# LIPASE-CATALYZED SYNTHESIS OF OMEGA-3 VEGETABLE OILS

By

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## ABSTRACT

The effects of temperature, reaction time, and substrate concentration on the incorporation of decanoic acid (DA) and alpha-linolenic acid (ALA), into cocoa butter, were compared, using an immobilized enzyme derived from *Rhizomucor miehei*. All variables had an effect on incorporation of DA and ALA into cocoa butter but effects were not equivalent for the two fatty acids. Thus, DA was not an adequate model fatty acid for the incorporation of ALA into cocoa butter. The highest ALA incorporation achieved was 77.3±1.3. Samples with ALA incorporated were prepared as "pure" and "blends", and these exceeded the milk and dark chocolate Canadian Food and Drug Regulation guidelines for products making omega-3 fatty acid content claims. The highest %TAG content, 97.3±1.0%, was achieved for the 11.9wt% "blend" sample. Differential scanning calorimetry suggested that both "pure" and "blend" samples contained mainly form IV and V, with much smaller quantities of form II polymorphs.

## LIST OF ABBREVIATIONS AND SYMBOLS USED

| | |
|---|---|
| ALA | alpha-Linolenic acid |
| BHT | Butylated hydroxytoluene |
| DA | Decanoic acid |
| DAG | Diacylglycerol |
| $dH_2O$ | Distilled water |
| DHA | Docosahexaenoic acid |
| DSC | Differential Scanning Calorimeter |
| EPA | Eicosapentaenoic acid |
| EPA-EE | Eicosapentaenoic acid ethyl ester |
| FAME | Fatty acid methyl ester |
| FID | Flame ionization detector |
| GC | Gas chromatography |
| HPLC | High Performance Liquid Chromatography |
| LCFA | Long-Chain Fatty Acids |

| | |
|---|---|
| LT-ELSD | Low Temperature Evaporative Light Scattering Detector |
| MAG | Monoacylglycerol |
| O | Oleic acid |
| P | Palmitic acid |
| PUFA | Polyunsaturated Fatty Acid |
| Prob>F | Probability value |
| $R^2$ | Coefficient of determination |
| RSM | Response Surface Methodology |
| S | Stearic acid |
| T8 | Tricaprylin |
| T10 | Tricaprin |
| T12 | Trilaurin |
| TAG | Triacylglycerol |

# CHAPTER 1 INTRODUCTION

Omega-3 fatty acids have been linked to many health benefits. Among these are heart health, brain and eye development and function, joint health, mood, behavior, cancer, diabetes, skin disorders, pregnancy, and lactation (Lee and Akoh, 1996 and Senanayake and Shahidi, 1999). Current consumer awareness of the health benefits of omega-3 consumption has increased the interest of using food as a delivery system for these fatty acids. This awareness has also peaked the demand for omega-3 enriched foods that have desirable nutritional, sensory, and functional characteristics. In recent years, the most common way of enriching foods with omega-3 fatty acids has been by using microencapsulated fish oils. The use of microencapsulation protects the fish oil from processing conditions and from interactions with the food matrix and storage environment (Shi and King, 2007). Current research in the field of structured lipids has made it possible to potentially incorporate omega-3 fatty acids into food products without the use of microencapsulation and could possibly prevent some of the unfavorable sensory characteristics associated with fish oil addition.

Structured lipids are triacylglycerol (TAG) molecules that have been modified to incorporate a new fatty acid in the glycerol backbone, thus producing a new TAG molecule (Osborn and Akoh, 2002). These structured lipids can be used for special functions in nutritional, food, and pharmaceutical applications (Xu, 2000). For instance, Liu et al. (2007) synthesized a cocoa butter analog using enzymatic interesterification of lard and tristearin with a *Rhizomucor miehei* derived lipase in a super critical carbon dioxide batch reactor (Liu et al., 2007). Structured lipids are most commonly produced using either chemical or enzymatic interesterification, which is the reaction between an ester and a fatty acid, an alcohol, or another ester (Xu, 2000 and 2003). Within interesterification reactions, enzymatic acidolysis is the most common method used to produce structured lipids. Enzymatic acidolysis is the reaction between a TAG and a fatty acid using a sn-1, 3 regiospecific lipase (Negishi et al., 2003). The acidolysis reaction is reversible and usually considered a two-step reaction involving hydrolysis and esterification, with diacylglycerol (DAG) as an intermediate and precursor to side reactions (Xu, 2003).

More recently, immobilized enzymes have started to gather more popularity in the production of structured lipids. The use of immobilized enzymes provides various advantages over the traditional chemical

interesterification methods and over typical enzymatic methods. The use of immobilized enzymes, although more expensive, provides reuse of the enzyme and decreased production of pollutants (Xu, 2000 and Goh et al., 1993). The use of immobilized enzymes also provides the opportunity for increased specificity because enzymatic lipases that have sn-1, 3 regiospecificity are available (Wongsakul et al., 2003).

In this study the immobilized enzyme being used is derived from *R. miehei,* a sn-1, 3 regiospecific lipase. It will be used to produce two types of structured lipids: one containing a short chain fatty acid, decanoic acid (DA), and the other containing an omega-3 fatty acid, alpha-linolenic acid (ALA). The incorporation of these fatty acid into a vegetable oil, in this case cocoa butter, will be compared, to test the possibility of using DA as a model for the incorporation of ALA into cocoa butter. Various molar concentrations, times, and temperatures will be examined to determine how these variable influence fatty acid incorporation into cocoa butter while producing the lowest amounts of by-products, DAG and monoacylglycerol (MAG), being produced. Samples will be prepared and statically tempered to determine the polymorphs present in ALA incorporated cocoa butter and if these are similar to those found in unmodified cocoa butter.

# CHAPTER 2 LITERATURE REVIEW

## 2.1 DEFINITION OF STRUCTURED LIPID

There are several definitions that can be applied to structured lipids. The simplest of these is a TAG molecule that has been restructured so that its fatty acids have had their positions changed on the glycerol backbone, but this definition does not include the addition of a new fatty acid (Kim and Akoh, 2006). For this reason, a more acceptable definition is the modification of a TAG molecule so that a new fatty acid is incorporated or the position of a fatty acid is changed on the TAG molecule thereby creating a new TAG molecule called a structured lipid (Osborn and Akoh, 2002). Structured lipids are usually produced by either chemical or enzymatic interesterification (Xu, 2000 and 2003).

## 2.2 ENZYMATIC INTERESTERIFICATION

Interesterification involves three types of reactions: acidolysis, glycerolysis, and transesterification. Acidolysis is the reaction between a fatty acid and a TAG molecule. Glycerolysis involves a reaction between glycerol and TAG, and transesterification is the reaction between two TAG molecules (Marangoni and Rousseau, 1995). Acidolysis using a sn-1, 3 regiospecific lipase

is the most common interesterification method used to synthesize structured lipids (Negishi et al., 2003). Enzymatic acidolysis is a reversible reaction in which DAG acts as an intermediate and precursor to side reactions (Figure 2.1). The substrate ratio in the reaction determines total product yield of the reaction when at equilibrium (Xu, 2003).

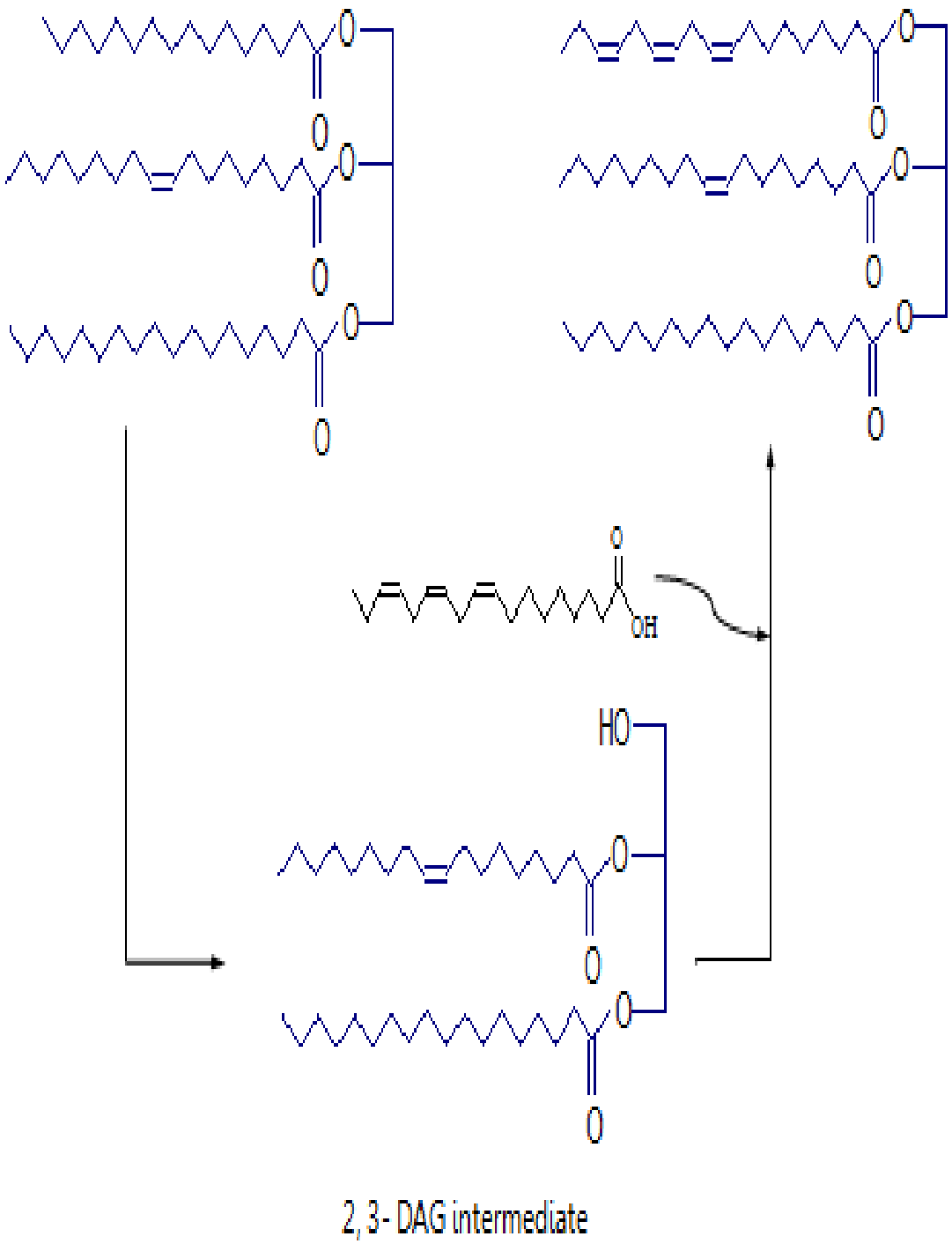


Figure 2.1 Enzymatic interesterification using acidolysis of cocoa butter with ALA, including a 2, 3- DAG intermediate.

Side reactions are inevitable and typically result in DAG formation. As the intermediates for the main reaction, DAG can also lead to acyl migration which occurs if the fatty acid at the sn-2 position migrates to the sn-1 position after the lipase has removed the original sn-1 fatty acid. Acyl migration then causes the formation of unwanted TAG by-products. Acyl migration and by-product formation is increased by higher temperature, reaction time, water content, water activity, and enzyme load. Researchers have also found that packed enzyme bed reactors produced less acyl migration than stirred tank reactors (Xu, 2000). In our experiment the formation of interesterification by-products was monitored using High Performance Liquid Chromatography (HPLC).

## 2.3 ADVANTAGES AND DISADVANTAGES OF IMMOBILIZED ENZYME USE

As opposed to chemical interesterification for the production of structured lipid, the use of immobilized enzymes is more recent and poses new advantages and disadvantages when compared to chemical methods. The immobilization of enzymes increases their thermostability over free enzymes, and may improve their mechanical strength, hydrophobic and hydrophilic characteristics, regeneration, and functionality after use (Zhang et al., 2001). The use of immobilized enzymes results in reduced environmental pollutants because smaller amounts of solvents and reactants are needed. Another

advantage is that lipases are available from a wide variety of sources and can be improved by genetic engineering (Xu, 2000).

In enzymatic interesterification, lower temperatures are used which may prevent the destruction of natural all *cis* n-3 PUFA by oxidation and *cis-trans* isomerization. Enzyme activity increases as temperature increases until a maximum is reached. Increases in temperature cause the kinetic energy of the enzyme's molecular framework to increase and the amount of molecules colliding with the enzyme also increase. This initially causes an increase in reaction rate but with sufficiently high temperatures the reaction rate begins to decrease as the enzyme is denatured. The explanation for this is that the bonds holding the three-dimensional shape of the enzyme are weak and therefore sensitive to temperature changes (Senanayake and Shahidi, 1999). Although using immobilized enzymes is more time consuming, the prevention of PUFA oxidation with the relatively low temperature used makes up for the additional time spent (Chen and Ju, 2000).

Researchers have also found that enzymes demonstrate specificity towards particular fatty acid structures. Wongsakul et al. (2003) found that when a two-step reaction involved the production of 2-MAG by alcoholysis of TAG with ethanol, using a sn-1, 3 regiospecific lipase, the 2-MAG obtained

could be esterified with a fatty acid using a sn-1, 3 regiospecific lipase resulting in the desired structured lipid. These researchers found that it was not necessary to use a sn-1, 3 regiospecific lipase because the fatty acid chain length specificity of the enzyme was more important than where it acted on the TAG structure. They also concluded that *Candida antarctica* fraction B discriminates DHA from EPA, so it can be used to selectively enrich for DHA, showing that the enzyme was more specific based on the chain length of the fatty acid instead of its position on the TAG molecule (Wongsakul et al., 2003). Shimada et al. (1996 and 2001) also found that lipases have stricter fatty acid specificity during esterification; he found this by raising the PUFA content of an oil. This was accomplished using *C. rugosa* lipase to selectively hydrolyze the non-PUFA ester bonds of the oil (Shimada et al., 1996 and 2001). Hamam and Shahidi (2007) examined the incorporation of short chain fatty acids into triolein and concluded that as fatty acid chain length decreases the amount of incorporation increases (Hamam and Shahidi, 2007). Paez et al (2003) found similar results in that caprylic acid incorporation was higher than oleic acid when incorporated into triolein (Paez et al., 2003).

The main disadvantage of enzyme use, especially immobilized enzymes, is cost. The immobilized enzyme is expensive, but it does have the advantage

that it can be reused which in the long run may be more cost effective (Goh et al., 1993).

## 2.4 CHOICE OF ENZYME USED

Many lipases are now available and their applications vary. The decision as to what enzyme is most appropriate for a specific application has been explored by many researchers.

