab, Temelli, and Curtis 2013) and reduction in the medium viscosity (Lubary 2011). Steytler et al. (1991) reported that the synthesis of butyl laurate with Novozym 435 was not significantly influenced by high pressure (500 bar). The effect of pressure on enzyme stability is not so significant in the range of 0 to 300 bar (Fornari and Stateva 2014) because within the pressure range of 100 to 400 bar which is typically used, only certain

reversible conformational changes occur in the enzyme (Rezaei, Temelli, and Jenab 2007) which is different to the irreversible denaturation that happens at very high pressures (Jenab 2013).

It is important to note that the enzymatic glycerolysis conducted at atmospheric conditions reached the 4±0.7% level of 1,3-DG after 8 h versus 1h at $SCCO_2$ pressures (Table 5. 3). These particular results indicate that at higher pressures, the effect of CO_2 solubilization in the lipid phase is substantial and therefore has an important impact on the production rate of 1,3-DG and total DG. This is in agreement with other studies which show the positive influence of pressure on increasing the reaction rate of DG production under $SCCO_2$ conditions compared to reactions that are conducted in hexane or under solvent-free conditions (Lubary 2011).

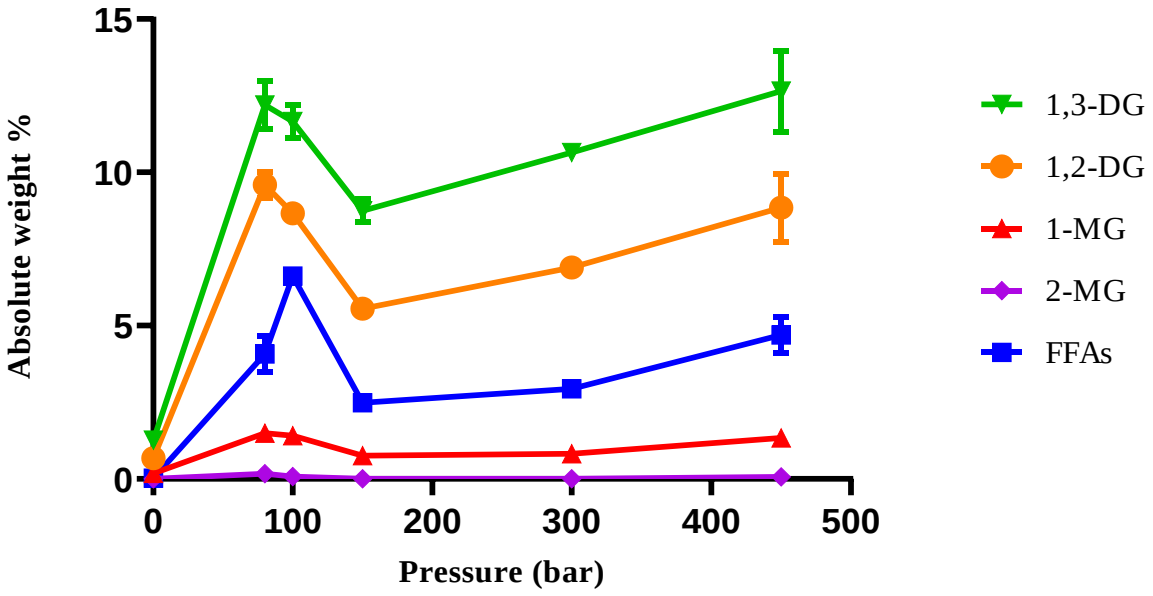


Figure 5. 4 Effect of pressure on the level of DG, MG, and FFA after lipase-catalyzed esterification between soybean oil and glycerol (2:1) with Novozym 435 at 3% (w/w of initial substrates) under $SCCO_2$ medium at 60 °C after 4 h

Jenab et al. (2013) reported the effect of pressure on enzymatic interesterification between canola oil and fully-hydrogenated canola oil in the $SCCO_2$ medium by changing the pressure between 10 and 250 bar at 65 °C. Their study showed the degree of interesterification increased with pressure from 10 to 100 bar and decreased with a further increase up to 250 bar (Jenab, Temelli, and Curtis 2013). Similar outcomes can be discovered in the literature for the synthesis of butyl butyrate from vinyl butyrate and 1-butanol under $SCCO_2$ conditions with Novozym 435 (Lozano et al. 2004). Ester synthesis decreased with pressure increase from 80 to 150 bar at any fixed temperature between 40 and 60 °C; the authors reported the best result was obtained at 60 °C and 80 bar (Lozano et al. 2004).

Moquin et al. (2006) preferred 100 bar because it is a pressure that can be reached without the use of a high-pressure pump and so results in a low energy consumption and a positive economic impact. Considering the content of 1,3-DG or total DG in SDO, the cost of the equipment and the reduction of energy consumption, 80 bar was the optimum operating pressure from the results of the present study.

5.5.2.2 EFFECT OF TEMPERATURE

Figure 5. 5 illustrates the influence of temperature upon the components of SDO, especially the level of 1,3-DG, for a set of experiments carried out at a constant pressure of 80 bar with 3% enzyme for 4h. The level of 1,3- and 1,2-DG increased with temperature from 40 to 60 °C (Figure 5. 5). The content of DG (22±1.1%) was maximized at 60 °C and increased significantly compared to DG level at 40 °C (Table 5. 4). This increase in DG level with increasing the temperature from 40 to 60 °C is probably due to a direct temperature effect on the reaction rate

and enzyme activation (Fornari and Stateva 2014). At 70 °C, there was a decrease in DG and 1-MG contents that could be attributed to the thermal denaturation of the enzyme (Habulin et al. 2007). However, there was a non-significant increase for FFA contents that could be due to soybean oil hydrolysis at temperatures higher than 60 °C (Figure 5. 5). Therefore, 60 °C was the optimal temperature to maximize the DG level for Novozym 435 activity in this reaction system in contact with the $SCCO_2$ medium.

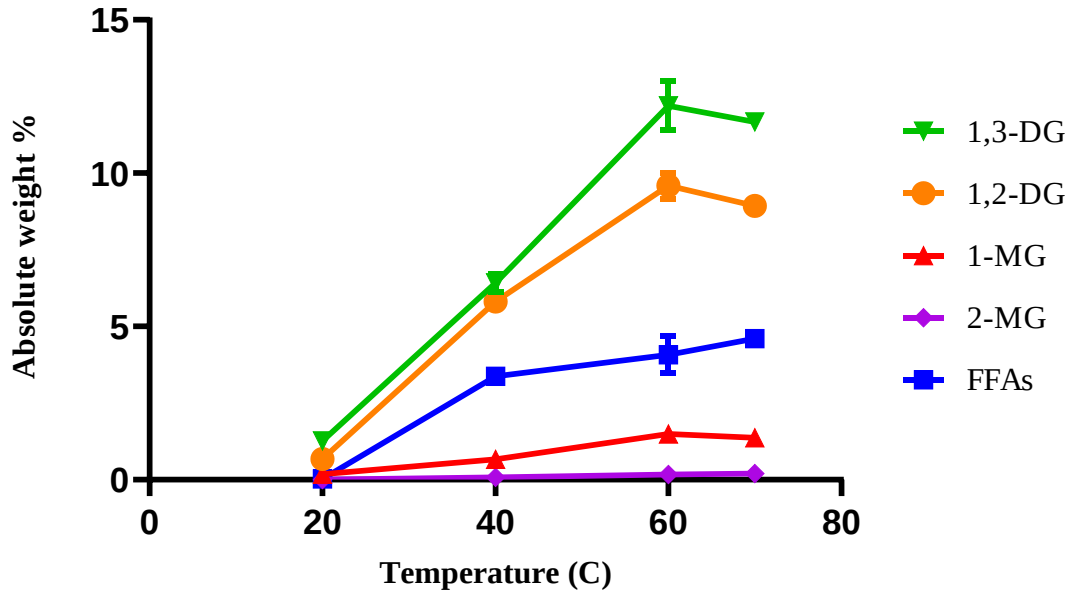


Figure 5. 5 Effect of temperature on the level of DG, MG, and FFA after lipase-catalyzed esterification between soybean oil and glycerol (2:1) with Novozym 435 at 3% (w/w of initial substrates) under $SCCO_2$ medium at 80 bar after 4 h

Habulin et al. (2007) reported that the esterification of citronellol with lauric acid increased as temperature increased from 50 to 60 °C. They reported that the reason the reaction rate decreased at 70 °C could be due to a change of the density or some other properties of the $SCCO_2$/solvent mixture. They also stated that temperatures above 60°C could deactivate the crude lipase due to

protein denaturation (Habulin et al. 2007). For the lipase-catalyzed esterification of lavandulol with acetic acid in contact with $SCCO_2$ the maximum conversion was obtained at 60 °C (Olsen et al. 2006).

5.5.2.3 EFFECT OF TIME

To assess the effect of reaction time on the synthesis of each component of SDO lipase-catalyzed esterification of soybean oil, the reaction was conducted at 60 °C and 80 bar and an enzyme load of 3% (weight of initial substrates). As shown in Table 5. 4, the total DG level reached its maximum level of about 30% after 12 h reaction. Kristensen, Xu and Mu (2005) reported that the best DG yield from glycerolysis of rapeseed oil was obtained after 7 hours of reaction with Novozyme 435 at atmospheric conditions. They continued the reaction time until 23 hours. However, the DG yield increased only 3.3% relative to the first yield (Kristensen, Xu, and Mu 2005). Dhake et al. (2011) evaluated the transesterification of citronellol and vinyl acetate at 80 bar and the maximum amount of the citronellol ester was obtained after 12 h reaction. Figure 5. 6 illustrates that the highest level of 1,3-DG (18±0.3%) was obtained after 12 hours of reaction. All the components of SDO were maximized after 12 h of reaction. However, only 1,2-DG and 1,3-DG levels were greater than 10%. As always, shorter reaction time with optimum yield is an advantage for any reaction in industry: considering the small differences between total DG yield of 8h (28±0.9%) and 12h (30±0.6%), the reaction time of 8 h was selected as the optimal reaction time.