Lipases can be classified into two groups random and specific. Random lipases have no regiospecificity while specific lipases have sn-1, 3 regiospecificity. Random lipases include those derived from *Candida rugosa*, *Geotrichum candidum*, and *Staphylococcus aureus*, while pancreatic lipase, *R. miehei* lipase, *Aspergillus niger* lipase, *Pseudomonas flourescens* lipase, and *Rhizopus arrhizus* lipase are considered specific lipases (Marangoni and Rosseau, 1995). Enzymes with sn-2 regiospecificity do not seem to exist.

Another factor to consider when choosing an enzyme is the range of temperatures that it is capable of functioning in. This is important because the optimum temperature of an interesterification reaction depends on the enzyme being used. The source of the enzyme must be considered, whether it is immobilized or not, what type of immobilization was used, and the reaction

mixture pH (Huang and Akoh, 1996). Enzyme selection is made more difficult because there is no clear consensus as to what the denaturation temperature of an immobilized enzyme is; however, for *R. miehei*, Xu (2000) stated that the reaction temperature should not exceed 70-80°C, for fear of enzyme denaturation (Xu, 2000).

In the present work, enzyme derived from *R. miehei* was chosen based on the work of Hamam and Shahidi (2007). This group investigated the incorporation of long chain fatty acid (LCFA) into triolein using *Candida antarctica, R. miehei, Pseudomonas* sp., and *Candida rugosa*. They found that the use of *R. miehei* resulted in the greatest incorporation of ALA into triolein (Hamam and Shahidi, 2007). Triolein, consisting of saturated 18 carbon (C) fatty acids, bears some similarity in structure to cocoa butter, which consists of one 16C saturated fatty acid, and two 18C fatty acids with one unsaturated and the other saturated; ALA was the target fatty acid for incorporation in the present work (Figure 2.2). Based on these findings, enzyme derived from *R. miehei* was chosen for the incorporation of ALA into cocoa butter in our current research. *R. miehei* derived lipase has an enzyme activity of 86.8U/g, in which 1U is equal to the amount of enzyme which releases 1μmol of stearic acid per minute at a pH of 8.0 and a temperature of 70°C (Fluka Analytical, 2010). This

means that if 100mg of lipase derived from *R. miehei* is used, approximately 0.002mg of stearic acid are freed per minute from a TAG molecule.

## 2.5 PREVIOUS RESEARCH CONDITIONS

Research in the interesterification field has examined several variables and variable parameters when performing enzyme-mediated reactions. Beside the variables explored in this study (mole ratio, time and temperature), past research in the area has also examined water activity, solvent used, enzyme load, and scale of reaction, which can range from test tube level to packed-bed reactors. Judging from the available literature, most researchers make the observation of mole ratio, time, and temperature effects a priority. Measurement of water content seems to be of greater importance when free enzymes were used or when the research team immobilized the enzyme as opposed to purchasing an immobilized enzyme. The solvent used can vary or solvent free systems can also be used; it must, however, be considered that lipases work better in liquid environments so if a viscous liquid, such as oil, is being used, it might be beneficial to use a solvent (Shimada, 2001). The enzyme load is usually dependant on the amount of substrates and this is also affected by the scale of reaction used. For instance, large bioreactors require larger amounts of substrates and other reaction components, while test-tube

scale reactors require fewer materials. Researchers change these variables and their parameters so that optimum conditions can be obtained for their specific study needs. The table (Table 2.1) below shows several reaction conditions through which researchers have acquired these optimum conditions for their specific incorporation needs; a more extensive account of variables affecting structured lipid formation using lipase-catalyzed reactions can be found in Xu (2000).

Table 2.1 Previous research conditions.

| Researcher(s) | Concentration | Time | Temperature | Enzyme |
|---|---|---|---|---|
| Hamam and Shahidi (2007) | 1:3 (fatty acid to triolein) | 24hrs | 45±2˚C | *R. miehei, C. antarctica, Pseudomonas sp., A. niger, C. rugosa* |
| Kim and Akoh (2006) | 1:6 (sesame oil: caprylic acid) | Continuous packed bed reactor 1.15L/min | 45˚C | *R. miehei* |
| Osborn and Akoh (2002) | Large scale batch reactor (1L Stearic acid and beef tallow) | 24hrs | 58±2˚C | *C. antarctica* and *R. miehei* |
| Zhang et al. (2001) | Large scale batch reactor (1-kg and 300-kg Palm stearin and coconut oil) | 6hrs | 60˚C | *T. lanuginosa* and *R. miehei* |
| Shimada, et al. (1996) | 1:2 (wt/wt of tuna oil/caprylic acid) | 48hrs | 30˚C | *Rhizopus delemar* |
| Lee and Akoh (1996) | 1:2 (T8, T10, T12 and EPA-EE) | 24hrs | 55˚C | *R. miehei* and *C. Antarctica* |

## 2.6 CHARACTERISTICS OF COCOA BUTTER

Cocoa butter properties vary depending on the geographical source, crop conditions, and the conditions present during roasting and pressing (Chichester and Mark, 1987). The cocoa butter TAG molecule is predominantly composed of palmitic acid (P), oleic acid (O), and stearic acid (S) attached to a glycerol backbone (Xu, 2000). The approximate total concentration of the fatty acids is palmitic acid (16:0) 24-30%, oleic acid (18:0) 33-39%, stearic acid (18:1) 30-36% (Gunstone, 1996). Oleic acid is mainly located in the sn-2 position, while stearic and palmitic acid are more commonly located in either the sn-1 or 3 positions, with the most common configuration being palmitic acid in the sn-1 position, oleic acid in the sn-2 position, and stearic acid in the sn-3 position (referred to as POS; Figure 2.2) (Xu, 2000). In cocoa butter, TAG molecules are most commonly present as POS 42.2%, SOS 24.2%, and POP 21.8%, with 11.8% being composed of other TAG molecules (Zarringhalami et al., 2010). The average molecular weight of a cocoa butter TAG molecule is 823.36 g/mol.

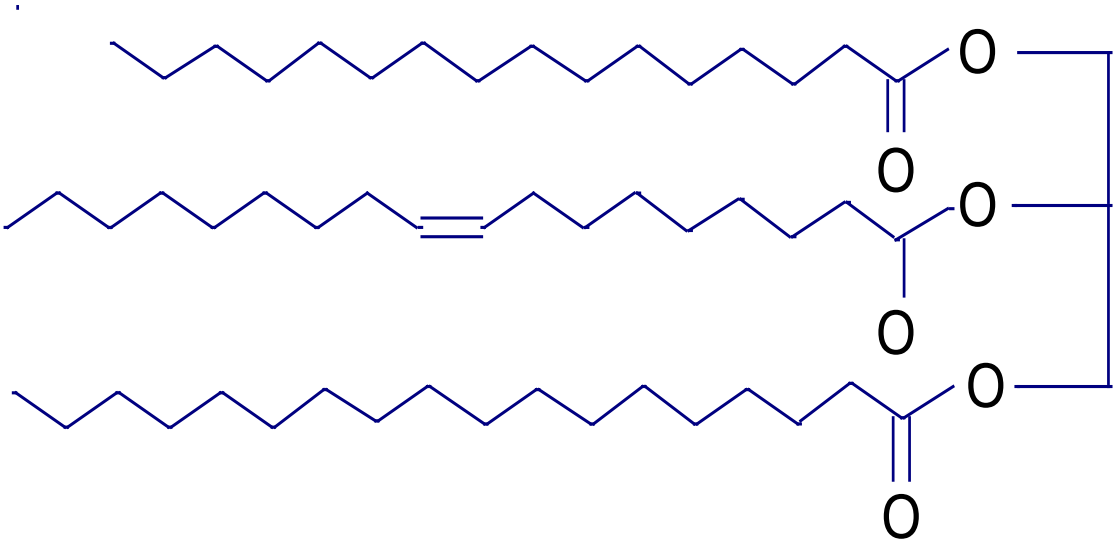

Figure 2.2 Cocoa butter TAG structure.

However, cocoa butter is not made up entirely of TAG molecules. Chaiseri and Dimick (1995) found that cocoa butter has approximately 99% simple lipids, 0.3–0.8% glycolipids, and <0.1–0.2% phospholipids when cocoa butter samples from six nations were analyzed. Simple lipids include TAG, free fatty acids, MAG, DAG, and sterols. The simple lipid fraction was composed of 96.8–98.0% TAG and other simple lipids accounted for 2.9–3.2%. This 2.9–3.2% was composed of 36.4–52.0 free fatty acids, 40.4–57.4% DAG, 3.8–6.4% sterols, and 1.1–1.4% MAG. Sterols were further divided into β-sitosterol (2.0–3.8 %), stigmasterol (1.1–1.8%), and campesterol (0.5–0.8%) (Chaiseri and Dimick 1995).

Cocoa butter has a melting range of 32-35˚C; this temperature is affected by the amounts and positions of the three fatty acids present (Xu, 2000; Chichester and Mark, 1987). The crystalline arrangement of the lipid molecules in cocoa butter is also affected by temperature. Cocoa butter has 6 polymorphic forms which start with I and end with VI. Form I is very unstable and melts around 17˚C; it then quickly transforms into form II which slowly converts into forms III and IV. If form IV is used to make chocolate, the result is a soft chocolate that will have little snap when broken. In a few days, form IV will transform into form V; this transformation will be faster at higher temperatures. Forms V and VI are the most stable and occur between 32-36˚C. These two forms are the most sought after when confectionary chocolate is being produced. Form V is usually the most desired because it is hard, provides a good snap, has a glossy sheen, and is relatively resistant to bloom. When this crystalline form is present in the melt and is poured into a mould it will contract well, which is desirable to chocolate makers (Beckett, 2008). To obtain form V crystals, chocolate is tempered, a process by which stable and unstable nuclei are formed by cooling the bulk of the chocolate to a melting point above that of form I. The walls of the heat exchanger have a lower temperature which leads to form I crystallization followed by transformation into form IV. The mixture is

then heated to a temperature above the melting point of form IV so that only form V is left. The tempered chocolate is then cooled so that complete solidification of form V is obtained, thus having the most stable product (Gunstone, 2001).

Tempering is an essential part of chocolate manufacturing and, without it the physical characteristics we expect when enjoying chocolate would be absent. This basic process described above actually involves a careful combination of time, temperature, and shear rate. Zarringhalami et al. (2010) described one such tempering process for dark chocolate samples that the researchers prepared. While stirring, the chocolate sample was cooled to 27-28˚C in 20 minutes, thus obtaining seed crystals. The sample was held at this temperature for several minutes which allowed crystals to form. Then the sample was heated to 30-31˚C therefore melting any unstable crystals present. The samples were then poured into moulds and cooled down to 10˚C where they were kept for 24 hours (Zarringhalami et al., 2010).

The effects of adding DA and ALA into the cocoa butter TAG molecule must be investigated. This can be accomplished by testing the melting range of the modified cocoa butter, using differential scanning calorimetry (DSC). If the cocoa butter polymorphism is changed, which would be expected, the physical

properties of chocolate products will be affected, including gloss, snap, contraction, heat resistance, melting point, and bloom-resistance (Osborn and Akoh, 2002).

However, cocoa butter is not the only ingredient in a chocolate product. Chocolate is a combination of several ingredients including sweetening agents, cocoa liquor, cocoa butter, cocoa powder, emulsifiers, and milk solids. The most popular types of chocolate are dark, milk, and white chocolate and the amounts of the other ingredients required depends on the desired product (Canadian Department of Justice, 2010b).

## 2.7 OMEGA-3 FATTY ACID LABEL CLAIMS

According to the Canadian Department of Justice, a food company can make the label claim that a food product contains omega-3 PUFAs if the food contains 0.3g or more omega-3 PUFAs per reference amount and stated serving size or 0.3g or more omega-3 PUFA per 100g if the food is a pre-packaged meal. Also, they require that the nutritional facts table include a declaration of omega-3 PUFA, omega-6 PUFA, and monounsaturated fatty acids (Canadian Department of Justice, 2010a). It is important to note that in order to meet the labeling claim requirements it might not be necessary to use the optimal

incorporation conditions, as determined experimentally. Instead the conditions that meet minimal omega-3 labeling claims can be used.

In order for a food to be considered a chocolate product several specifications must be met and one of these is cocoa butter content. The Canadian Department of Justice, Food and Drug Regulations, state that for a product to be considered milk chocolate it must contain 25% total cocoa solids of which no less than 15% is cocoa butter. For a product to be considered a dark chocolate, no less than 35% is total cocoa solids of which no less than 18% is cocoa butter (Canadian Department of Justice, 2010b). However, the wording used by the Canadian Department of Justice can be unclear and lead to misinterpretations of the regulations which occurred during this research study. Originally the minimum omega-3 fatty acid incorporation needed to meet labeling claims was calculated as being 21wt% for milk chocolate and 11.9wt% for dark chocolate. Yet, the actual minimum omega-3 fatty acid incorporation needed was quite different. For a milk chocolate product, a minimum of 5.3 wt% omega-3 fatty acids are required to meet omega-3 fatty acid labeling claims. For dark chocolate a minimum of 4.2wt% omega-3 fatty acids must be incorporated. These regulations and their interpretation must be considered when calculating the amount of omega-3 fatty acids that must be incorporated

to meet labeling claims for omega-3 fatty acids. In order to determine these amounts a series of calculations were performed and are included in appendix I.

## 2.8 RESPONSE SURFACE METHODOLOGY

RSM is composed of mathematical and statistical methods used to develop an experimental plan (Xu, 2002). This method makes the evaluation of multiple parameters, alone or in combination, possible. The main advantage of using RSM is that fewer experiments are needed to gather enough information to obtain statistically acceptable results and it is normally used when optimizing an experimental model. A quadratic polynomial model is usually used to predict responses, and after experimental responses are evaluated a mathematical relationship can be established between the variables and responses. The RSM software allows for response surfaces and contour plots to be generated so that the shape of the response surfaces can be characterized and the optimum can be located. This method also makes it possible for the significance of variables to be determined. RSM does have some limitations because the model is derived from the variables specified and extrapolation is not advisable (Xu et al. 1998a and 2002). Optimization using RSM is very useful in enzymatic reaction experiments because there is no model that can fully predict the results of these complex systems. Another factor to consider is that factors tested have

interactions with each other that influence the results obtained and using RSM allows the relationships of the variables to be evaluated (Xu, 2003).

## CHAPTER 3 OBJECTIVES

The objectives of this study were 1) to compare the incorporation of a short chain fatty acid, DA, with that of an omega-3 fatty acid, ALA, into a vegetable oil, cocoa butter, using an immobilized enzyme derived from *R. miehei* while observing the effects of reaction temperature, time, substrate concentration, and enzyme load; 2) to monitor the formation of reaction by-products; 3) to create modified cocoa butter incorporating ALA that meets label claims for omega-3 PUFA; and 4) characterize the chemical and physical properties of the modified cocoa butter.