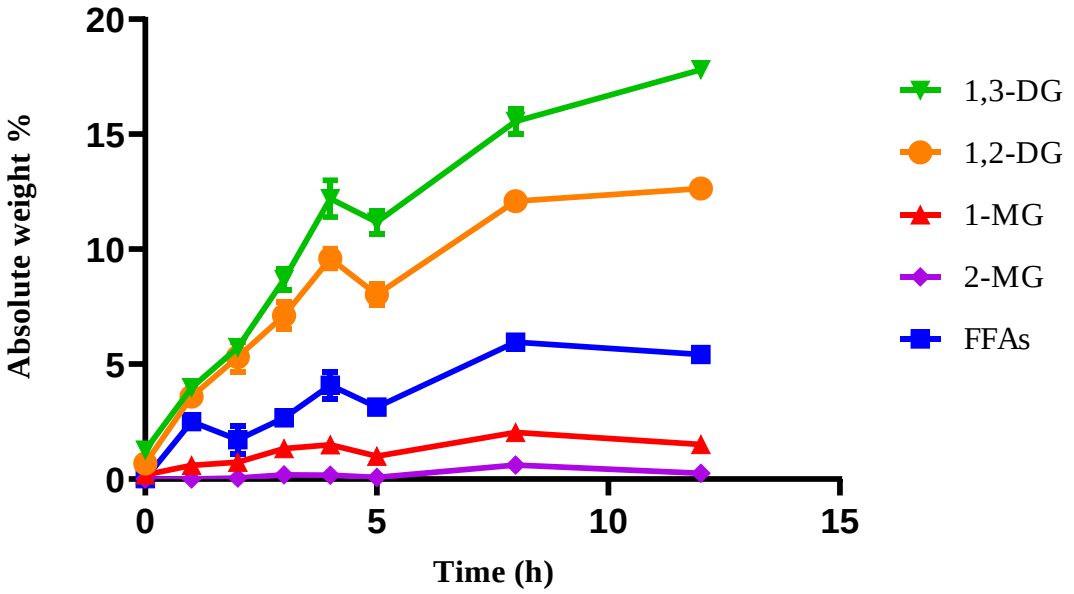


Figure 5. 6 Effect of reaction time on the level of DG, MG, and FFA after lipase-catalyzed esterification between soybean oil and glycerol (2:1) with Novozym 435 at 3% (w/w of initial substrates) in contact with $SCCO_2$ at 60 °C and 80 bar

5.5.2.4 EFFECT OF ENZYME LOAD

The effect of Novozym 435 addition at 3, 6.5, and 10% (w/w of total substrates) on the PEG and FFA contents of SDO was studied at 60 °C and 80 bar. As shown in Figure 5. 7 the 1,3-DG reached its maximum level of 21±1.0% after 4 h for enzyme loads of 10%. It needed about 8 h to reach 16±0.5% when the enzyme load was lower at 3%. The amount of total DG level increased significantly from 29±0.7% with the enzyme load of 6.5% to 36±1.9% with the enzyme load of 10% (Table 5.4). Based on these results, the enzyme load of 10% was chosen as an optimum load. Similar results from the literature showed that the optimum range of the enzyme load is between 5 and 15% (Jenab et al. 2014; Sun et al. 2012).

Further addition of enzyme (more than 15%) did not increase the yield of fatty acid ester in an alcoholysis reaction of coconut oil (Sun et al. 2012), but it did speed up the reaction rate (Lee et al. 2015). High concentration of enzyme can increase the viscosity of the reaction medium (Jenab 2013) which results in saturation in product yield for the reaction (Lee et al. 2015). This causes limitation in availability of the substrate to bind with the active site of the excess enzyme (Jenab 2013).

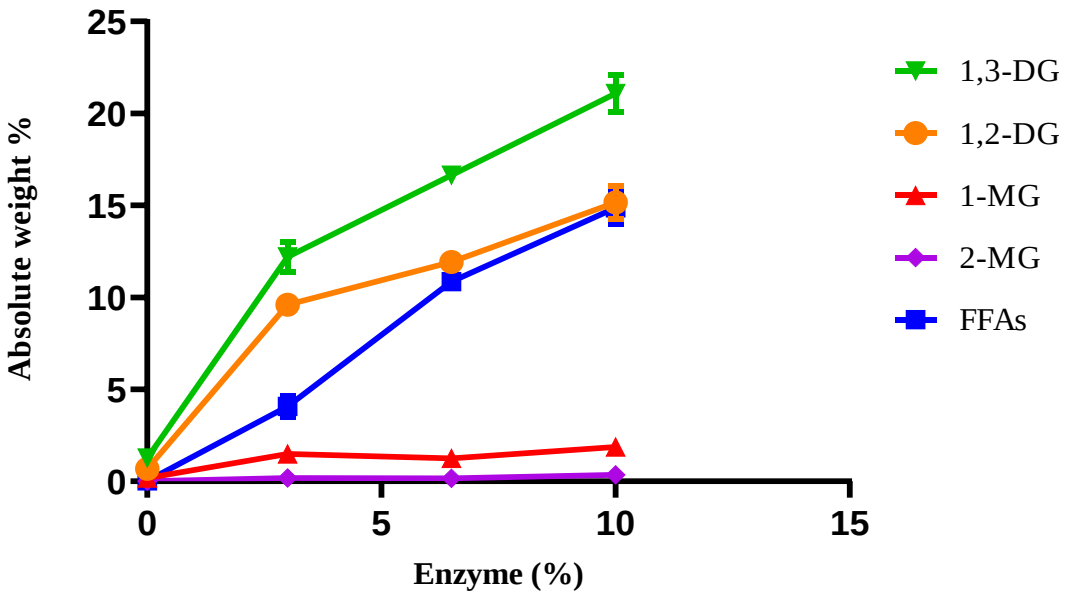


Figure 5. 7 Effect of enzyme load on the level of DG, MG, and FFA after lipase-catalyzed esterification between soybean oil and glycerol (2:1) in contact with $SCCO_2$ at 60 °C and 80 bar after 4 h reaction

5.6 MATHEMATICAL MODEL DERIVATION

In order to find a mathematical model for the DG value, averaged from different replications of the same experimental conditions, in terms of four independent variables (time, pressure, temperature and enzyme load), a set of analyses was conducted on the experimental data listed in Table 5. 1. The independent variables were first converted to a set of values in the range of [-1, +1]. The idea of theses level of assigned values (from-1to1) was obtained from the study of Haar et al (2015). The results are shown in Table 5. 5.

Table 5. 5 Variables, values and assigned values for mathematical modelling

Variables	**Symbol**	**Value (x_i)**	**Assigned Value ($x_{i,n}$)**
Time (h)	x_1	1; 2; 3; **4**; 5; 8; 12	-1; -0.5; -0.25:**0**; 0.25; 0.5; 1
Pressure (bar)	x_2	**80**; 100; 150; 300; 450	**0**; 0.05; 0.19; 0.59; 1
Temperature (°C)	x_3	40; **60**; 70	-1.0; **0**; 1.0
Enzyme content % (w/w)	x_4	**3**; 6.5; 10	**0**; 0.5; 1.0

The reason behind the selection of the assigned values for the independent variables is to force a certain value of each variable to be zero (bolded values shown in Table 5. 5). The specific value for each independent variable is the one used in other experiments to investigate the effect of other variables on the DG value. For instance, the assigned value of the pressure at 80 bar is equal to zero because this pressure was used when the other three variables were changed. Then, the relationship between the DG values and assigned values of each independent variable is found in Eq. (5. 3)

$$DG_i = f_i(x_{i,n}) + b_i \quad i = 1, 2, 3, 4 \tag{5. 3}$$

where b_i $(i = 1, 2, 3, 4)$ are constant values and n represents for assigned values.

According to Table 5. 5, there is a relationship between each independent variable (x_i) and the corresponding assigned values $(x_{i,n})$:

$$x_{i,n} = g_i(x_i) \qquad i = 1, 2, 3, 4 \tag{5. 4}$$

Having obtained the functions $g_i(i = 1, 2, 3, 4)$ using a least squares technique, they are used in the set of Eq. (5. 3) to derive a unique multivariable function for DG level in SDO in terms of all four independent variables.

$$DG = (f_1 o g_1)(x_1) + (f_2 o g_2)(x_2) + (f_3 o g_3)(x_3) + (f_4 o g_4)(x_4) + (\frac{b_1+b_2+b_3+b_4}{4}) \tag{5. 5}$$

where $(f_i o g_i)$ is the composition of functions f_i and g_i. Details of the functions are provided in Appendix A. 2. For the DG values obtained from the experiment, the following mathematical model was found based on the above procedure (Eq. (5. 6)).

$$DG_{model} = \sum_{i=1}^{4} a_i {x_i}^4 + \sum_{i=1}^{4} b_i {x_i}^3 + \sum_{i=1}^{4} c_i {x_i}^2 + \sum_{i=1}^{4} d_i x_i + e \tag{5. 6}$$

The coefficients a_i, b_i, c_i and d_i $(i = 1,2,3,4)$ are defined in Table 5. 6, and $e = 20.30313$.

Table 5. 6 Values of coefficients defined in the mathematical model

a_1	-3.93182×10^{-5}	b_1	1.95177×10^{-3}	c_1	-2.00486×10^{-1}	d_1	4.37491
a_2	0	b_2	-1.52591×10^{-6}	c_2	1.39836×10^{-3}	d_2	-0.36994
a_3	-1.56204×10^{-5}	b_3	2.14459×10^{-3}	c_3	-8.49066×10^{-2}	d_3	0.77547

5.7 MODEL VERIFICATION

As known, the R^2 is a measure of merit for the regression model which indicates how well the model explains the behaviour of the system. In order to verify the validity of the model, the R^2 value was calculated using the following equation:

$$R^2 = \frac{S_t - S_r}{S_t} \quad (5.\ 7)$$

where,

$$S_t = \sum_{j=1}^{N} (DG_{exp._j} - \overline{DG})^2 \quad (5.\ 8)$$

$$\overline{DG} = \frac{\sum_{j=1}^{N} DG_{exp._j}}{N} \quad (5.\ 9)$$

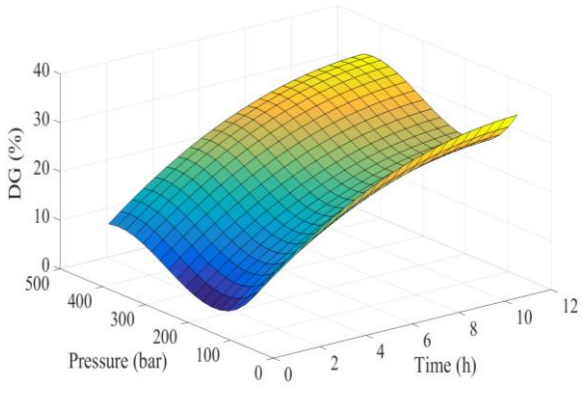
$$S_r = \sum_{j=1}^{N} (DG_{exp._j} - DG_{model_j})^2 \quad (5.\ 10)$$

The values of DG obtained from the model are listed in Table 5. 7. Based on the results, $R^2$is equal to 0.94 which confirms that the derived model is satisfactorily valid for the experimental results and can be used to determine the DG value in SDO in terms of other values of the independent variables. However, the model may be valid only for the values of independent variables inside the range specified in Table 5. 5.

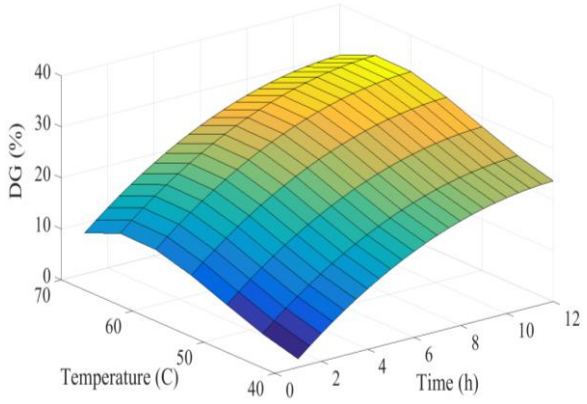
Table 5. 7 DG results obtained from experiment and model with different variables during enzymatic glycerolysis performed for model validation

j	**DG value obtained from Experiment ($DG_{exp._j}$)**	**DG value obtained from model (DG_{model_j})**
1	7.6±0.4	10.9
2	11.0±0.8	14.7
3	15.8±1.0	18.1
4	21.8±1.1	21.1
5	19.2±1.0	23.8
6	27.6±0.9	29.8
7	30.4±0.6	33.0
8	20.3±0.6	18.0
9	14.3±0.6	13.4
10	17.6±0.4	16.2
11	21.5±2.4	20.2
12	12.3±0.2	11.9
13	20.6±0.5	18.3
14	28.6±0.7	28.3
15	36.2±1.9	35.5

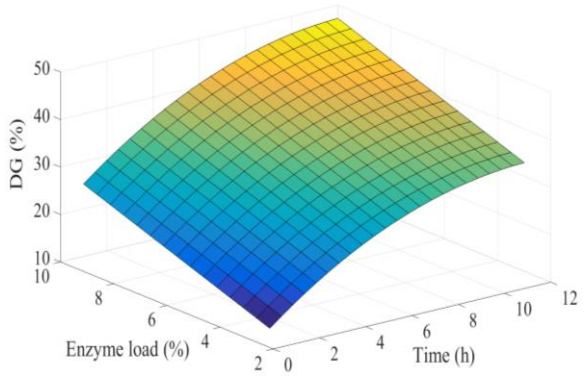
Having obtained a mathematical model for the DG value, the effect of the independent variables on the DG yield was pictorially shown by plotting the DG in terms of the combination of two variables. As a result, six plots were obtained (Figure 5. 8).


DG (%)
Pressure (bar)
Time (h)

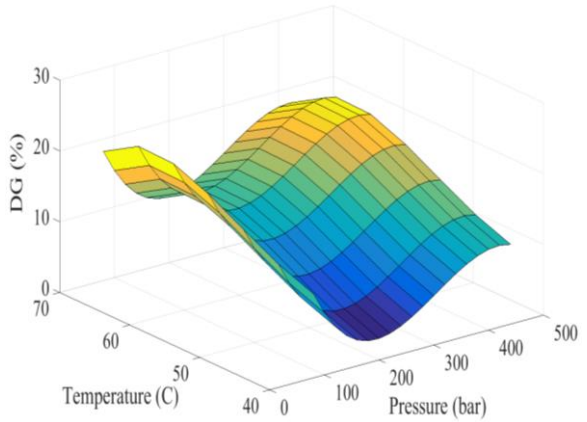
(a)


DG (%)
Temperature (C)
Time (h)

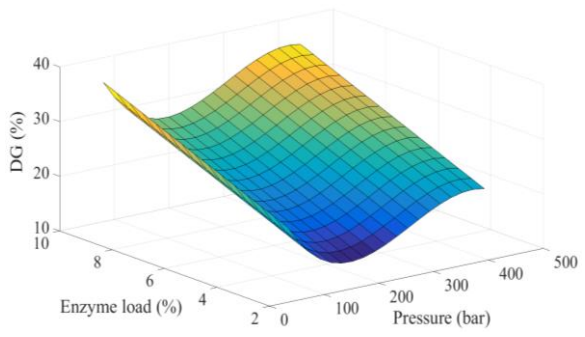
(b)


DG (%)
Enzyme load (%)
Time (h)

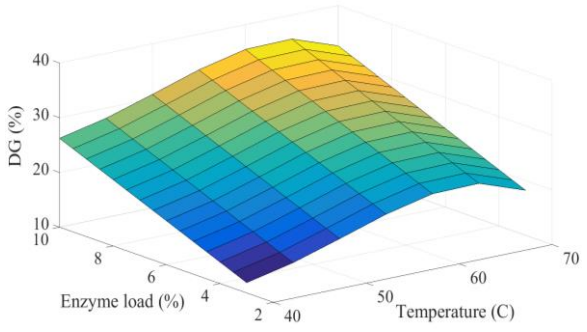
(c)


DG (%)
Temperature (C)
Pressure (bar)

(d)


DG (%)
Enzyme load (%)
Pressure (bar)

(e)


DG (%)
Enzyme load (%)
Temperature (C)

(f)

Figure 5. 8 Three-dimensional surface plot of the DG yield [%(w/w)] obtained from the model depending on:

(a) pressure (bar) and time (h) (temperature = 60 °C; enzyme Load = 3%)

(b) temperature (°C) and time (h) (pressure = 80 bar; enzyme load =3%)

(c) enzyme load (%) and time (h) (temperature = 60 °C; pressure = 80 bar)

(d) temperature (°C) and pressure (bar) (enzyme Load = 3%; time = 4 h)

(e) enzyme load (%) and pressure (bar) (temperature = 60 °C; time = 4 h)

(f) enzyme load (%) and temperature (°C) (pressure = 80; time = 4 h)

With reference to Figure 5. 8, the time and enzyme load affect the DG yield in an ascending manner, *i.e.*, with longer time and greater enzyme load, the higher the DG value; however, there is no dramatic increase in the DG value after 8 h. With respect to the pressure and temperature, it is clearly shown that the pressure of 150 bar minimizes the DG value in the determined range. On the other hand, the temperature of 60 °C results in the maximum DG level compared to other temperatures in the range (40-70 °C).

5.8 VALIDATION

Based on the plots obtained from the mathematical model, the DG value should be a maximum at a time of 12 h, the pressure equal to 80 bar, a temperature of 60 °C and an enzyme load of 10% (w/w) of total substrates. Since the DG value did not change dramatically after 8 h, this time was chosen, instead of 12 h, to conduct one more experiment aimed at confirming the strength of the model for maximizing DG content. Therefore, the final experiment was performed based on x_1=8 h, x_2=80 bar, x_3=60 °C and x_4=10%, and the DG value obtained was

equal to 43.7%. As anticipated, the result of this experiment matched well with that of the mathematical model because the corresponding DG value obtained from the model equals 44.1%.