# CHAPTER 4 INFLUENCE OF REACTION CONDITIONS ON INCORPORATION OF UNUSUAL FATTY ACIDS INTO COCOA BUTTER

## 4.1 INTRODUCTION

Current consumer awareness of the health benefits of omega-3 consumption has increased the interest of using foods as a delivery system for these essential fatty acids. One way to incorporate omega-3 fatty acids into lipids is by creating structured lipids, thus obtaining value-added fats and oils. Structured lipids can be created to change the physical characteristics of a lipid or to improve its nutritional quality. They can be formed using enzymatic interesterification, where a lipase is used to catalyze an acyl exchange reaction (Osborn and Akoh, 2002). The most widely used interesterification reaction for the production of structured lipids is acidolysis, which involves the incorporation of a new fatty acid into TAG, thus synthesizing a structured lipid (Ciftci et al., 2009a and Akoh et al., 2002). Research in interesterification reactions from a functional and nutritional point of view is very popular because it makes it possible to create value-added foods (Ciftci et al., 2009a). However, when structured lipid are being manufactured it is important to determine if by-products, such as DAG and MAG, are being formed and in what amounts. These by-products are unwanted in the final product because they can lead to

undesirable changes to the physical properties of lipids (Chaiseri and Dimick, 1995).

Enzymatic interesterification has been used to create cocoa butter equivalents and substitutes using other lipids as the starting material, thus creating a structured lipid (Osborn and Akoh, 2002). Cocoa butter is the preferred fat in chocolate and confectionery industries due to its physical characteristics in these products and it has the greatest influence on chocolates' physical and chemical properties when compared to other ingredients. It is responsible for chocolates' melt-in-mouth feel, its snap when bit into, contraction, heat resistance, gloss, and bloom resistance (Abigor et al., 2003; Ciftci et al., 2009b; and Osborn and Akoh, 2002). TAG species found in cocoa butter depend on the cocoa bean origin; the most common TAGs found are POS, SOS, and POP (Zarringhalami et al., 2010). The fatty acids present in cocoa butter and their combinations in the TAG molecule give cocoa butter its characteristic crystallization properties, which in turn give it its specific melting characteristics (Osborn and Akoh, 2002). In this study instead of creating a cocoa butter equivalent or substitute, a structured lipid was made using cocoa butter as the starting material and incorporating an omega-3 fatty acid, thus

creating an omega-3 cocoa butter that could eventually be used to create chocolate and confectionary products rich in omega-3 fatty acids.

In order to develop an experimental plan, RSM was used. It makes the evaluation of multiple parameter effects possible, and it accounts for their effects in combination or alone. However, the greatest advantage of using RSM is that the number of experiments that need to be conducted is reduced, while still obtaining enough information for statistically acceptable results (Xu et al., 1998a). This allows the researcher to save time and money. Most, if not all, past research conducted using RSM, in the structured lipid field, aims to optimize reaction conditions. In the work being presented, optimal conditions were not necessarily wanted because it would lead to a very high fatty acid incorporation which would result in other difficulties if the modified cocoa butter was used directly in a chocolate or confectionary product. Instead, RSM was used to develop model equations which demonstrate how the variables affect the incorporation of DA and ALA. DA was chosen as a comparison fatty acid to test its capacity of being an appropriate kinetic predictor of ALA incorporation. The main reason for choosing DA was that it was readily available and more economical than ALA.

This study focuses on the incorporation of two fatty acids, DA and ALA, into cocoa butter using an immobilized lipase derived from *R. miehei*. The aim was to investigate, by means of RSM, the effects of mole ratio, time and temperature on the degree of incorporation of the two fatty acids. A face centered design was used to develop the experimental plan and to determine how the variables influenced the incorporation of the fatty acids. The formation of by-products in the ALA-incorporated cocoa butter was also investigated.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Experimental Design

Response surface methodology (RSM) was used to optimize the experimental conditions of the interesterification reaction whereby either DA or ALA were incorporated into cocoa butter. A face centered experimental design was used to establish the relationship between the incorporation of the fatty acid into cocoa butter and the three experimental variables (Cocoa butter: fatty acid ratio, temperature, and time). This experimental design required 17 experiments, with 8 experimental points located in the corners of the cube, 6 experimental points in the center of the cube faces, and 3 points located in the center of the cube. This is considered a 3 factor, 3 level face-centered cubic

design. The fitted model was used to study the optimal experimental conditions using the amount of incorporation obtained from the variables tested.

In this experiment, the generation and data analysis of the face centered experimental design were performed using the experimental design software Design Expert Version 7.1.6 (Minneapolis, MN, USA).

### 4.2.2 Materials

Cocoa butter was obtained from the Nestle R & D Center Inc (Marysville, OH, USA). DA was purchased from Sigma Aldrich (Oakville, ON, Canada), ALA was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA), and all solvents and reagents (Optima grade) were purchased from Fischer Scientific (Ottawa, ON, Canada). Immobilized enzyme derived from *R. miehei*, was purchased from Fluka Analytical, a Sigma Aldrich company (Oakville, ON, Canada).

### 4.2.3 Interesterification reaction

Lipase immobilized from *R. miehei* (100mg) was placed in 20mL screw top glass vials to which 1: 3, 1: 6, or 1: 9 molar concentration of either cocoa butter : DA or cocoa butter : ALA solutions were added as solutions of cocoa butter, DA, and ALA followed by the addition of hexane to make a total of 5mL of solution. In order to prepare the cocoa butter solution cocoa butter was first

purified by dissolving in hexane, filtering over anhydrous sodium sulfate, evaporating the hexane in a rotary evaporator (Buchler instruments flash evaporator, Fort Lee, New Jersey, USA), and storing under $N_2$ in the freezer. Cocoa butter solution was made at the time of the experiment in a 100mg cocoa butter / mL of hexane concentration using a volumetric flask. DA solution was made by dissolving 3.14g of DA in 50mL of hexane in a 50mL volumetric flask, which was then stored for use at room temperature away from sunlight. ALA solution was made by dissolving 2.53g of ALA in 25mL of hexane in a 25mL volumetric flask, which was then stored for use at room temperature from sunlight. The vials were flushed with $N_2$ before being capped. The samples were then placed in a shaking incubator at 100rpm for 6, 18, or 30 hours at 40, 50, or 60˚C (Table 4.1).

Table 4.1 Experimental variables and parameters for the RSM face centered experimental design.

| Run | Time (hrs) | Temperature (˚C) | Molar Concentration (Cocoa butter: fatty acid) |
|---|---|---|---|
| 1 | 6 | 40 | 1:3 |
| 2 | 6 | 40 | 1:9 |
| 3 | 18 | 40 | 1:6 |
| 4 | 30 | 40 | 1:3 |
| 5 | 30 | 40 | 1:9 |
| 6 | 6 | 50 | 1:6 |
| 7 | 18 | 50 | 1:3 |
| 8 | 18 | 50 | 1:6 |
| 9 | 18 | 50 | 1:6 |
| 10 | 18 | 50 | 1:6 |
| 11 | 18 | 50 | 1:9 |
| 12 | 30 | 50 | 1:6 |
| 13 | 6 | 60 | 1:3 |
| 14 | 6 | 60 | 1:9 |
| 15 | 18 | 60 | 1:6 |
| 16 | 30 | 60 | 1:3 |
| 17 | 30 | 60 | 1:9 |

After the appropriate amount of time elapsed, the samples were removed from the incubator, the enzyme was filtered out, and the filtrate was collected in

50mL glass centrifuge tubes. The enzyme was rinsed using 5mL of hexane and this too was collected in the 50mL centrifuge tube. Once the filtrate was collected, the enzyme was rinsed again, allowed to air dry and then stored in the refrigerator (4°C) for subsequent use. To the filtrate, which contained the modified cocoa butter, excess free fatty acids, and the hexane used to rinse the enzyme, 5mL of 1:1 acetone: ethanol was added, along with 3 drops of phenolphthalein indicator. The solution was then flushed with $N_2$, capped, and vortexed. The sample solution was neutralized by titrating with 0.5M NaOH until the first hint of pink appeared and remained. This resulted in two layers forming; the top layer was then removed and placed in a 20mL glass screw top test tube to which 1mL of distilled $H_2O$ ($dH_2O$) was added. The test tube was then capped and vortexed. The two layers were allowed to separate and the top layer was passed over a bed of anhydrous sodium sulfate and collected in a pre-weighed 10mL graduated glass centrifuge tube. The samples were then evaporated in a 40°C water bath under a stream of $N_2$. The masses of the test tubes were recorded and the amount of lipid recovered was determined. The samples were then dissolved in hexane at a concentration of 25mg of lipid/mL of hexane. Samples were stored under $N_2$ in the freezer until further testing was required.

A series of experiments were conducted in order to determine the optimal mass of enzyme to be used for all subsequent runs of the interesterification reaction. The following set of variable parameters was used: molar ratio of 1:3 (cocoa butter : DA), for 6 hours, at 40°C temperature. The enzyme amount was varied to determine the effect of enzyme load on incorporation of DA. The enzyme amounts used ranged from 50-250mg of lipase immobilized from *R. miehei*; this mass includes the immobilized enzyme support. Statistical analysis of this data was performed using SPSS (version 11.0.1) package program with a 95% confidence interval.

### 4.2.4 Fatty acid methyl ester (FAME) production

In order to determine quality and structure of fatty acids present in the lipid recovered, they were converted into FAMEs using the Hilditch procedure and analyzed using GC-FID.

The dissolved recovered lipid (12.5mg) was placed in a 10mL glass graduated screw top centrifuge tube; hexane was then evaporated in a 40°C water bath under a stream of $N_2$. To this, 1.5mL of methylene chloride with 0.01% BHT and 3.0mL of Hilditch reagent was added, which was prepared by mixing 100mL of dry methanol with 1.5mL of sulfuric acid. The sample was

then flushed with $N_2$, capped, and vortexed. The test tubes were placed on a heating block at 100˚C for one hour. Samples were allowed to cool to room temperature on the laboratory bench. When the samples had cooled, 3mL of hexane and 1mL of $dH_2O$ were added and the tube was capped, vortexed, and centrifuged for 2-5 minutes. The top layer was removed to a 10mL glass test tube and the hexane extraction was then repeated two more times using 1mL of hexane. To this tube, 2mL of $dH_2O$ was added. The sample was then capped, vortexed, and centrifuged for 2 minutes. The top layer was then removed and placed in a 10mL glass centrifuge tube to which a scoop of anhydrous sodium sulfate was added; the tube with sample and sodium sulfate was shaken, and left to settle. The solvent was then transferred to a pre-weighed 10mL glass test tube and evaporated in a 40˚C water bath under a stream of $N_2$. Total mass of FAME obtained was then determined and hexane added to a total concentration of 10mg FAME/mL of hexane. The sample was then transferred to a GC vial and any remaining FAME stored in a 5mL glass screw top vial, flushed with $N_2$, capped, sealed with sealing film, and stored in a freezer.

### 4.2.5 Gas chromatography – Flame Ionization detector (GC-FID) of FAME

FAMEs were separated on a DB-23 polar capillary column (30m x 0.25mm ID flexible fused silica column) with 50% cyanopropyl polysiloxane coating

installed in a Perkin Elmer autosystem GC equipped with an autosampler and a flame ionization detector. Samples (10mg/mL) were introduced using a 5µL syringe, which delivered 1µL of sample, to an injector held at a constant 250˚C temperature. Helium was used as a carrier gas at a flow rate of 1.0mL/minute and the Helium split flow was set at a rate of 100mL/minute to obtain a split ratio of about 1:100. Air and hydrogen flow rates to the detector were 450mL/min and 45mL/min, respectively. The FID was maintained at 270˚C. The oven was initially held at 153˚C for 2 minutes; the temperature was then increased 2.3˚C/minute until 174˚C was reached and was held for 0.2 minutes. The temperature was again increased at a rate of 2.5˚C/minute to 210˚C and held for 2 minutes or until 24:1 eluted. The total run time was approximately 32 minutes (Budge et al., 2006).

### 4.2.6 High Performance Liquid Chromatography – Normal phase (HPLC)

Samples of ALA incorporated cocoa butter were diluted to approximately 0.25mg/ml in 2:1 $CHCl_3$ : MeOH, after which they were filtered. An HPLC with autosampler (Thermofisher Scientific, Mississauga, ON) and a low temperature evaporative light scattering detector (LT-ELSD; Sedere North York, ON) was used for the analysis. A Gain of 8 on the ELSD was used along with a 5µL injection volume. The mobile phase used had a flow rate of 2mL/min and a run time of

10 min as shown in Table 4.2. The detector temperature was set at 30˚C and the column was held at 20±1˚C. Individual peaks were identified by comparing their retention times to that of tristearin, 1, 2-distearin, and 1, 3-distearin. Standard curves were also developed using these standards to calculate the %TAG, %1, 2-DAG, and %1, 3-DAG in the samples. These were prepared the same way as the samples at a concentration of 0.25mg/ml in 2:1 $CHCl_3$: MeOH with injection volumes ranging from 2.5-25µL, depending on the standard.

The HPLC was purchased from Thermofisher Scientific and is a Thermo Finnigan Surveyor with autosampler and uses a Sedere LT-ELSD Sedex 80 (Mississauga, ON, Canada). A Waters spherisorb silica column (5µm particles; 250mm x 4.6mm) was used. Solvents and reagents (Optima grade) were purchased from Fischer Scientific (Ottawa, ON, Canada).

Table 4.2 Composition of mobile phase used for gradient elution with HPLC analysis.

| Time (min) | Hexane (%) | Tert-Butyl Methyl Ether (%) |
|---|---|---|
| 1.0 | 98.0 | 2.0 |
| 5.0 | 100.0 | 0.0 |
| 7.0 | 100.0 | 0.0 |
| 8.0 | 98.0 | 2.0 |
| 10.0 | 98.0 | 2.0 |

## 4.3 RESULTS

### 4.3.1 Effect of Enzyme load

Under the specific conditions tested, enzyme load did not have a significant effect on fatty acid incorporation (Figure 4.1). ANOVA did not indicate a significant effect of enzyme mass on DA incorporation. Therefore, 100mg was chosen as the enzyme load for all further testing.