5.9 CONCLUSION

This study addressed the feasibility of producing a soybean oil-based DAG oil (SDO) under $SCCO_2$ using enzymatic glycerolysis, in a sustainable manner, without any purification. This production procedure has a potential of being used in food manufacturing where there is a challenge in production of structured, healthier and "greener," fats rather than the available *trans* and saturated fat-laden food products. The most important conclusions are summarized as follows

1- The analysis results show that ^{31}P-NMR is an effective tool for analysis of the monoglycerides, diglycerides and free fatty acid contents of soybean oil and SDO. Due to the high accuracy of ^{31}P-NMR, the calculation of relative changes of total DG in soybean oil and SDO was possible.

2- The maximum level of total DG obtained under the $SCCO_2$ conditions was 7 times higher than the total maximum DG level obtained from atmospheric conditions after 8h of reaction. Therefore, $SCCO_2$ is a useful reaction medium to produce the SDO with high DG levels through the enzymatic glycerolysis of soybean oil.

3- Glycerolysis in $SCCO_2$ with Novozym 435 can produce high levels of DG without generating significant amounts of MG.

4- The optimal reaction condition to obtain the maximum 1,3-DG content, 60% of total DG (44% of SDO) occurred at 60 °C, 80 bar and 8 h of reaction time with an

immobilized enzyme load of 10% (w/w of total substrates). The commercial lipase, Novozym 435, was very specific in the synthesis of DG under $SCCO_2$ conditions, particularly 1,3-DG, which is the healthier isomer than 1,2-DG.

5- A mathematical model was developed and validated which can be used to determine the DG content in SDO, in terms of four different independent variables (pressures, temperatures, times and enzyme loads) defined in a specific range for each variable.

6- Overall, this research contributed to the development of a unique, relatively fast and eco-friendly methodology for the production and analysis of value-added DAG oil from glycerol, a sustainable biomaterial source since glycerol is a byproduct of biodiesel production.

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CHAPTER 6

OVERALL CONCLUSIONS

6.1 CONCLUSIONS

The findings of the studies presented in this thesis deliver essential ideas for more sustainable alternative processes of synthesis and more practical techniques of analysis of structured lipids (SL) that possess functional and nutraceutical applications.

SLs are crucial complex lipid mixture because of their widespread application in the food, pharmaceutical, cosmetic and biodiesel industries (Kleiner and Akoh 2018). In the first study, different NMR methodologies were screened in order to find green, fast, accurate and reliable analytical techniques for analysis of the structured lipids. In addition, to prove that the composition of the SLs was influenced by enzyme and supercritical carbon dioxide, two different time course studies were designed under special consideration of maintaining mild reaction condition, such as conducting the reaction under low temperature to improve the health benefits of the product, and the economic and ecological benefits of the process (in the second and third studies).

In the first study, a comprehensive nuclear magnetic resonance (NMR) analysis of a high monoester mixture of soybean oil (HMMS) was conducted using ^{1}H, ^{13}C and ^{31}P spectroscopy. ^{1}H-NMR spectroscopy was effective for quick detection of the fatty acid composition, some plant sterols, glycerol esters and propylene glycol mono- and di-esters contained within the HMMS. It has previously been shown that ^{1}H-NMR can identify the fatty acid (FA) types in different vegetable oils in less than one minute (Castejon et al. 2014). Different regions of ^{13}C-

NMR spectra provide unique analytical information. The glycerol region was informative in providing the quantification necessary to conduct a yield conversion calculation. Details on fatty acid composition and the distribution of each FA at different positions of the triglycerides (TGs) was obtained by analyzing the carbonyl region. The olefinic region informed on unsaturated fatty acid composition, such as oleic acid, linoleic acid and linolenic acid of the acylglycerols of HMMS. The aliphatic region provided information on PUFA, MUFA and SFA of the mono- and di-esters within the HMMS using C16 data. The ^{31}P-NMR spectroscopy detected and determined the absolute amounts of 1-MG, 1,2-DG 1,3-DG and 2-MG. The ^{31}P-NMR, with more sensitivity than ^{13}C-NMR, demonstrated that there were significant signals for the PEGs of the HMMS which were not detectable with carbon NMR. In order to prove this research provides promising information for other studies, the analytical ability of ^{1}H-, ^{13}C- and ^{31}P-NMR for direct analysis of the un-purified products that are obtained from the enzymatic alcoholysis was assessed and compared to each other. The results verified that ^{31}P-NMR spectroscopy is a practical methodology with high-reproducibility for the precise detection and quantification of MGs, DGs and other minor compounds such as tocopherol and Free FAs.

The first study's findings informed on the selection of an appropriate nucleus for NMR analysis of PEG contents and FA composition and the distribution of FAs between the *sn*-2 and *sn*-1,3 positions on the TGs in order to conduct the second study. Thus, ^{31}P-NMR and ^{13}C-NMR were preferred for both quantification and qualification analyses to investigate PEG content and fatty acid composition within TGs of the HMMS of a time course study of an alcoholysis reaction in $SCCO_2$ conducted with and without enzyme.

Data from the second study demonstrated that enzymatic alcoholysis is more effective than non-enzymatic alcoholysis for the production of structured lipids in $SCCO_2$, and therefore the third study was conducted with the enzyme in $SCCO_2$ to produce the DAG enriched oil.

The third study addresses the feasibility of producing sustainably soybean oil-based DAG oil (SDO) by enzymatic glycerolysis in a solvent-free system without any purification. The first objective of this study was to compare the production of SDO under both atmospheric and $SCCO_2$ pressures to know how effective $SCCO_2$ is when Novozym 435 catalyzes the glycerolysis of soybean oil. ^{31}P-NMR was used for precise analysis of SDO because ^{31}P-NMR is a versatile technique for a rapid analysis of PEGs, FFA, and tocopherols concurrently and is an eco-friendly experiment, since it requires very little solvent in contrast to chromatographic analytical methodologies. The results showed that $SCCO_2$ provides a better reaction medium compared to atmospheric conditions for Novozym 435, which was very specific in the synthesis of DG, particularly 1,3-DG, in the glycerolysis of soybean oil. As the second objective, different independent variables including pressure, time, temperature and enzyme load were used to optimize the production of SDO where the amounts of DG (the main products) were to be maximized, and the by-products MG and FFA were to be minimized. The optimal reaction conditions were 60 °C, 80 bar and 8 h of reaction time with an immobilized enzyme load of 10% (w/w) of total substrates to obtain the SDO with 44% total DG. A mathematic model derived from these four independent variables was developed in order to maximize the amount of the DG. The model was satisfactorily validated and can be used by scientists and manufacturers to determine the DG value in SDO as a function of the pressure, temperature, time, and enzyme load.

The present thesis provides useful information for quick and accurate analysis and production of "greener" sustainable structured lipids to formulate healthier alternatives to *trans* and saturated fats for food manufacturers who must respond to consumer demand to provide equivalent or improved products compared to existing ones.

6.2 LIMITATIONS

The following areas would be considered as the main limitations of the current research project:

1. Since the findings of the present research are aimed at food manufacturers, conducting an enzymatic alcoholysis under $SCCO_2$ in a continuous packed bed reactor is required in order to provide continuous operation and greater performance compared to batch reactors. Therefore, it will be useful to compare the results of the enzymatic alcoholysis under $SCCO_2$ conditions when conducted in a continuous packed bed reactor with a batch reactor.
2. Since experimental conditions should be identical for good experimental design, we used fresh enzyme. However, it is valuable to run a test to know the extent of reusability and the stability of the immobilized enzyme for this enzymatic alcoholysis under $SCCO_2$ conditions.

6.3 FINAL REMARKS

Evaluating the ability of ^{1}H-, ^{13}C- and ^{31}P- NMR analyses of the un-purified products obtained directly after the enzymatic reaction showed the advantages of each technique for a specific purpose. These NMR spectroscopy techniques, using different nuclei, can be applied for every step of industrial production of other similar complex lipid mixtures. NMR spectroscopy can be

used for direct analysis of a substrate mixture before reaction, during the reaction, after the reaction, and the final products after purification. The current research thesis offers promising information which is practical for researchers in the field of NMR analysis and enzymatic interesterification under $SCCO_2$ pressure. The outcome of the methodology section regarding the alcoholysis reaction is the development of production methods for other structured lipids in a $SCCO_2$ system. The findings of the influence of enzyme and $SCCO_2$ on acyl glycerol composition and fatty acid profiles in the final reaction products will aid in the production of high yields of products of interest in a shorter reaction time. A high yield of favorable products in a shorter reaction time can be achieved by knowing how enzyme and $SCCO_2$ influence acyl glycerol composition and fatty acid profile in the final reaction products. Furthermore, the derivation of a mathematical model is useful for creating equations for other products of the reaction, such as a model for minimizing the amount of FFA or MGs, by following the mathematical model extraction steps. There are remarkable signs of progress in utilizing immobilized lipases in interesterification reactions on a commercial scale since manufacturers have become aware of the importance of acting and responsibility for using green technology to make low *trans*-fat products. Conducting enzymatic alcoholysis under $SCCO_2$ is a modern technology, which is a practical alternative to conventional methods using toxic chemical catalysts. Based on the observations of this research, Novozym 435 works under $SCCO_2$ conditions, thus enzymatic interesterification is a beneficial technology for production of SLs. Moreover, enzymatic glycerolysis under $SCCO_2$ produces greater amounts of DG compared to the same reaction under atmospheric conditions. The substantial yield of specific products of the lipid mixture and the considerable reduction in reaction time lead to significant energy and cost savings. This thesis is focused on greener alternative processes of interesterification operations

and accurate techniques of analysis of SLs. Integration of these methodologies contributes to the development of complex lipid mixtures with more functional and nutraceutical applications in contrast to non-ecofriendly techniques.