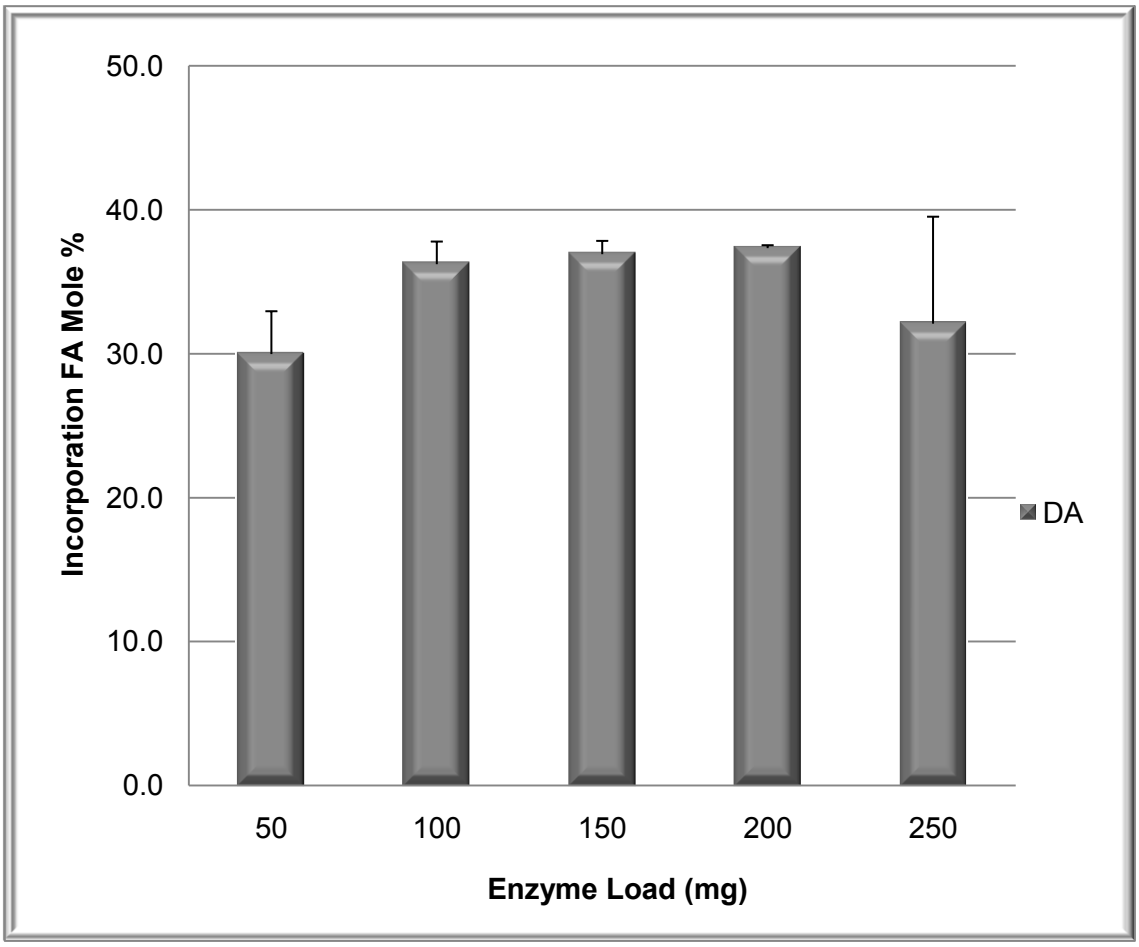


Figure 4.1 Variation of DA incorporation with immobilized enzyme load, using a 1:3 molar concentration (cocoa butter: DA), at 40°C, for 6 hours . Each bar represents average mole% ± standard deviation, n=3.

### 4.3.2 Optimization of reaction parameters

The highest and lowest incorporations for both fatty acids were obtained using the same variable combinations (Table 4.3). The highest incorporation for both DA and ALA was achieved with the following testing parameters: a 1:9 molar ratio, for 30 hrs, at 60˚C, which are the greatest values for the

parameters tested. DA had an incorporation of 68.2±1.1 mole% and ALA had an incorporation of 77.3±1.3 mole%. The lowest incorporation levels for DA were 44.8±7.5 mole% which was obtained with a 1:3 molar ratio, 30 hours, and 60˚C. The lowest ALA incorporation was obtained at 1:3 molar ratio, 6 hours, and 40˚C (41.6±0.6 mole%).

Table 4.3 Incorporation of DA and ALA into cocoa butter as average Mole% ± standard deviation (n=3). Entries in bold indicate the lowest and highest incorporation values.

| Sample | | | DA | | ALA | |
|---|---|---|---|---|---|---|
| **Molar concentration (Cocoa butter : fatty acid)** | **Time (hrs)** | **Temperature (˚C)** | **Average (Mole%)** | **St Dev (Mole%)** | **Average (Mole%)** | **St Dev (Mole%)** |
| **1:3** | **6** | **40** | **45.8** | **±1.4** | **41.6** | **±0.6** |
| 1:9 | 6 | 40 | 55.0 | ±0.4 | 51.1 | ±0.4 |
| 1:6 | 18 | 40 | 62.2 | ±1.8 | 57.9 | ±0.8 |
| 1:3 | 30 | 40 | 49.1 | ±2.1 | 51.3 | ±1.9 |
| 1:9 | 30 | 40 | 66.1 | ±1.0 | 68.2 | ±1.5 |
| 1:6 | 6 | 50 | 53.8 | ±3.5 | 58.3 | ±0.04 |
| 1:3 | 18 | 50 | 48.7 | ±2.4 | 52.5 | ±0.1 |
| 1:6 | 18 | 50 | 64.3 | ±1.4 | 67.5 | ±0.7 |
| 1:6 | 18 | 50 | 64.0 | ±2.9 | 66.4 | ±0.8 |
| 1:6 | 18 | 50 | 61.2 | ±1.1 | 66.4 | ±0.6 |
| 1:9 | 18 | 50 | 67.3 | ±2.0 | 72.2 | ±0.1 |
| 1:6 | 30 | 50 | 64.5 | ±0.5 | 69.0 | ±0.1 |
| 1:3 | 6 | 60 | 45.1 | ±3.0 | 47.1 | ±0.2 |
| 1:9 | 6 | 60 | 58.1 | ±1.5 | 64.9 | ±0.4 |
| 1:6 | 18 | 60 | 67.8 | ±0.5 | 69.2 | ±0.4 |
| 1:3 | 30 | 60 | 44.8 | ±7.5 | 53.6 | ±0.2 |
| **1:9** | **30** | **60** | **68.2** | **±1.1** | **77.3** | **±1.3** |

A quadratic model best fit the data obtained for both DA and ALA (Table 4.4). The quadratic model showed an insignificant lack-of-fit ($p>0.05$), a low standard deviation (Mole %), and a high R-squared value, all desired results. Significant lack-of-fit would be shown by a low probability value (Prob>F), below 0.05. Our model has lack-of-fits of 0.40 and 0.21, for DA and ALA incorporation, respectively. This indicates that the model fits the data. The good fit is further supported by the R-squared value which indicated that over 97% of the variance in the data was explained by the models. When the residuals for each of the fatty acids incorporation (Mole %) data were plotted, these demonstrated a randomly scattered configuration around zero indicating that the model describes the data well (Figures 4.2a and b).

Table 4.4 Model statistics for fatty acid incorporation in a quadratic model.

| Fatty acid Incorporated | Standard Deviation (Mole %) | R-Squared | Lack-Of-Fit test Prob>F |
|---|---|---|---|
| DA Incorporation | 2.15 | 0.97 | 0.40 |
| ALA Incorporation | 1.20 | 0.99 | 0.21 |

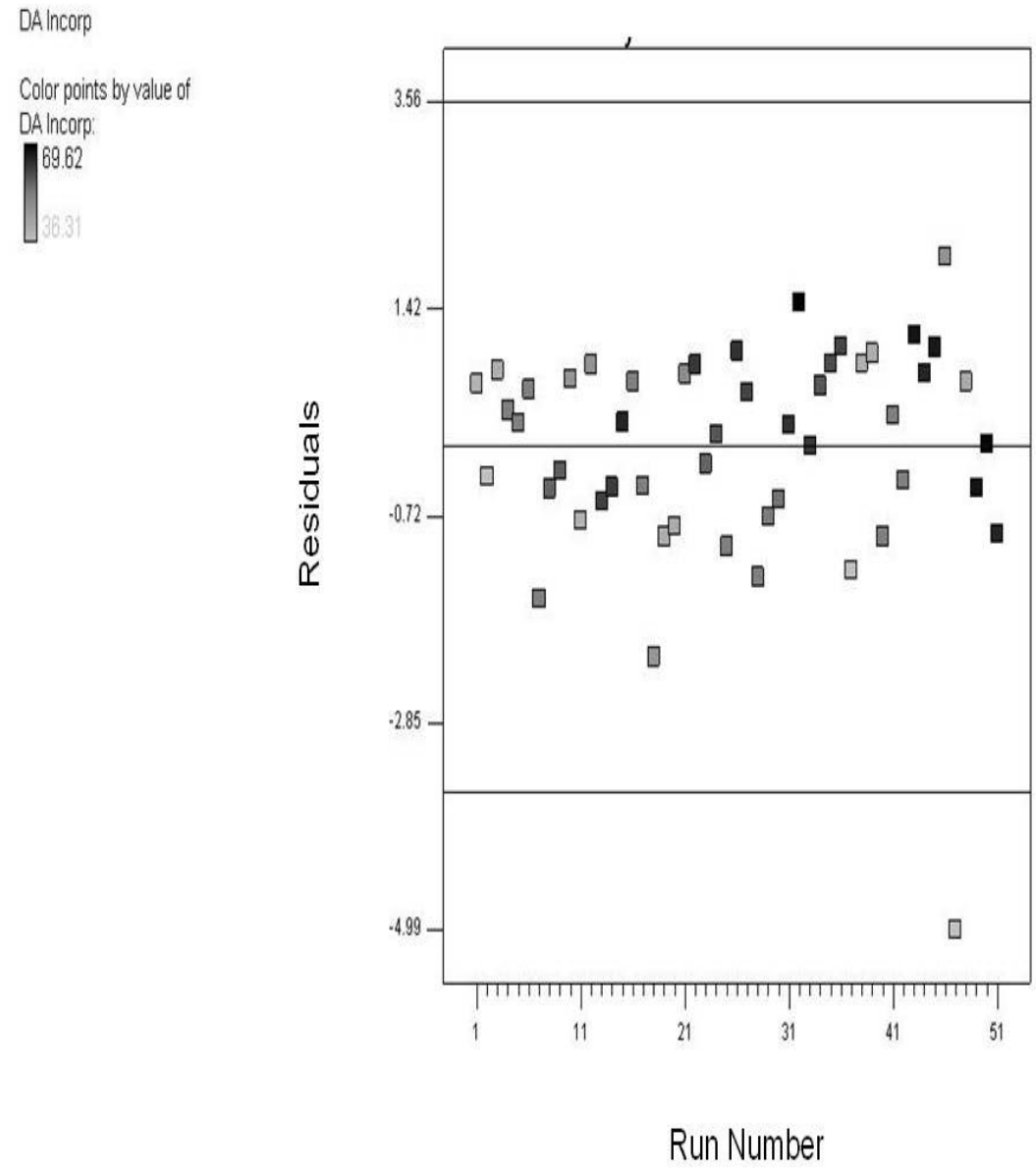


Figure 4.2a Residual values for DA mole% incorporation. Run numbers refer to those in Table 4.1 and values shown are for all triplicates of these runs.

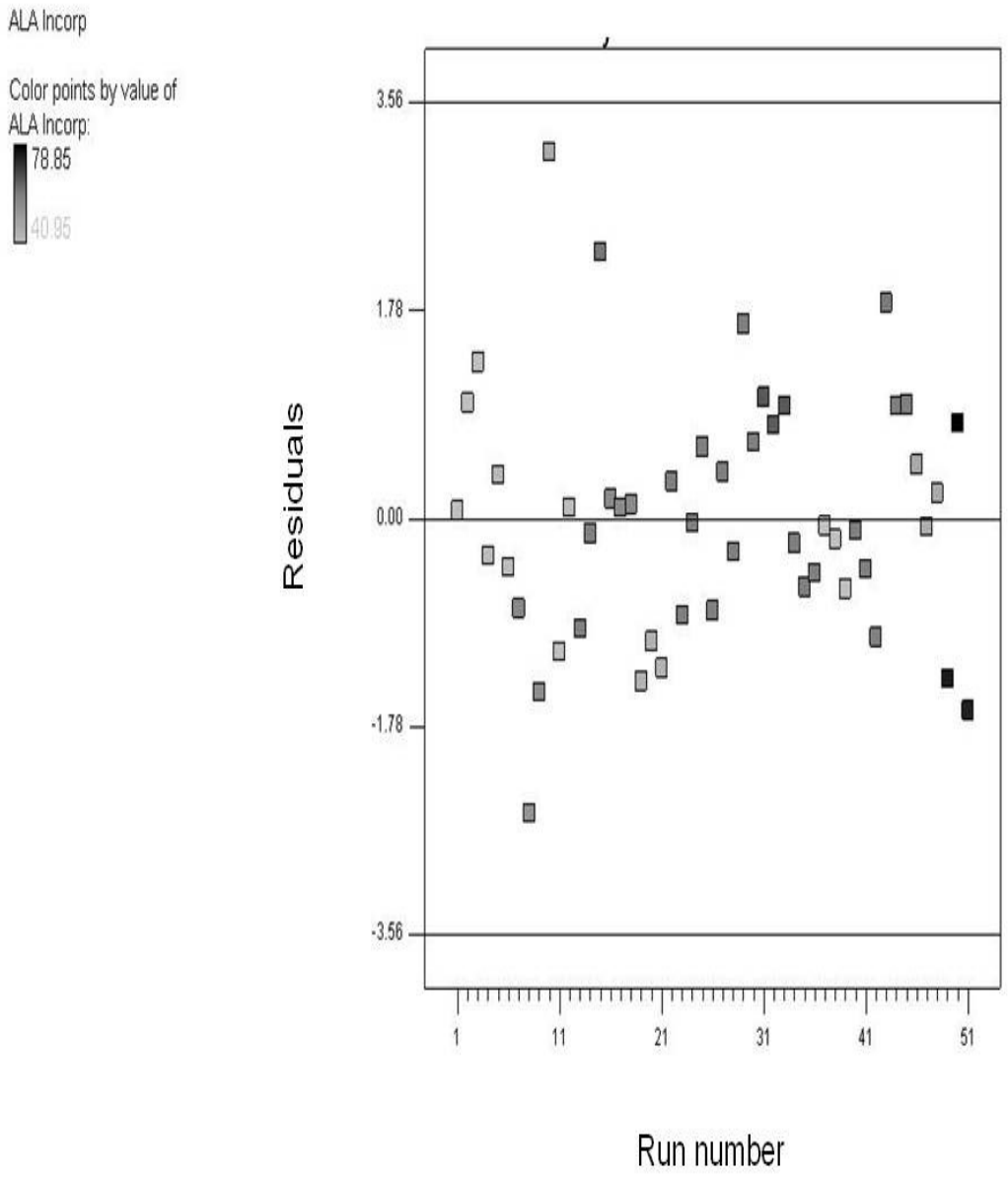


Figure 4.2b Residual values for ALA mole% incorporation. Run numbers refer to those in Table 4.1 and values shown are for all triplicates of these runs.

ANOVA also suggests a significant model for the incorporation of both fatty acids with $F=27$, $P<0.01$ and $F=122$ $P<0.01$ for DA and ALA, respectively. These values imply that the model is significant and specifies which variables are significant. For the DA incorporation model, it was noted that molar ratio and time had a significant effect on incorporation ($F=142$, $P<0.0001$ and $F=26$, $P<0.0001$, respectively), along with the interaction of these two variables and their squares (Table 4.5). The ALA incorporation model, however, was not identical. For ALA incorporation, the three variables tested (molar ratio, time, and temperature) all had significant effects, as did their interactions and squares (Table 4.5); the only insignificant variable ($p>0.05$) was the combination of time and temperature with a Prob>F value of 0.053.

Table 4.5 ANOVA for response surface using the quadratic model for fatty acid incorporation.

| Source | Sum of squares | | Mean square | | F value | | Prob>F | |
|---|---|---|---|---|---|---|---|---|
| | DA | ALA | DA | ALA | DA | ALA | DA | ALA |
| Model | 1149 | 1581 | 127 | 175 | 27 | 122 | 0.0001 | <0.0001 |
| Molar ratio | 660 | 766 | 660 | 766 | 142 | 534 | <0.0001 | <0.0001 |
| Time | 121 | 317 | 121 | 317 | 26 | 221 | 0.0014 | <0.0001 |
| Temperature | 3 | 175 | 3 | 175 | 0.7 | 122 | 0.42 | <0.0001 |
| Molar ratio and Time | 41 | 22 | 41 | 22 | 8 | 15 | 0.020 | 0.0056 |
| Molar ratio and Temperature | 13 | 28 | 13 | 28 | 2 | 19 | 0.13 | 0.0030 |
| Time and Temperature | 2 | 7 | 2 | 7 | 0.5 | 5 | 0.48 | 0.053 |
| Molar ratio$^2$ | 93 | 43 | 93 | 43 | 20 | 30 | 0.0029 | 0.0009 |
| Time$^2$ | 60 | 20 | 60 | 20 | 12 | 14 | 0.0087 | 0.0067 |
| Temperature$^2$ | 3 | 21 | 3 | 21 | 0.8 | 15 | 0.40 | 0.0058 |

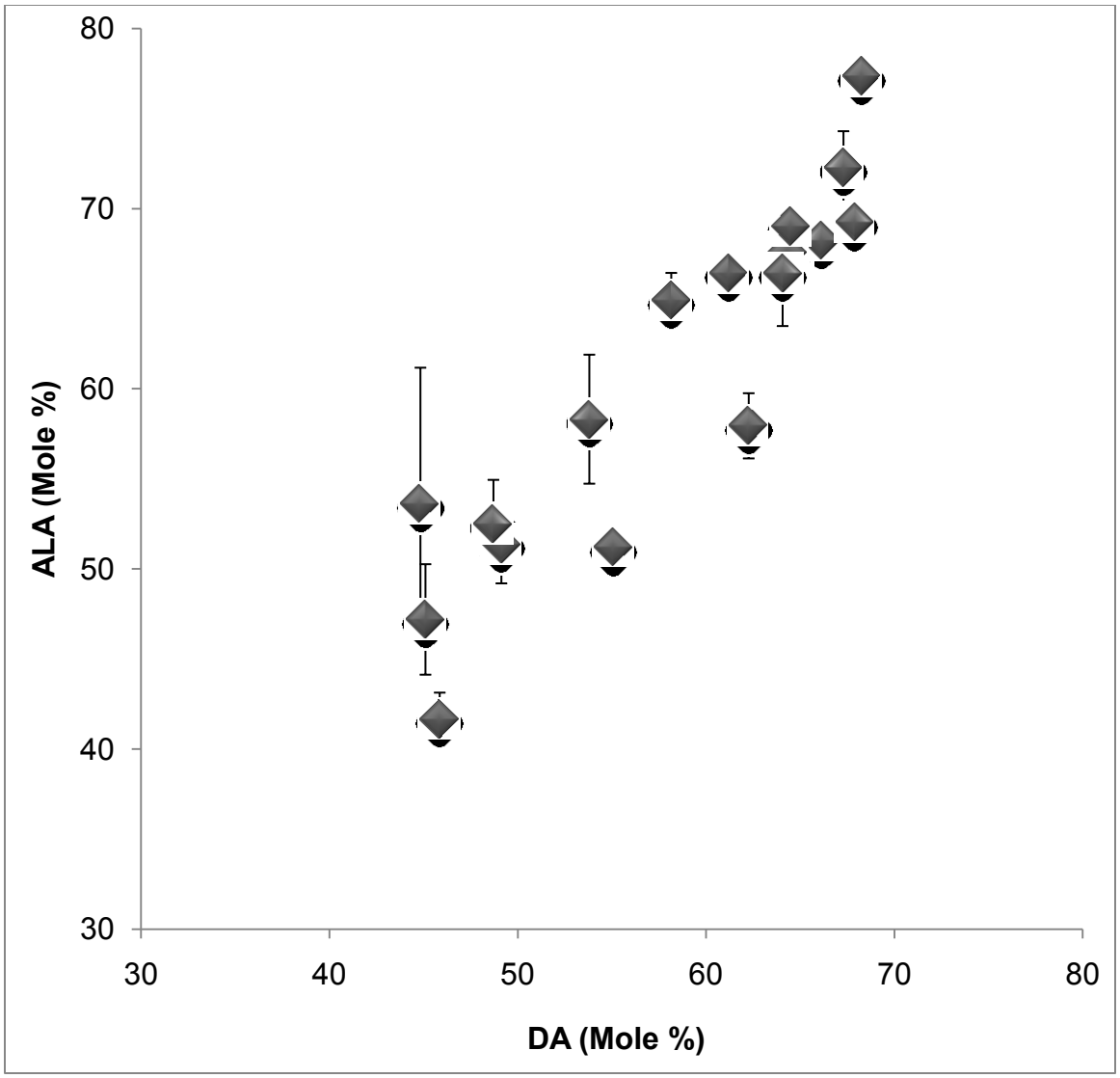


Figure 4.3 DA vs. ALA average ± standard deviation (Mole %) incorporation, n=3.

RSM allows for response surfaces and contour plots to be generated so that the shape of the response surfaces can be characterized and the optimum located (Xu, 2002). These plots allow for the relationship between variables and their effects on the response to be easily visualized. For example, for DA

incorporation, as time increased, incorporation increased (Figure 4.4); a similar effect was observed for molar ratio, where incorporation also increased as higher molar ratios were used (Figure 4.4). Similar results can be seen in Figure 4.5 for ALA incorporation; again as time and molar ratio increased so did ALA incorporation. It should however, be noted that an optimum cannot be determined because the plots do not reach a clear maximum. They do however, seem to reach a plateau, without showing a decrease at less favorable variable combinations (Figure 4.5).

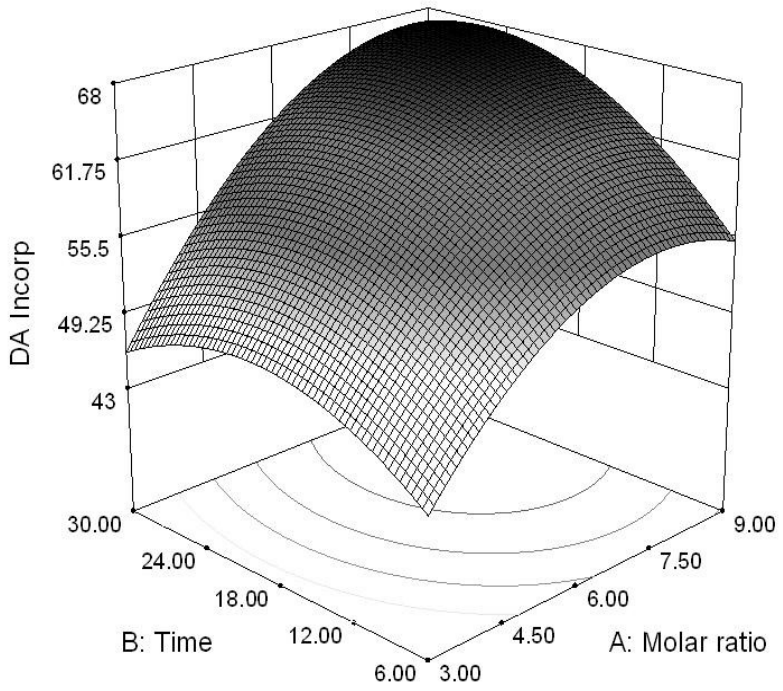


Figure 4.4 Response surface for the incorporation of DA showing the effects of time and molar ratio.

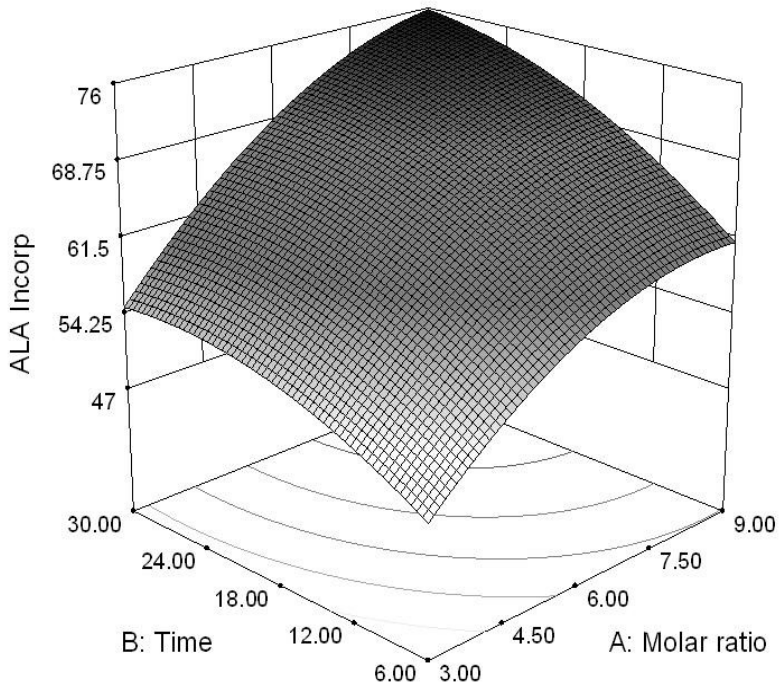


Figure 4.5 Response surface for the incorporation of ALA showing the effects of time and molar ratio.

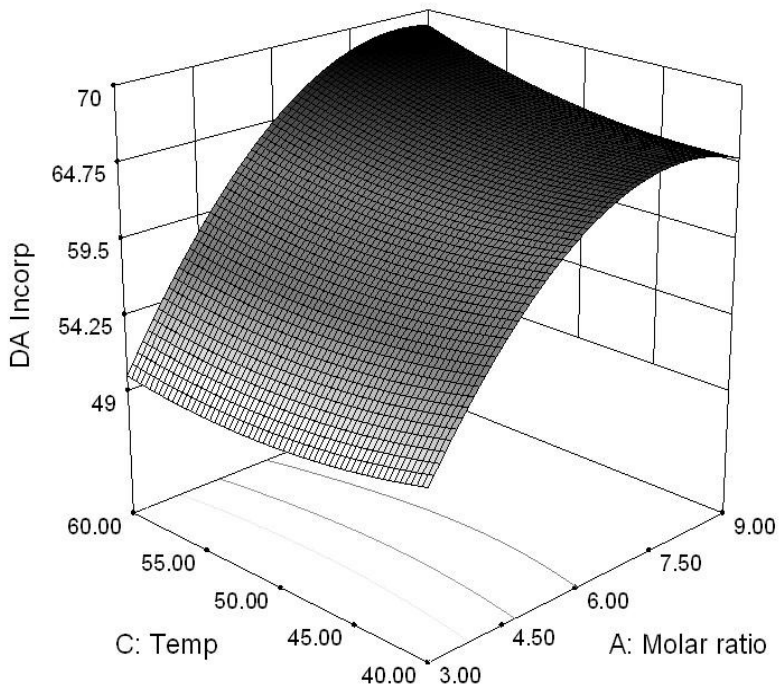


Figure 4.6 Response surface for the incorporation of DA showing the effects of temperature and molar ratio.

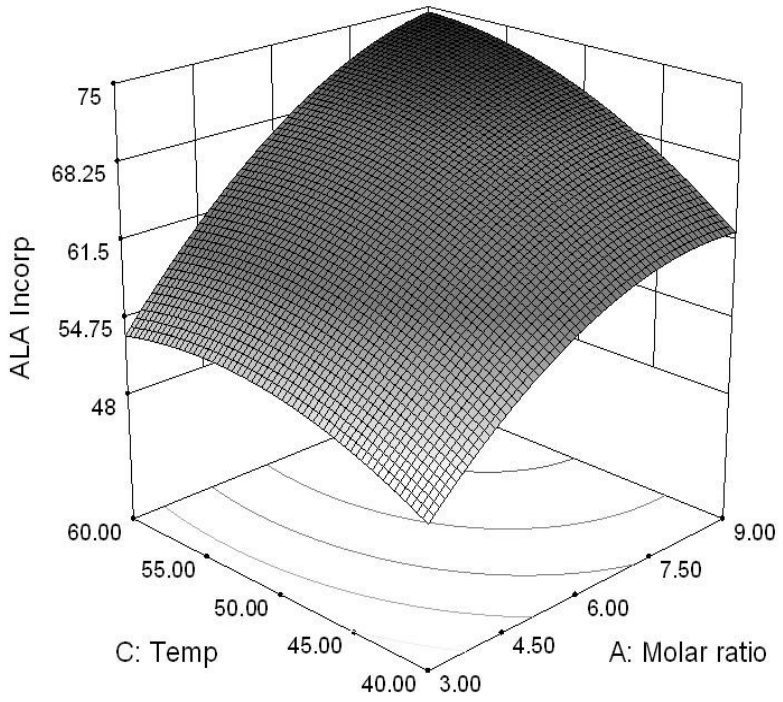


Figure 4.7 Response surface for the incorporation of ALA showing the effects of temperature and molar ratio.

However, response surfaces differ when temperature is considered. For DA incorporation, temperature was not significant; however for ALA incorporation it did have a significant effect. In Figure 4.6, DA incorporation relative to temperature and molar ratio is shown and it can be seen that as the temperature is increased, there is no significant change in the amount of DA

incorporated, while as molar ratio is increased there is an increase in the incorporation of DA. For ALA, however, there is an obvious increase in incorporation as temperature and molar ratio increase (Figure 4.7). Again it must be noted that an optimum was not reached which is seen by a lack of a peak; therefore maximum DA and ALA incorporation was not accomplished.

For each fatty acid model, RSM creates a factorial equation which can be used to predict incorporation under conditions other than those tested; these equations are shown below for both fatty acids. The ALA incorporation equation will be used in the following chapter.