6.4 HIGHLIGHTS OF THIS STUDY

- ^{31}P-NMR is a reliable sensitive method, detects low level of all partially esterified glycerols, free fatty acids and tocopherols of un-purified structured lipids and quantifies them with an excellent accuracy and repeatability.

- Different reaction times provide various compositions of PEGs and FAs in a lipase-catalyzed interesterification (LIE) system. A short reaction time of 4h provided the highest 2-MG yield, of 21%, and the highest amount of polyunsaturated fatty acid (PUFA) (65%) in the triglycerides (TGs) of HMMS.

- $SCCO_2$ is absolutely critical for the effective use of Novozyme 435 for the production of high levels of DGs, particularly 1,3-DG, through enzymatic glycerolysis.

- A model was developed, and nicely validated, to define the composition of diacylglycerol-enriched structured lipids with enhanced DG levels.

6.5 FUTURE DIRECTIONS

Future studies should be oriented toward the processes before and after the enzymatic reaction in $SCCO_2$. It could help to establish plants with greener production lines from oil extraction

processes to the process of production of food or pharmaceutical products. $SCCO_2$ extraction can be used to extract oil from any oil sources such as oilseeds, oily fruits etc. However, $SCCO_2$ extraction also needs an optimization of the different independent variables such as pressure, time, temperature, co-solvent percentage or crushed seed size to give the best yield of oil. In addition, $SCCO_2$ extraction can be applied after enzymatic reaction to obtain some highly pure products of interest such as pharmaceutical and nutraceutical products or food additives.

6.6 REFERENCES

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APPENDICES

APPENDIX 1: SUPPORT INFORMATION FOR CHAPTER 3

Table A. 1. 1 ^{1}H-NMR chemical shifts and peak multiplicity of linoleic acid, mono-, di- and tri-olein as model pure compounds and propylene glycol mono- and –di-esters

Proton	Multiplicity	Compound	Chemical shift (ppm)								
			β-Sitosterol*	Stigmasterol*	1-MP†	2-MP†	1,2-DP†	1,3-Diolein	1-Monoolein	Triolein	Linoleic acid
H18	singlet	Axial CH3	0.67								
H18	singlet	Axial CH3		0.69							
CH_2CH_3	triplet	OL chains						0.87	0.87	0.87	
CH2CH3	triplet	LO chains									0.88
CH3	doublet	All acyl chains			1.18						
CH3	doublet	All acyl chains				1.21					
–(CH2)n-	envelope	All acyl chains						1.30	1.30	1.30	
CH3	doublet	All acyl chains					1.31				
OCOCH2CH2	multiplet	All acyl chains						1.62	1.62	1.60	1.62
CH2CH=CH	multiplet	OL chains						2.00	2.00	2.00	
CH2CH=CH	multiplet	LO chains									2.04
OCOCH2CH2	triplet	All acyl chains of TG								2.30	
OCOCH2CH2	triplet	All acyl chains of FA, MG and 1,3-DG						2.34	2.34		2.34
CH=CHCH2CH=CH	multiplet	LO chains									2.76
CH2OH	broad	Glycerol of 1-MG/*sn*-3							3.59		
CHOH	multiplet	Glycerol of 1-MG/*sn*-2							3.92		
CHOH	broad	Glycerol of 1,3-DG/*sn*-2						4.07			
CH2OCO	doublet of doublet	Glycerol of 1,3-DG/*sn*-1,3						4.13			
CH2OCO	doublet of doublet	Glycerol of TG/*sn*-1,3								4.14	
CH2OCO	doublet of doublet	Glycerol of 1-MG/*sn*-1							4.15		
CH2OCO	doublet of doublet	Glycerol of 1,3-DG/*sn*-1,3						4.18			
CH2OCO	doublet of doublet	Glycerol of TG/*sn*-1,3								4.28	
CHOCO	quintet	Glycerol of TG/*sn*-2								5.25	
CH=CH	multiplet	All acyl chains						5.34	5.34	5.34	5.28-5.40

* These data are taken from study of Suttiarporn et al (Suttiarporn et al. 2015) and †These data are taken from study of Andries et al (Andreis, Meić, and Veksli 1983)

Table A. 1. 2 ^{13}C-NMR chemical shifts and position of each chain of standard linoleic acid, mono-, di-, and tri-olein

Component name	Carbon	Chemical shifts (ppm)			
		1,3-Diolein	Monoolein	Triolein	Linoleic acid
OL	TG/*sn*-1,3			62.08	
OL	TG/*sn*-2			68.90	
OL	1,3-DG/*sn*-2	68.11			
OL	1,3-DG/sn-1/*sn*-3	64.90			
	1-MG/*sn*-1		63.33		
OL	1-MG/*sn*-3		65.08		
	1-MG/*sn*-2		70.23		
LO	FA/C1				180.48
OL	1-MG/C1		174.31		
OL	1,3-DG/C1	173.72			
OL	TG/C1/*sn*-1			173.09	
OL	TG/C1/*sn*-2			172.69	
LO	FA/C13				130.14
LO	FA/C9				129.95
LO	FA/C10				128.03
LO	FA/C12				127.86
OL	all acylglycerols/ C9	129.57	129.66	129.62	
OL	all acylglycerols/ C10	129.87	129.99	129.91	
OL, LO	(MG, DG, TG, FA)/C2	33.97	34.10	34.11	34.07
OL, LO	(MG, DG, TG, FA)//C16	31.81	31.86	31.85	31.49
OL, LO	(all acylglycerols)/C12	29.66	29.72	29.71	
LO	(all acylglycerols)/C7	29.56	29.64	29.64	
OL	(MG, DG, TG)/C14	29.43	29.48	29.47	29.55
OL	(MG, DG, TG)/ C13, C15	29.22	29.28	29.27	29.31
OL	(all acylglycerols, FA)/C5	29.06	29.12	29.12	29.10
OL, LO	(all acylglycerols, FA)/C4	29.00	29.06	29.05	29.00
OL	(all acylglycerols)/C8, C11	27.11	27.18	27.16	27.16
OL, LO	(all acylglycerols)/C11			24.82	25.59
OL, LO	(all acylglycerols)/C3	24.77	24.85	24.78	24.61
OL	(all acylglycerols)/C17	22.56	22.63	22.62	
LO	all acylglycerols)/C17				20.54
OL, LO	(all acylglycerols)/C18	14.00	14.06	14.03	14.01

Table A. 1. 3 ^{13}C-NMR chemical shifts and position of each chain of a high monoester mixture of soybean oil and soybean oil

Component Name	Chemical shifts (PPM)		Carbon position
	Soybean oil	HMMS	
			Glycerol carbons
Any chain	-	16.25	2-MP/*sn*-3
Any chain	-	16.54	1,2-DP/*sn*-3
Any chain	-	18.87	1,2-Propanediol/ *sn*-3
Any chain	-	19.19	1-MP/*sn*-3
Any chain	62.11	62.11	TG/*sn*-1,3
Any chain	-	65.39	1,2-DP/*sn*-1
Any chain	-	65.90	2-MP/*sn*-1
Any chain	-	66.08	1-MP/*sn*-2
Any chain	-	67.98	1,2-DP/*sn*-2
Any chain	-	68.05	1,2-Propanediol/ *sn*-2
Any chain	-	68.23	1,2-Propanediol/ *sn*-1
Any chain	68.92	68.89	TG/*sn*-2
Any chain	-	69.42	1-MP/*sn*-1
Any chain	-	71.77	2-MP/*sn*-2
			Olefinic carbons
LN	127.14	127.11	TG/C15
LN	127.77	127.75	TG/C10
LO	127.92	127.90	TG/C12
LO	128.09	128.06	TG/C10
LN	128.24	128.22	TG/C13
LN	128.29	128.26	TG/C12
OL	129.71	129.68	Acylglycerols/C9
LO	129.97	129.96	Acylglycerols/C9
OL	129.99	129.98	Acylglycerols/C10
LO	130.20	130.17	TG/C13
LN	130.23	130.18	TG/C9
LN	131.93	131.90	TG/C16
	-		**Carbonyl carbons**
LO+LN	172.79	172.79	TG/*sn*-2/C1
OL	172.82	172.80	TG/*sn*-2/C1
SFA	-	172.87	TG/*sn*-2/C1
LO+LN	173.19	173.20	TG/*sn*-1,3/C1
OL	173.20	173.21	TG/*sn*-1,3/C1
SFA	173.23	173.24	TG/*sn*-1,3/C1
	-	173.47	1,2-DP/C1
	-	173.92	1-MP/2-MP/C1