Equation 1

DA incorporation = 49.84 + (7.289 * Molar ratio) + (1.329 * Time) – (1.290 * Temperature) + (0.06307 * Molar ratio * Time) + (0.04289 * Molar ratio * Temp) – (4.661e-003 * Time * Temperature) – (0.6550 * Molar ratio$^2$) – (0.03290 * Time$^2$) + (0.01175 * Temperature$^2$)

Equation 2

ALA incorporation = -57.82 + (4.348 * Molar ratio) + (1.296 * Time) + (3.050 * Temperature) + (0.04634 * Molar ratio * Time) + (0.06273 * Molar ratio * Temperature) – (8.193e-003 * Time * Temperature) – (0.4500 * Molar ratio$^2$) – (0.01930 * Time$^2$) – (0.02859 * Temperature$^2$)

### 4.3.3 Determination of interesterification by-products

HPLC with external calibration was used to determine the amounts of TAG, 1, 3-DAG, and 1, 2-DAG present in the samples. The standard curves had good fit (Figures 4.8, 4.10, and 4.12); this can be seen by their coefficients of determination ($R^2$) values, of 0.99, 0.99, and 0.99, for TAG, 1, 3-DAG, and 1, 2-DAG, respectively. For the three standard curves a cubic equation was developed and their residual values are shown in Figures 4.9, 4.11, and 4.13 for TAG, 1, 3-DAG, and 1, 2-DAG, respectively. The residual plots demonstrate that the data is randomly scattered which further demonstrates the goodness of fit of the standard curves. MAG was not detected in any sample.

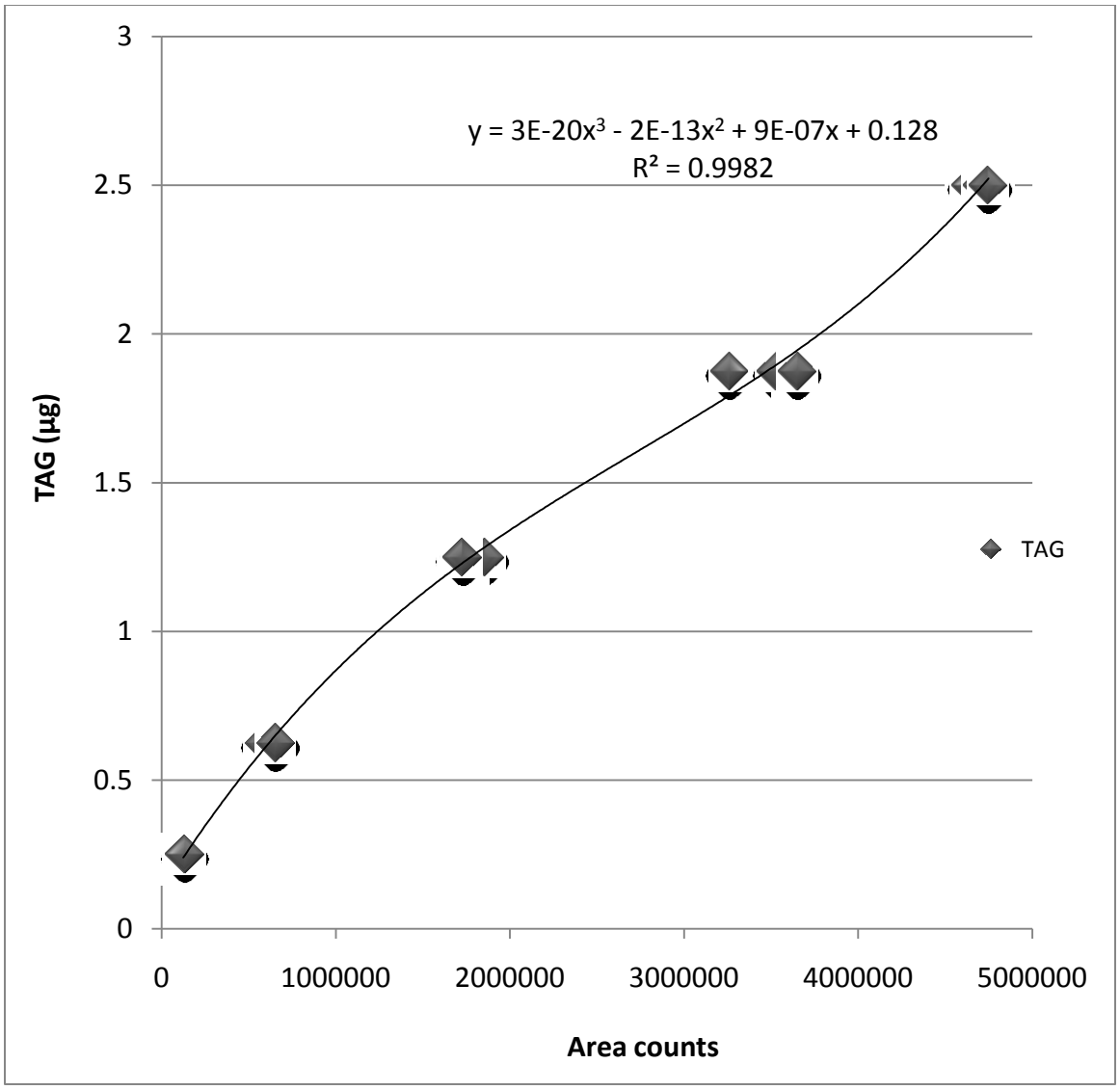


Figure 4.8 Standard curve for TAG determination using HPLC. Each point represents the µg TAG.

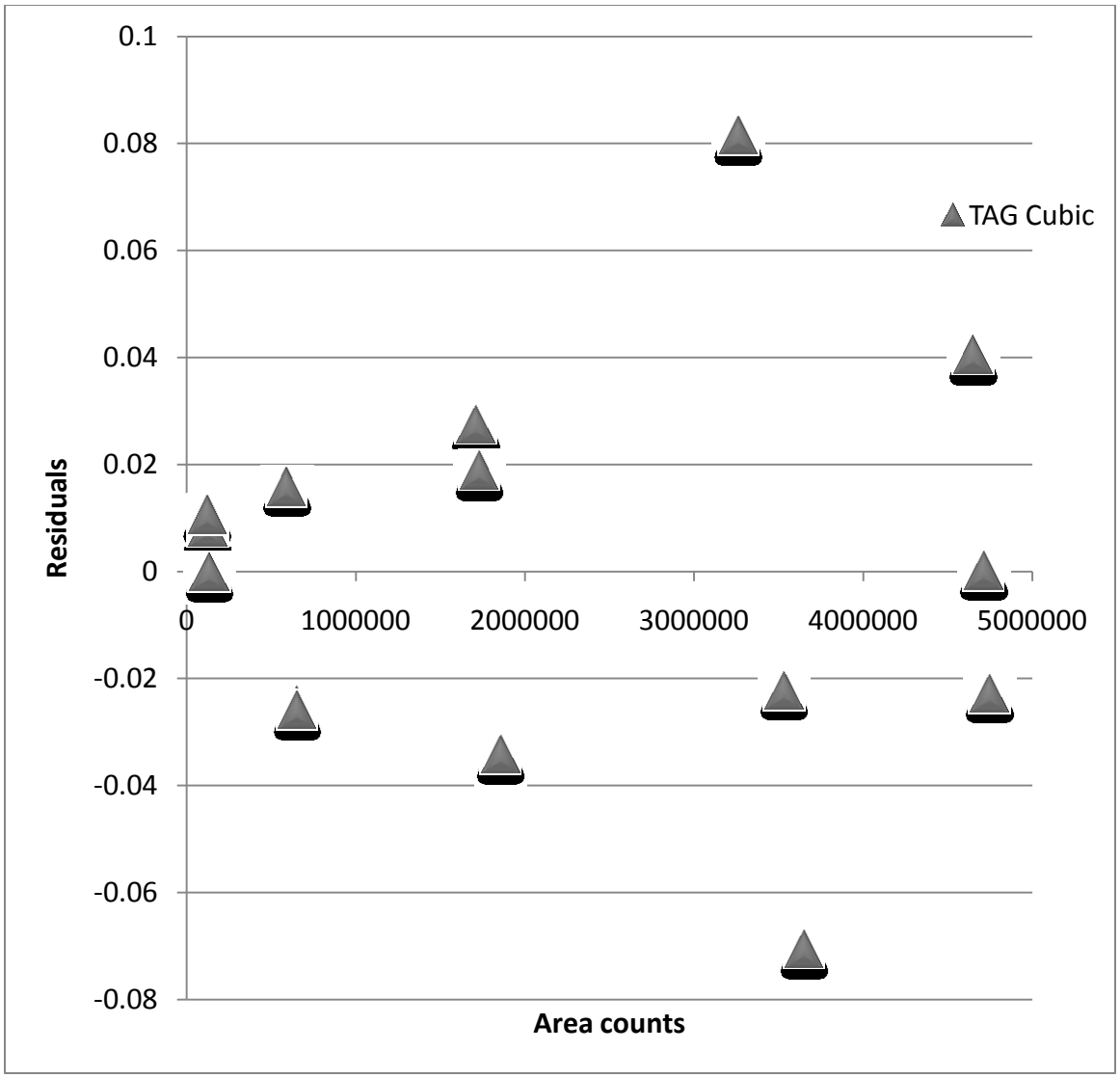


Figure 4.9 Residual values for the TAG standard curve.

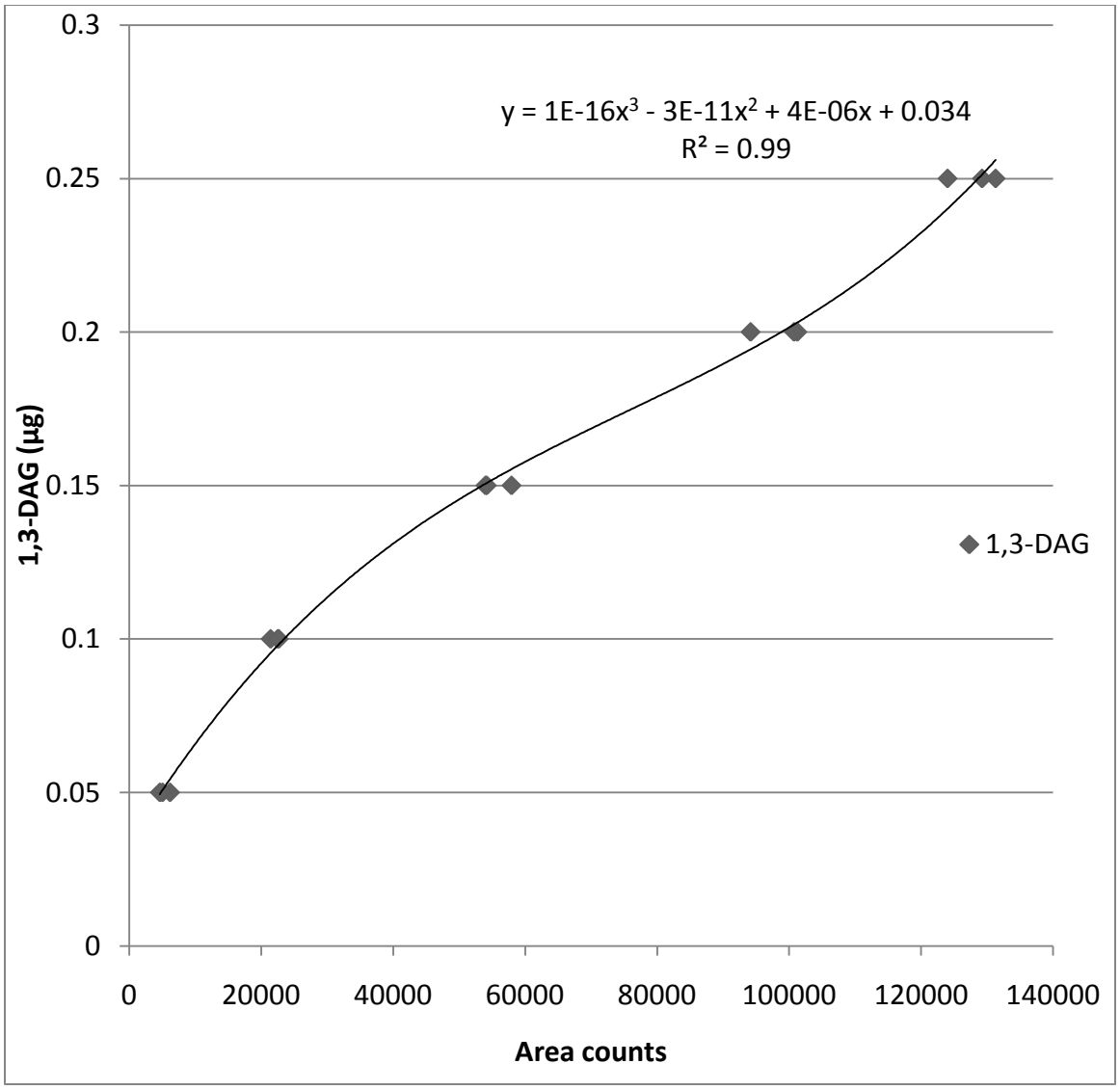


Figure 4.10 Standard curve for 1, 3-DAG determination using HPLC. Each point represents the µg 1, 3-DAG.

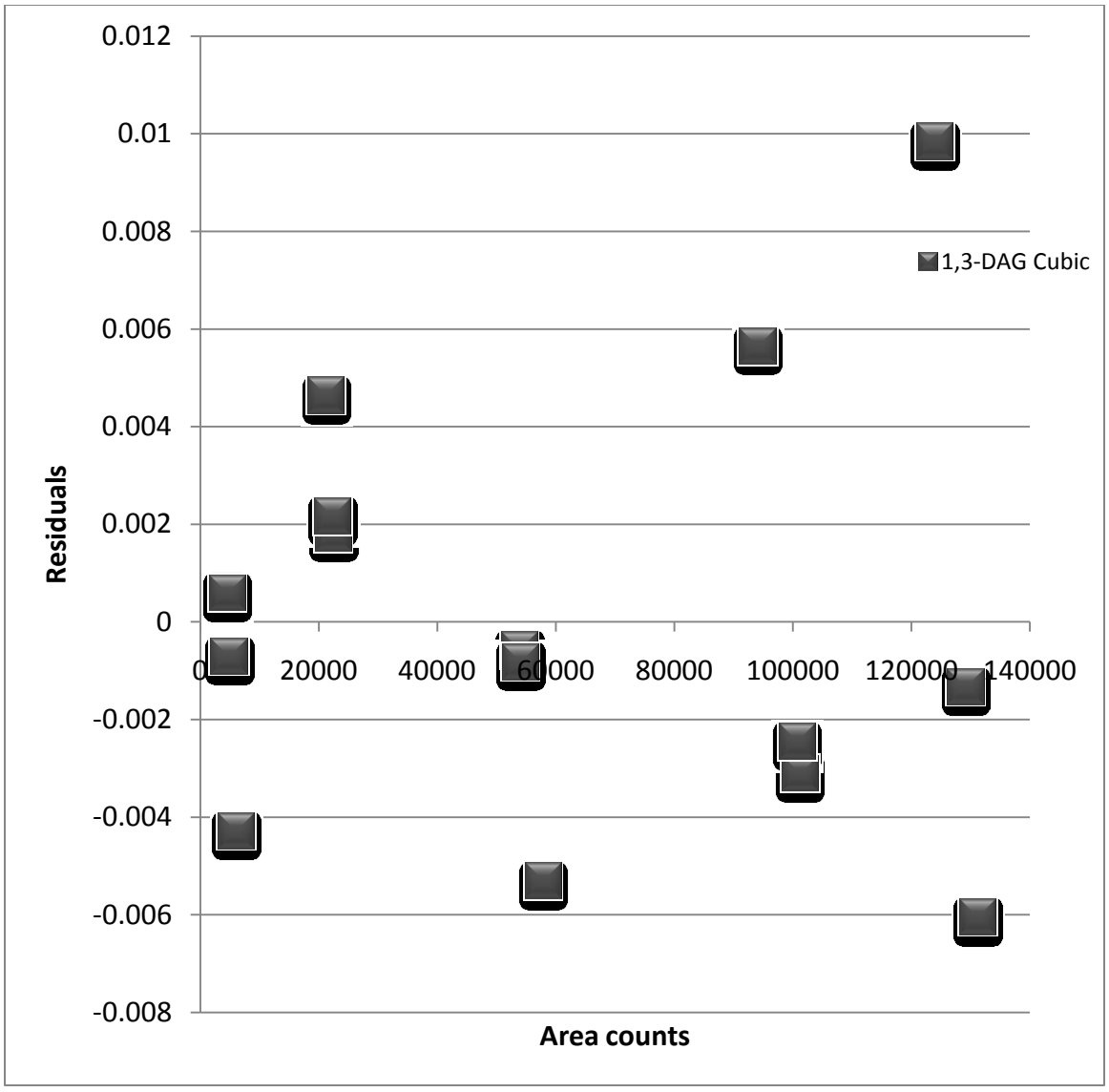


Figure 4.11 Residual values for the 1, 3-DAG standard curve.

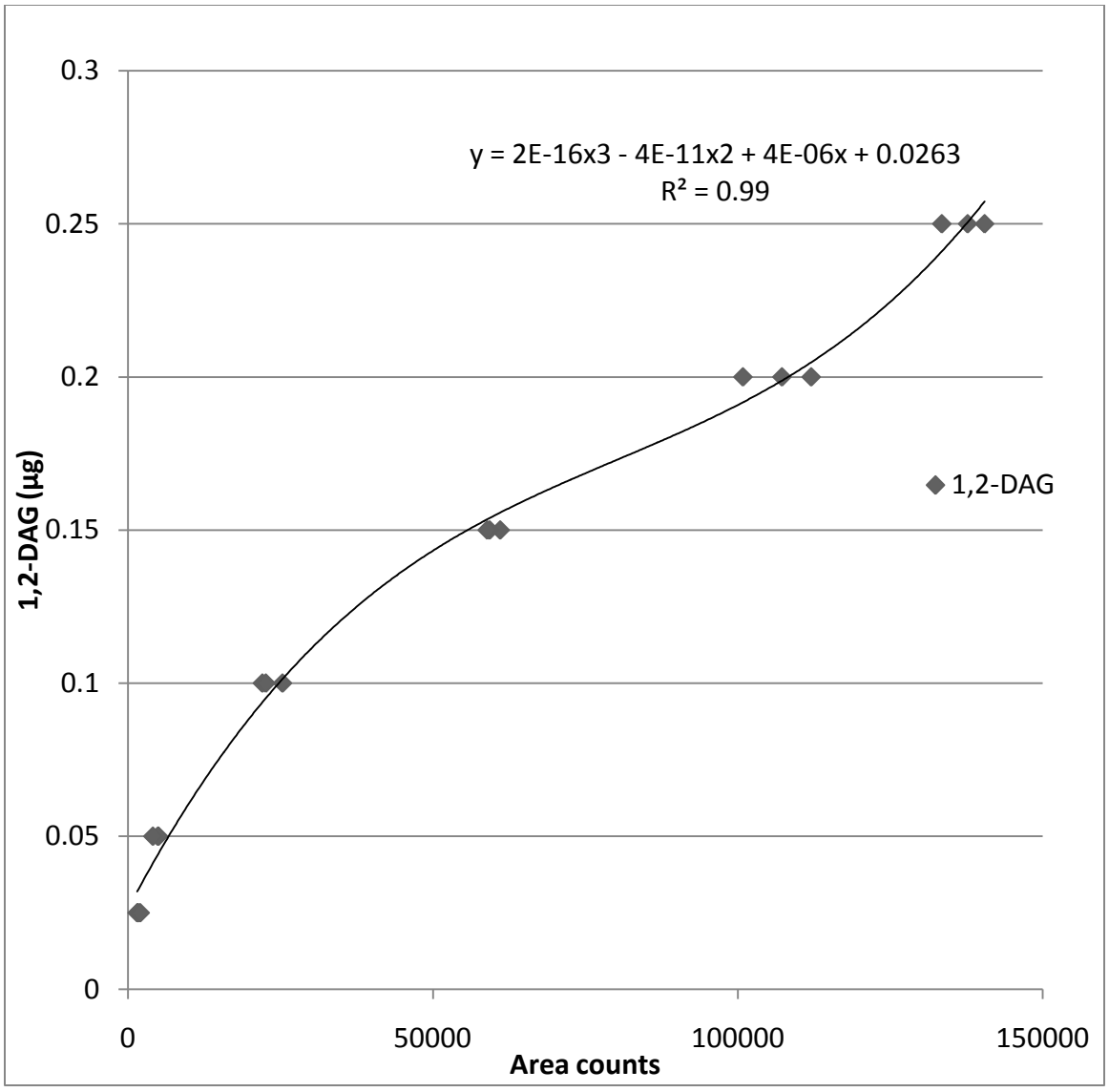


Figure 4.12 Standard curve for 1, 2-DAG determination using HPLC. Each point represents the µg 1, 2-DAG.

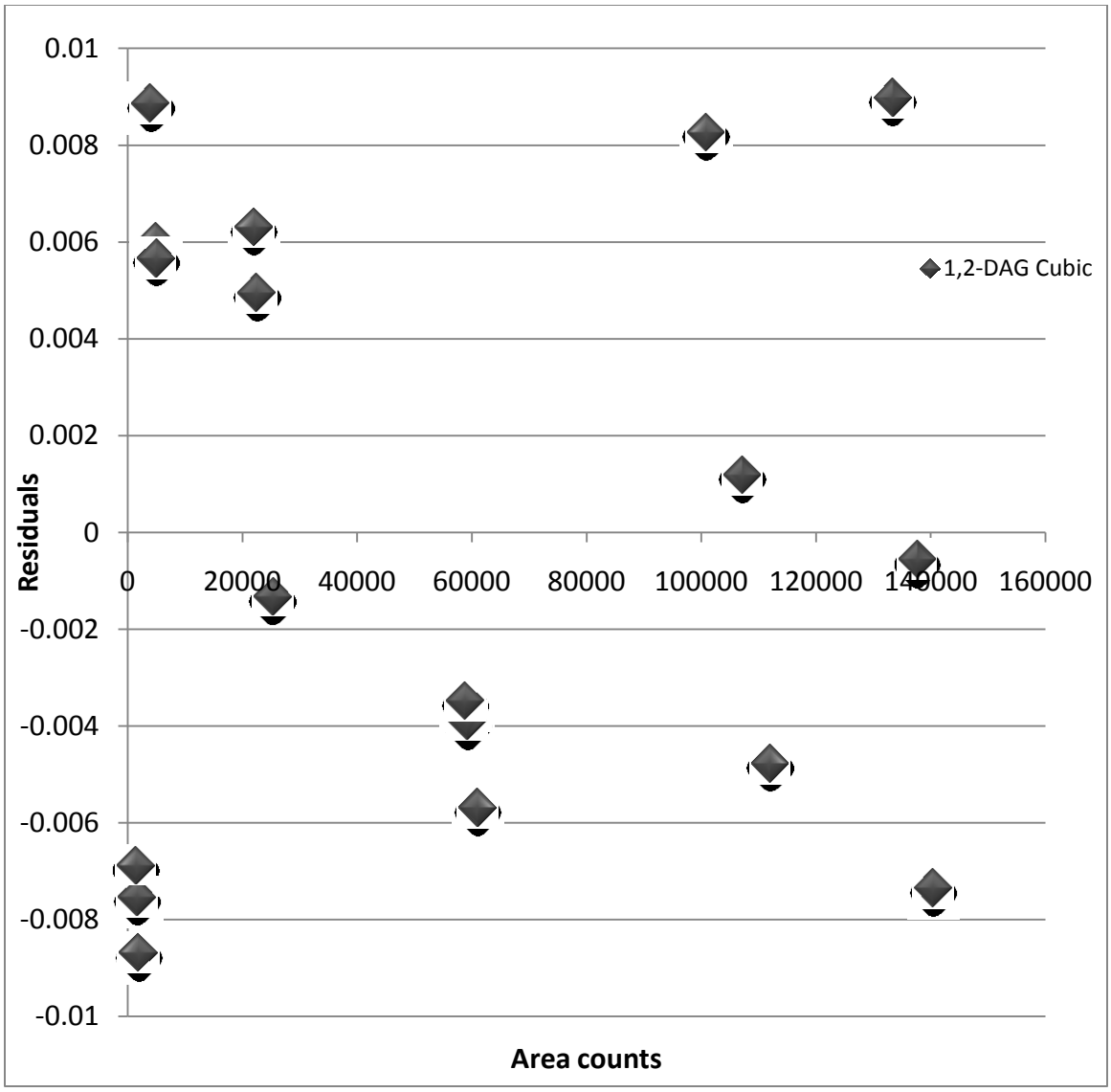


Figure 4.13 Residual values for the1, 2-DAG standard curve.

The highest %TAG (90.2±1.6) was found when using the following testing parameters: 1:3 molar ratio for 6 hours and at 40˚C (Table 4.6). In order to determine the lowest %TAG obtained, ANOVA analysis was performed, comparing the results obtained for the 3 center points (79.0±1.7, 77.3±2.7,

78.7±2.7), along with the experiments performed at: 1:3 molar ratio, 18 hours, 50˚C (76.4±2.1); 1:6 molar ratio, 30 hours, 50˚C (79.4±4.0); 1:6 molar ratio, 18 hours, 60˚C (76.4±2.8) and 1:3 and 1:9 mole ratio, 30 hours, 60˚C (77.3±1.3 and 78.1±1.4) (Table 4.6). No significant difference was found between the 5 experiments ($p>0.05$); thus, the conditions leading to the lowest %TAG cannot be specified; all produce equivalent results.

Table 4.6 Interesterification products identified using HPLC (average % ± standard deviation %, n=3).

| Mole ratio (Cocoa butter: ALA) | Time (hrs) | Temp (°C) | Average %TAG ± St Dev | Average %1,2-DAG ± St Dev | Average %1,3-DAG ± St Dev | %1,2-DAG: %1,3-DAG |
|---|---|---|---|---|---|---|
| 1:3 | 6 | 40 | 90.2±1.6 | 7.2±1.3 | 2.5±0.6 | 1:2.9 |
| 1:9 | 6 | 40 | 90.0±3.6 | 6.6±1.9 | 3.3±1.7 | 1:2.0 |
| 1:6 | 18 | 40 | 87.4±0.4 | 9.8±1.3 | 2.8±1.0 | 1:3.5 |
| 1:3 | 30 | 40 | 81.7±4.8 | 15.2±4.0 | 3.1±0.9 | 1:4.9 |
| 1:9 | 30 | 40 | 82.4±3.1 | 14.6±2.9 | 3.0±0.3 | 1:4.9 |
| 1:6 | 6 | 50 | 83.3±1.4 | 14.7±1.1 | 1.9±0.3 | 1:7.7 |
| 1:3 | 18 | 50 | 76.4±2.1 | 20.2±1.5 | 3.4±0.7 | 1:6.0 |
| 1:6 | 18 | 50 | 79.0±1.7 | 19.3±1.5 | 1.7±0.3 | 1:11.3 |
| 1:6 | 18 | 50 | 77.3±2.7 | 19.2±1.0 | 3.4±1.7 | 1:5.6 |
| 1:6 | 18 | 50 | 78.7±2.7 | 18.9±2.0 | 2.4±0.7 | 1:7.8 |
| 1:9 | 18 | 50 | 81.5±0.7 | 16.9±0.6 | 1.6±1.3 | 1:10.5 |
| 1:6 | 30 | 50 | 79.4±4.0 | 17.9±3.0 | 2.7±1.0 | 1:6.6 |
| 1:3 | 6 | 60 | 80.6±0.6 | 17.2±0.6 | 2.2±0.4 | 1:7.8 |
| 1:9 | 6 | 60 | 81.0±1.0 | 16.5±1.4 | 2.5±0.8 | 1:6.6 |
| 1:6 | 18 | 60 | 76.4±2.8 | 22.4±3.6 | 1.3±0.9 | 1:17.2 |
| 1:3 | 30 | 60 | 77.3±1.3 | 19.6±1.2 | 3.2±0.1 | 1:6.1 |
| 1:9 | 30 | 60 | 78.1±1.4 | 19.5±1.1 | 2.4±0.3 | 1:8.1 |