Component Name	Chemical shifts (PPM)		Carbon position
	Soybean oil	HMMS	
			Aliphatic carbons
LO	14.09	14.07	Acylglycerols/*sn*-1,3/*sn*-2/C18
OL	14.13	14.11	Acylglycerols/*sn*-1,3/*sn*-2/C18
LN	14.30	14.28	Acylglycerols/*sn*-1,3/*sn*-2/C18
LN	20.57	20.56	Acylglycerols/*sn*-1,3/*sn*-2/C17
LO	22.60	22.58	Acylglycerols/*sn*-1,3/*sn*-2/C17
OL+SFA	22.71	22.69	Acylglycerols/*sn*-1,3/*sn*-2/C17
LN	24.86	24.85	Acylglycerols/*sn*-1,3/*sn*-2/C3
LO+OL	24.90	24.89	Acylglycerols/*sn*-1,3/*sn*-2/C3
SFA	-	24.99	Acylglycerols/*sn*-1,3/*sn*-2/C3
	-	25.04	1-MP/C3
	-	25.06	1,2-DP/C3
	-	25.12	2-MP/C3
LN	25.55	25.54	Acylglycerols/ *sn*-1,3/*sn*-2/C14
OL +LN+LO	25.66	25.64	Acylglycerols/ *sn*-1,3/*sn*-2/C11
LO	-	27.18	Acylglycerol / *sn*-1,3/*sn*-2/C8
OL	27.22	27.21	Acylglycerol / *sn*-1,3/*sn*-2/C8
LN	-	27.23	Acylglycerol/ *sn*-1,3/*sn*-2/C8/
LN+OL+LO	29.07	29.06	Acylglycerols/*sn*-2/C4
SFA	29.11	29.10	Acylglycerols/*sn*-2/C4
OL+LO+LN+SFA	29.14	29.13	TG/C5
LO+LN	29.35	29.33	Acylglycerol/*sn*-1,3/C6/C15
OL	29.38	29.35	Acylglycerol/*sn*-1,3/C13/C15
OL + SFA	29.56	29.60	AcylglycerolC6
OL	29.63	29.62	TG/C14
LN	29.64	29.63	Acylglycerol/*sn*-1,3/C7
LO	29.69	29.71	Acylglycerol/*sn*-1,3/C7
OL	29.73	29.73	Acylglycerol/*sn*-1,3/C7
OL	29.79	29.77	Acylglycerol/*sn*-*1*,3/C12
LO+LN	31.55	31.53	Acylglycerol/*sn*-1,3/*sn*-2/C16
OL	31.94	31.91	Acylglycerol/*sn*-1,3/*sn*-2/C16
SFA	31.96	31.93	Acylglycerol/*sn*-1,3/*sn*-2//C16
OL+LO+LN+SFA	34.04	34.05	Acylglycerol/C2
LN	-	34.14	Acylglycerol/C2
LO	-	34.17	Acylglycerol/C2
OL	34.20	34.20	Acylglycerol/C2
SFA	-	34.25	Acylglycerol/C2
	-	34.27	1-MP/C2
	-	34.49	1,2-DP/ *sn*-1/C2
	-	34.56	2-MP/C2

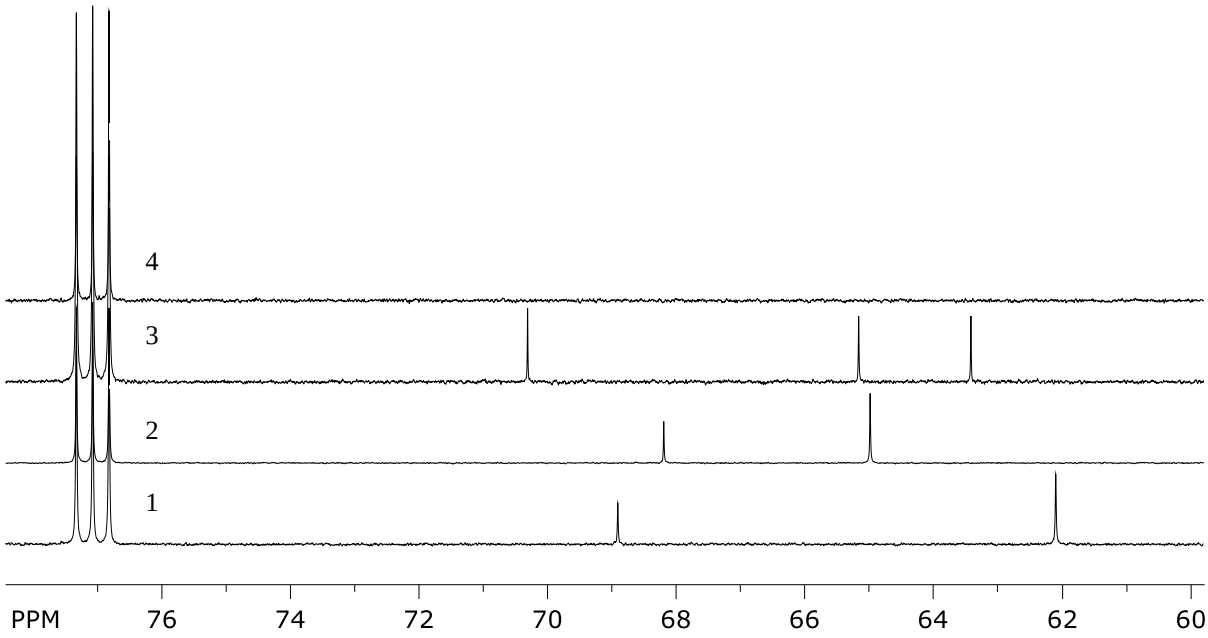

Figure A. 1. 1 ^{13}C-NMR spectra of glycerol carbons of model pure compounds. (1) triolein, (2) 1,3-diolein, (3) 1-monoolein (4) linolenic acid. Spectrograms are referenced to chloroform peak at 77.07 ppm

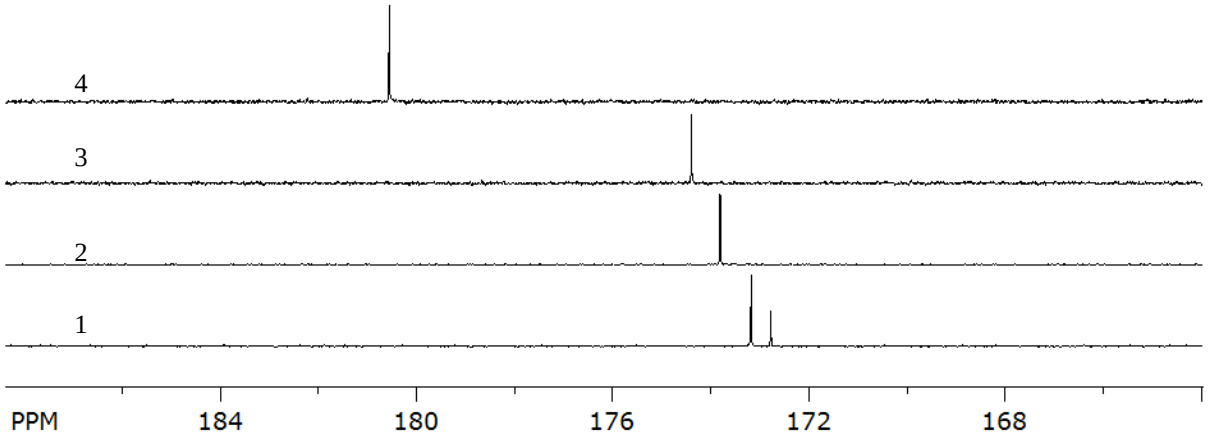

Figure A. 1. 2 ^{13}C-NMR spectra of carbonyl carbons of model pure compounds. (1) triolein, (2) 1,3-diolein, (3) 1-monoolein (4) linolenic acid. Spectrograms are referenced to chloroform peak at 77.07 ppm

Table A. 1. 4 ^{31}P-NMR chemical shifts of standard of mono- and di-olein

Compound	Chemical shifts (ppm)	
	1,3-Diolein	Monoolein
1,3-DG	146.69	
1-MG/*sn*-1		146.42
1-MG/*sn*-2		147.72

Table A. 1. 5 ^{1}H-NMR chemical shifts and multiplicity of components of a high monoester mixture of soybean oil

Component Name	Chemical shifts (PPM)	Multiplicity	Proton
β-Sitosterol	0.67	singlet	CH_3 H18
Stigmasterol	0.69	singlet	CH_3 H18
OL chains	0.87	triplet	$CH_2C\underline{H}_3$ $J_{18,17}$ = 7.07 Hz
LO chains	0.88	triplet	$CH_2C\underline{H}_3$ $J_{18,17}$ = 6.98 Hz
LN chains	0.98	triplet	$CH_2C\underline{H}_3$ $J_{18,17}$ = 7.55 Hz
1-MP	1.18	doublet	CH_3
2-MP	1.21	doublet	CH_3
Any acyl chains + 1,2-DP	1.29	envelope	$-(C\underline{H}_2)_n-$
Any acyl chains	1.60	multiplet	$OCOCH_2C\underline{H}_2$
OL chains	2.03	multiplet	$C\underline{H}_2CH=CH$
LO + LN chains	2.05	multiplet	$C\underline{H}_2CH=CH$
Any acyl chains of TG	2.29	triplet	$OCOC\underline{H}_2CH_2$ $J_{2,3}$ = 7.52 Hz
Any acyl chains of 1,2-DG	2.30	triplet	$OCOC\underline{H}_2CH_2$ $J_{2,3}$ = 7.55 Hz
Any acyl chains of MG + 1,3-DG	2.34	triplet	$OCOC\underline{H}_2CH_2$ $J_{2,3}$ = 7.51 Hz
LO chains	2.76	multiplet	$CH=CHC\underline{H}_2CH=CH$
LN chains	2.80	multiplet	$CH=CHC\underline{H}_2CH=CH$
Glycerol of 1-MG	3.59	doublet of doublet	3́*a-C$\underline{H}_2$OCO $J_{3́a,3́b}$ = 10.86 Hz $J_{3́a,2}$= 6.62 Hz
Glycerol of 1,2-DG	3.72	broad	3́a, 3́b-C$\underline{H}$2OCO
Glycerol of 1-MG	3.85	doublet	2́-C$\underline{H}$OCO
Glycerol of 1,3-DG	4.06	broad	2́-C$\underline{H}$OH
Glycerol of 1-MG	4.10	doublet of doublet	1́a-C$\underline{H}_2$OCO $J_{1́a,1́b}$ = 12.43 Hz $J_{1́a,2́}$ = 6.00 Hz
Glycerol of 1,3-DG	4.13	doublet of doublet	1á, 3 á-C$\underline{H}$2OCO $J_{1́a,1́b}$ = 11.91 Hz $J_{1́a,2́}$ = 5.92 Hz
Glycerol of 1,3-DG	4.18	doublet of doublet	1́b, 3́b-C$\underline{H}$2OCO $J_{1́a,1́b}$ = 11.33 Hz $J_{1́a,2́}$ = 4.35 Hz
Glycerol of 1,2-DG	4.23	doublet of doublet	1́a-C$\underline{H}$2OCO $J_{1́a,1́b}$ = 11.95 Hz $J_{3́a,2́}$ = 5.59 Hz
Glycerol of TG	4.29	doublet of doublet	1́a, b -C$\underline{H}$2OCO $J_{3́a,3́b}$ = 11.90 Hz $J_{3́a,2́}$ = 4.35 Hz
Glycerol of 1,2-DG	5.07	quintets	2́-C$\underline{H}$OCO
Glycerol of TG	5.27	quintets	2́-C$\underline{H}$OCO $J_{1́a,2́}$ = 5.88 Hz
Any acyl chains	5.28-5.40	multiplet	C$\underline{H}$=C$\underline{H}$ (m)

*1, 2 and 3 represent *sn*-1, *sn*-2 and *sn*-3 positions, respectively. Primed letters indicate glycerol carbons and unprimed ones denote acyl chain carbons of TGs, DGs and MGs

APPENDIX 2: SUPPORT INFORMATION FOR CHAPTER 5

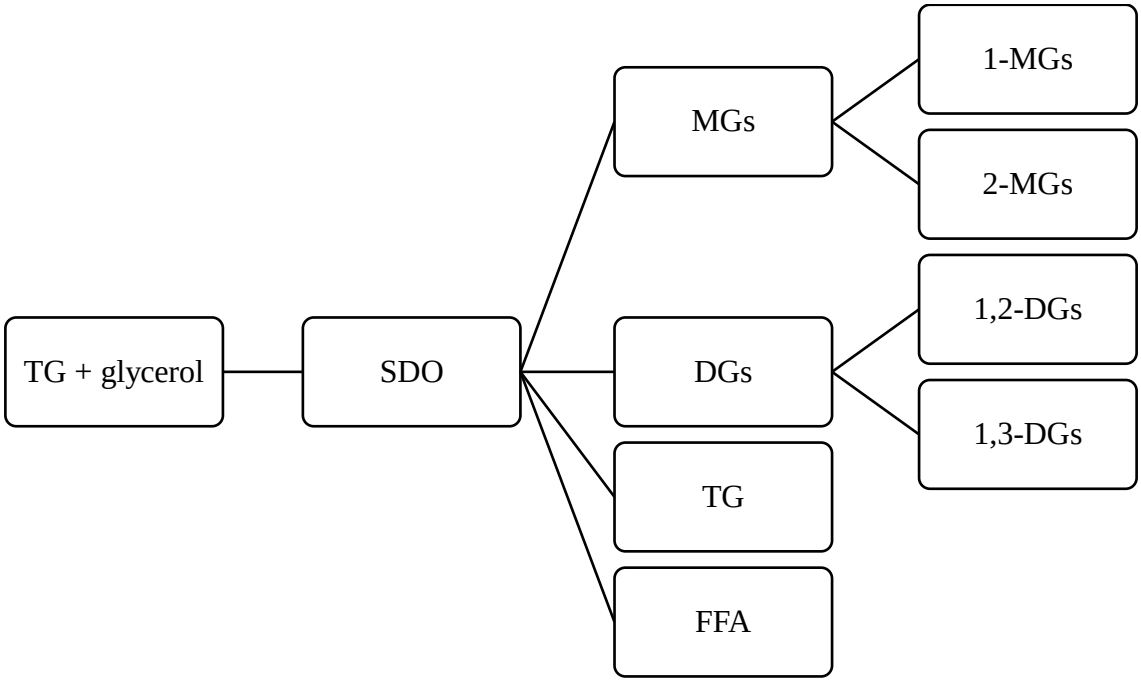

Figure A. 2. 1 The products of enzymatic alcoholysis of TG (soybean oil). 1-MGs (1-monoglycerides), 2-MGs (2-monoglycerides), 1,2-DGs (1,2-diglycerides), 1,3-DGs (1,3-diglycerides)

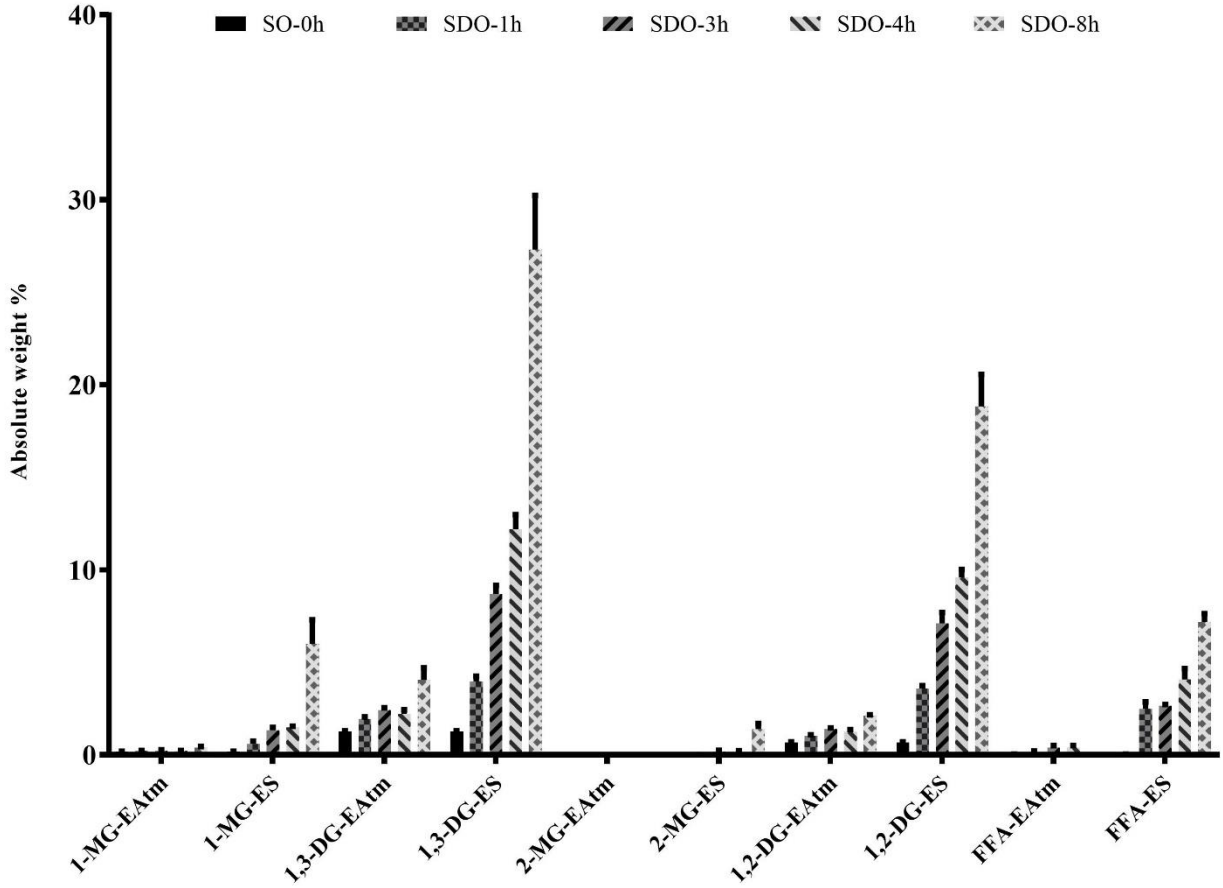

Figure A. 2. 2 Time monitoring of the content of MGs, DGs and free fatty acid (FFA) of SDO has obtained from enzymatic glycerolysis reaction at atmospheric (E. Atm.) and $SCCO_2$ (E. S.) conditions

To derive a mathematical model for DG value in Soy based DAG-enriched oil (SDO), the following steps were applied to the experimental data:

1- Average value of output (e.g. DG) of similar experiments was taken and Table A. 2. 1 was constructed.

Table A. 2. 1 Experimental data including values of independent variables and average values of total DG

No.	Time (x_1) (h)	Pressure (x_2) (bar)	Temperature (x_3) (°C)	Enzyme load (x_4) (%)	Total DG* (%)
1	1	80	60	3	7.6
2	2	80	60	3	11
3	3	80	60	3	15.8
4	4	80	60	3	21.8
5	5	80	60	3	19.2
6	8	80	60	3	27.6
7	12	80	60	3	30.4
8	4	100	60	3	20.3
9	4	150	60	3	14.3
10	4	300	60	3	17.6
11	4	450	60	3	21.5
12	4	80	40	3	12.3
13	4	80	70	3	20.6
14	4	80	60	6.5	28.6
15	4	80	60	10	36.2

* Total DG value is the mean value of DGs taken from 2 experiments with the same values of x_1, x_2, x_3 and x_4.

2- Table A. 2. 1 was rearranged to investigate the effect of each variable on the total DG value separately (see Tables A. 2. 2-A. 2. 5):

Table A. 2. 2 Investigation of effect of time on total DG

Time (x_1) (h)	Pressure (x_2) (bar)	Temperature (x_3) (°C)	Enzyme load (x_4) (%)	Total DG (%)
1	80	60	3	7.6
2	80	60	3	11
3	80	60	3	15.8
4	80	60	3	21.8
5	80	60	3	19.2
8	80	60	3	27.6
12	80	60	3	30.4

Table A. 2. 3 Investigation of effect of pressure on total DG

Time (x_1) (h)	Pressure (x_2) (bar)	Temperature (x_3) (°C)	Enzyme load (x_4) (%)	Total DG (%)
4	**80**	60	3	21.8
4	**100**	60	3	20.3
4	**150**	60	3	14.3
4	**300**	60	3	17.6
4	**450**	60	3	21.5

Table A. 2. 4 Investigation of effect of temperature on total DG

Time (x_1) (h)	Pressure (x_2) (bar)	Temperature (x_3) (°C)	Enzyme load (x_4) (%)	Total DG (%)
4	80	**40**	3	12.3
4	80	**60**	3	21.8
4	80	**70**	3	20.6

Table A. 2. 5 Investigation of effect of enzyme load on total DG

Time (x_1) (h)	Pressure (x_2) (bar)	Temperature (x_3) (°C)	Enzyme load (x_4) (%)	Total DG (%)
4	80	60	**3**	21.8
4	80	60	**6.5**	28.6
4	80	60	**10**	36.2

3- To negate the effect of other variables when investigating dependency of the total DG to a certain independent variable, a set of values between [-1, +1] was assigned to the independent variables as shown in Tables A. 2. 6-A. 2. 9.

Table A. 2. 6 Assigned values to time

Time (x_1)	Assigned value ($x_{1,n}$)
1	-1
2	-0.5
3	-0.25
4	**0**
5	0.25
8	0.5
12	1

Table A. 2. 7 Assigned values to pressure

Pressure (x_2)	Assigned value ($x_{2,n}$)
80	**0**
100	0.05
150	0.19
300	0.59
450	1

Table A. 2. 8 Assigned values to temperature

Temperature (x_3)	Assigned value ($x_{3,n}$)
40	-1
60	**0**
70	1

Table A. 2. 9 Assigned values to enzyme load

Enzyme (x_4)	Assigned value ($x_{4,n}$)
3	**0**
6.5	0.5
10	1

Note that the zero value ($x_{i,n} = 0$) was assigned to a specific value of x_i ($i = 1,2,3,4$). For example, $x_{1,n} = 0$ was given to time of 4 h, because the time equals 4 h in the experiments investigating the effect of other variables (pressure, temperature or enzyme load) on the DG level

(see Tables A. 2. 2-A. 2. 5). Combining Tables A. 2. 6-A. 2. 9 with Tables A. 2. 2-A. 2. 5, leads to:

Table A. 2. 10 Changes in DG value with respect to time based on assigned values

$x_{1,n}$	$x_{2,n}$	$x_{3,n}$	$x_{4,n}$	DG_1
-1	0	0	0	**7.6**
-0.5	0	0	0	**11**
-0.25	0	0	0	**15.8**
0	0	0	0	**21.8**
0.25	0	0	0	**19.2**
0.5	0	0	0	**27.6**
1	0	0	0	**30.4**

Table A. 2. 11 Changes in DG value with respect to pressure based on assigned values

$x_{1,n}$	$x_{2,n}$	$x_{3,n}$	$x_{4,n}$	DG_2
0	**0**	0	0	**21.8**
0	**0.05**	0	0	**20.3**
0	**0.19**	0	0	**14.3**
0	**0.59**	0	0	**17.6**
0	**1**	0	0	**21.5**

Table A. 2. 12 Changes in DG value with respect to temperature based on assigned values

$x_{1,n}$	$x_{2,n}$	$x_{3,n}$	$x_{4,n}$	DG_3
0	0	**-1**	0	**12.3**
0	0	**0**	0	**21.8**
0	0	**1**	0	**20.6**

Table A. 2. 13 Changes in DG with respect to enzyme load based on assigned values

$x_{1,n}$	$x_{2,n}$	$x_{3,n}$	$x_{4,n}$	DG_4
0	0	0	**0**	**21.8**
0	0	0	**0.5**	**28.6**
0	0	0	**1**	**36.2**

4- According to Tables A. 2. 10- A. 2. 13, a single-variable function can be found for each group of DG values (*i.e.*, DG_i , $i = 1,2,3,4$) in terms of each independent variable with assigned values. Using the Least Square technique, the functions were found considering $R^2 \geq 0.95$:

Eq. (A1): $DG_1 = f_1(x_{1,n}) + b_1 \quad ; \; x_{2,n} = x_{3,n} = x_{4,n} = 0$

Eq. (A2): $DG_2 = f_2(x_{2,n}) + b_2 \quad ; \; x_{1,n} = x_{3,n} = x_{4,n} = 0$

Eq. (A3): $DG_3 = f_3(x_{3,n}) + b_3 \quad ; \; x_{1,n} = x_{2,n} = x_{4,n} = 0$

Eq. (A4): $DG_4 = f_4(x_{4,n}) + b_4 \quad ; \; x_{1,n} = x_{2,n} = x_{3,n} = 0$

where, b_1, b_2, b_3 and b_4 are constant values.

5- Since each regression model $[f_i(x_{i,n}) \; ; \; i = 1,2,3,4]$ is obtained based on one single variable while other variables are set to zero, the general model (a function of multi-variables) can be obtained as follows:

Eq. (A5): $DG = f_1(x_{1,n}) + f_2(x_{2,n}) + f_3(x_{3,n}) + f_4(x_{4,n}) + \frac{b_1+b_2+b_3+b_4}{4}$

6- As illustrated in Eq. (A5), the multi-variable function is in terms of the assigned values. According to Tables A. 2. 6-A. 2. 9, one can find the relationship between the assigned values and their corresponding actual values for each independent variable. Therefore,

the following functions were found, using the Least Square technique, considering $R^2 \geq$ 0.95:

Eq. (A6): $x_{1,n} = g_1(x_1)$: based on Table A6

Eq. (A7): $x_{2,n} = g_2(x_2)$: based on Table A7

Eq. (A8): $x_{3,n} = g_3(x_3)$: based on Table A8

Eq. (A9): $x_{4,n} = g_4(x_4)$: based on Table A9

7- Substituting Eqs. (A. 2. 6-A. 2. 9) in Eq. (A5) results in:

Eq. (A10):

$$DG_{model} = f_1\big(g_1(x_1)\big) + f_2\big(g_2(x_2)\big) + f_3\big(g_3(x_3)\big) + f_4\big(g_4(x_4)\big) + \frac{b_1 + b_2 + b_3 + b_4}{4}$$

where, $f_i\big(g_i(x_i)\big) = (f_i o g_i)(x_i)$ is the composition of functions f_i and g_i.

composition of functions f_i and g_i.

Table A. 2. 14 Effect of temperature (°C) on DG yield

Study	Range	Optimum	DG yield and reaction route
(Singh and Mukhopadhyay 2014)	10-50	40	39%, glycerolysis
(Yamane et al. 1994)	45-60	48	87% after purification, glycerolysis
(Watanabe et al. 2003)	30-60	50	84% after purification, esterification
(Miranda et al. 2013)	40-60	60	50%, glycerolysis
(Lee et al. 2014)	40-60	60	30%, ethanolysis
(Voll et al. 2011)	40-70	70	25%, glycerolysis
(Fregolente et al. 2010)	-	70	53%, glycerolysis

Table A. 2. 15 Effect of enzyme load (wt%) on DG yield

Study	Range	Optimum	DG yield and reaction route
(Eom et al. 2010)	1-20 of substrates weight	10	39%, glycerolysis
(Voll et al. 2011)	2.5-15 of oil weight	15	25%, glycerolysis
(Liu et al. 2012)	1-15 of oil weight	10	53%, glycerolysis
(Miranda et al. 2013)	5-15 of oil weight	15	50%, glycerolysis
(Singh and Mukhopadhyay 2014)	1-30 of oil weight	2.5	39%, glycerolysis
(Lubary, et al. 2011)	3-10 of oil weight	3	33%, ethanolysis
(Lee et al. 2014)	2-5 of oil weight	3	38%, ethanolysis

Table A. 2. 16 Effect of time (h) on DG yield

Study	Range	Optimum	DG yield and reaction route
(Liu et al. 2012)	1-24	12	53%, glycerolysis
(Watanabe et al. 2003)	0.5-6	4	84% after purification, esterification
(Singh and Mukhopadhyay 2014)	2-6	5	39%, glycerolysis
(Lee et al. 2014)	-	2	38%, ethanolysis
(Saberi et al. 2011)	-	7	90% after purification, glycerolysis

Table A. 2. 17 Effect of pressure (bar) on DG yield in different studies

Study	Range	Optimum	DG yield and reaction route
(Shin et al. 2012)	-	120	10%, hydrolysis
(Lee et al. 2014)	70-100	80	20%, ethanolysis
(Moquin et al. 2006)	100-300	100	17%, glycerolysis-hydrolysis

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