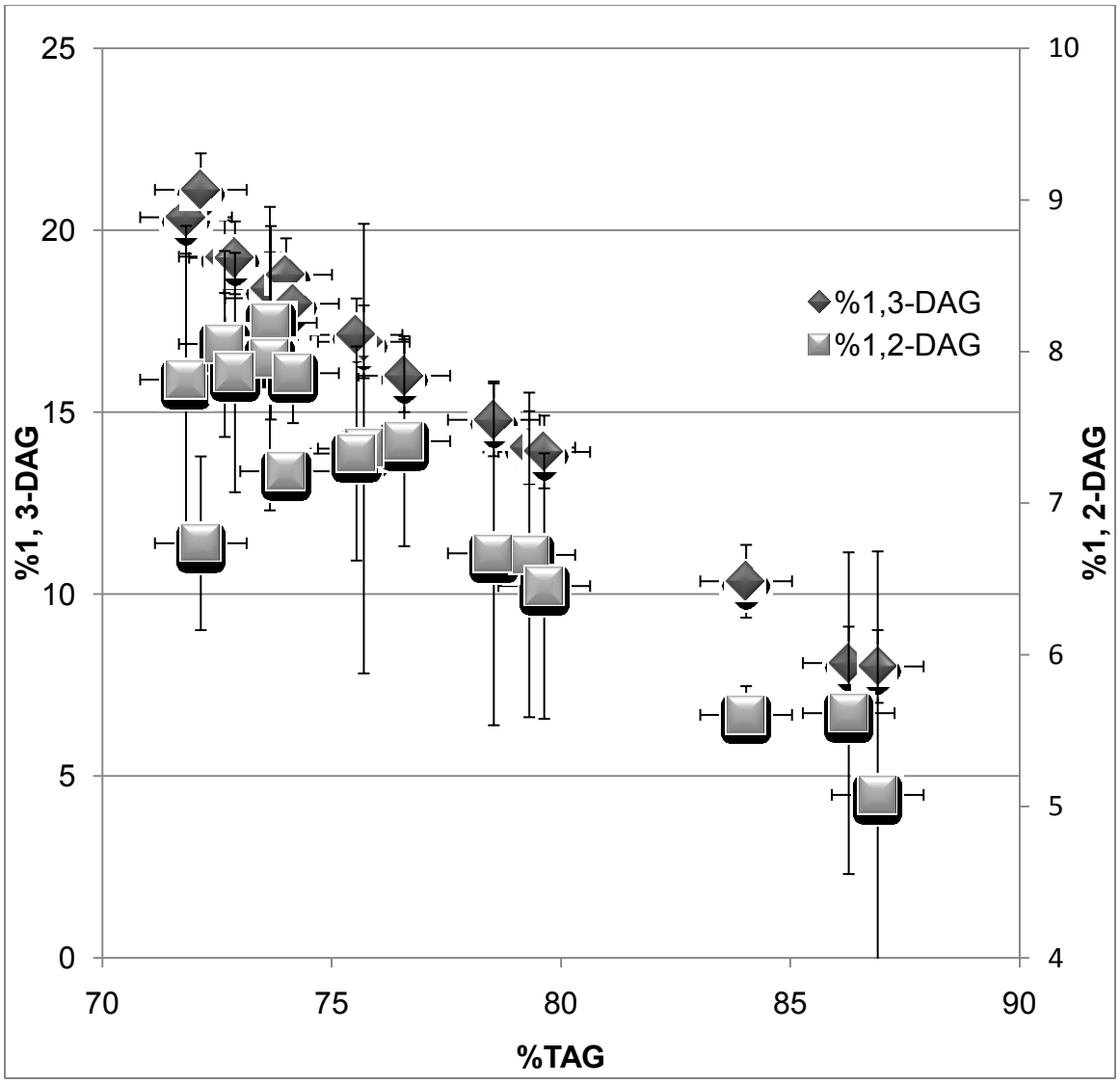


Figure 4.14 Comparison of Average %TAG ± standard deviation vs. %1, 3-DAG and %1, 2-DAG ± standard deviation (n=3) for enzymatic interesterification reaction by-product formation.

After statistical analysis using Design-Expert software to model TAG formation, it was found that a quadratic model best fit the results obtained for %TAG produced. The quadratic model showed an insignificant lack-of-fit

($p>0.05$), a low standard deviation (%TAG), and a high R-squared value, all desired results (Table 4.7). The residual value plot for %TAG produced in a quadratic model showed that the residuals were randomly scattered around zero, indicating that the model being used appropriately describes the data (Figure 4.15).

Table 4.7 Model statistics for %TAG produced in a quadratic model.

| **Interesterification product** | **Standard Deviation (%TAG)** | **R-Squared** | **Lack-Of-Fit test Prob>F** |
|---|---|---|---|
| **%TAG produced** | 1.74 | 0.9299 | 0.1822 |

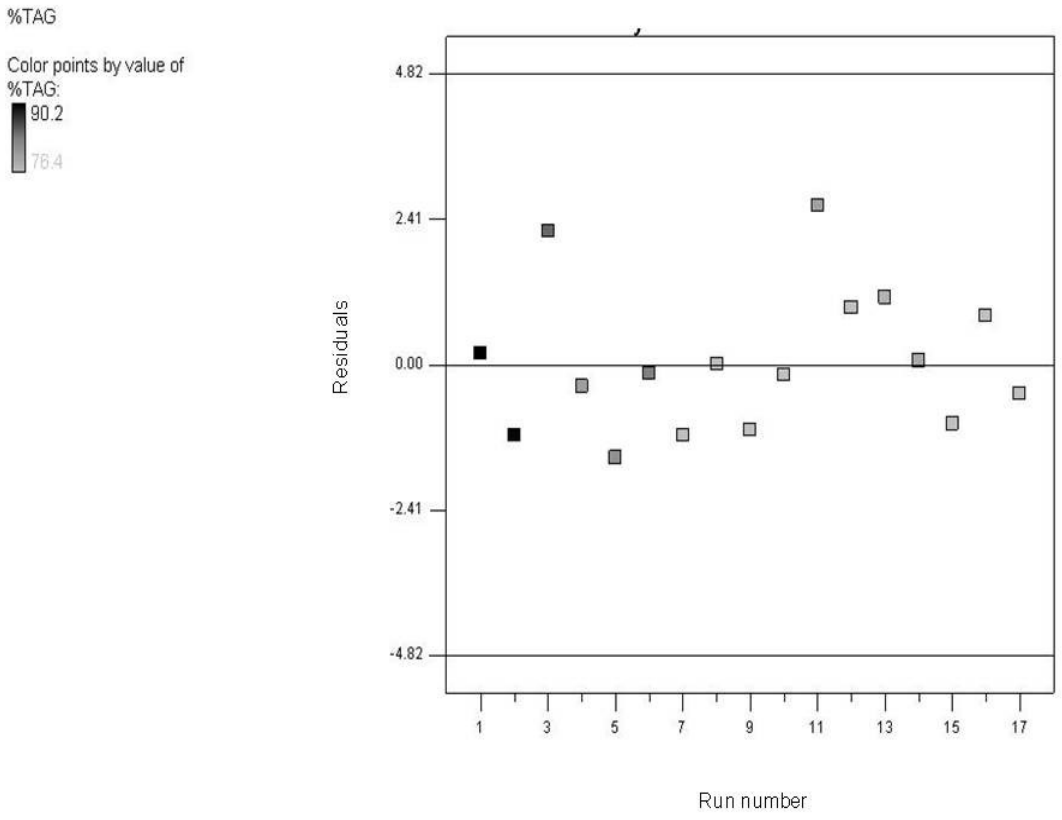


Figure 4.15 Residual values for %TAG in a quadratic model.

ANOVA suggest a significant model for %TAG production in cocoa butter into which ALA was incorporated (F=10.3, P=0.0028). For this model, it was noted that time and temperature were significant variables (F=23, P=0.0021 and F=48, P=0.0002, respectively) along with their squares (Table 4.8). However, molar ratio was not significant (F=1.52 and P=0.26).

Table 4.8 ANOVA for response surface using the quadratic model for %TAG produced.

| Source | Sum of squares | Mean square | F value | Prob>F |
|---|---|---|---|---|
| Model | 283 | 31 | 10 | 0.003 |
| Molar ratio | 5 | 5 | 2 | 0.26 |
| Time | 69 | 69 | 23 | 0.002 |
| Temperature | 147 | 147 | 48 | 0.0002 |
| Molar ratio and Time | 0.2 | 0.2 | 0.07 | 0.80 |
| Molar ratio and Temperature | 0.06 | 0.02 | 0.02 | 0.89 |
| Time and Temperature | 12 | 12 | 4 | 0.084 |
| Molar ratio$^2$ | 0.7 | 0.7 | 0.2 | 0.64 |
| Time$^2$ | 10 | 10 | 3 | 0.12 |
| Temperature$^2$ | 16 | 16 | 5 | 0.056 |

Time and temperature had an obvious effect on %TAG production; increases in both variables resulted in decreased %TAG production (Table 4.8).

As expected from ANOVA analysis, there was no apparent increase in the %TAG produced as the mole ratio was increased from 1:3 to 1:9 (cocoa butter: fatty acid), (Figure 4.16 and 4.17).

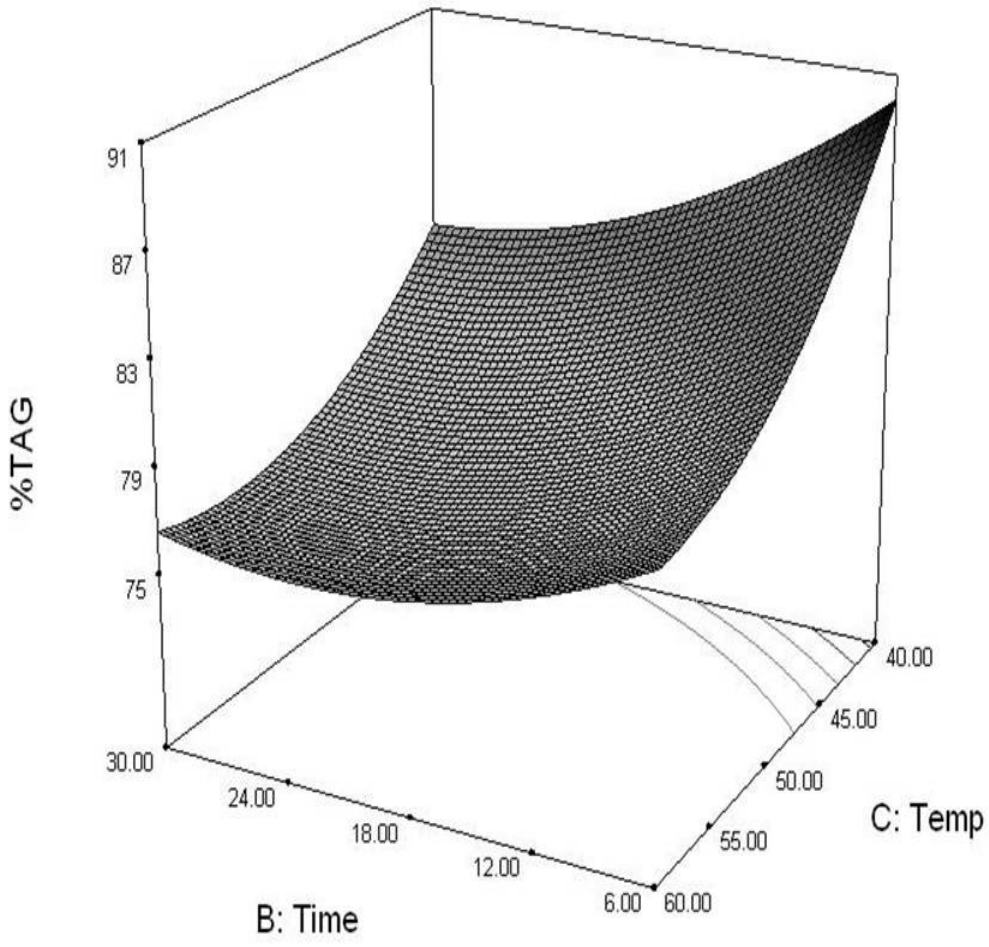


Figure 4.16 Response surface of %TAG showing effects of time and temperature.

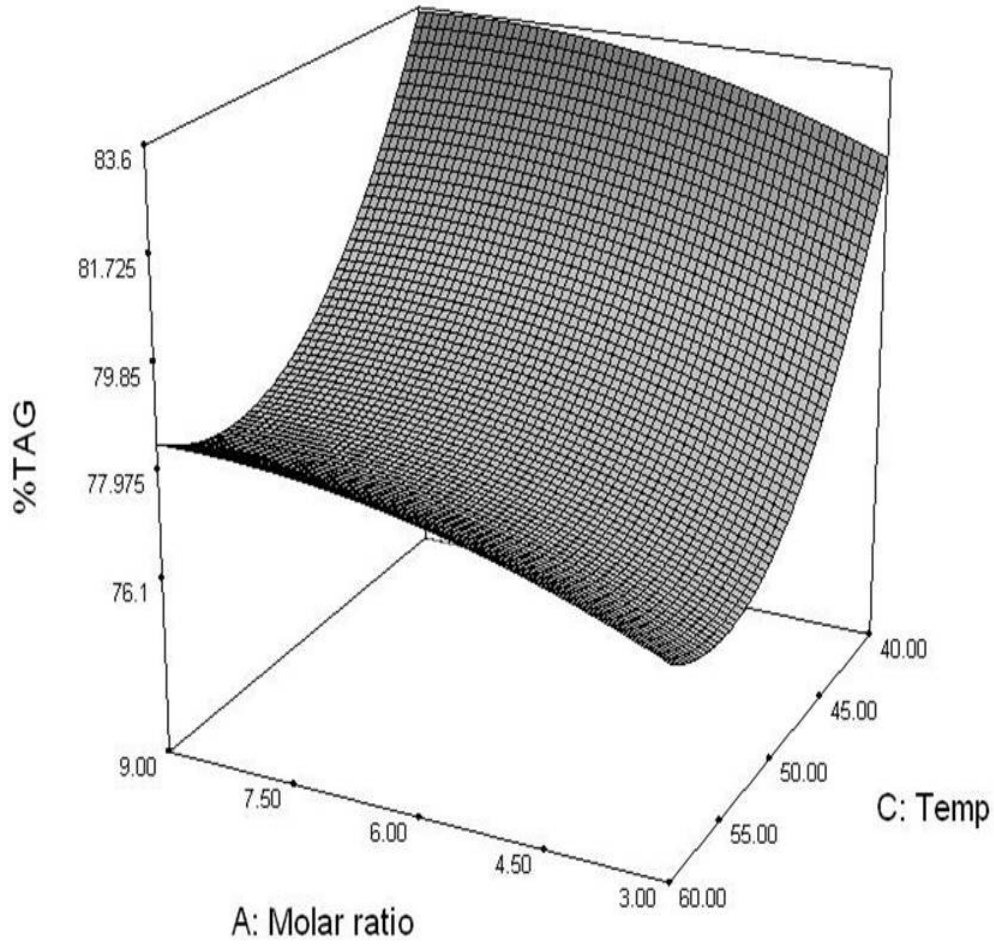


Figure 4.17 Response surface of the %TAG showing effects of molar ratio and temperature.

## 4.4 DISCUSSION

When this research was begun its primary objective was to determine if DA could be used as a model fatty acid for the incorporation of ALA into cocoa butter. DA was chosen as a comparison fatty acid because it was readily

available and more economical than ALA. To test this, RSM was used to develop an experimental plan where 3 variables, molar ratio, time, and temperature, were tested at 3 levels. The parameters of each variable were considered before testing began.

### 4.4.1 Effect of enzyme load

To determine the most appropriate enzyme load, a series of experiments were performed (Figure 4.1) and through ANOVA, it was found that there was no significant difference between the enzyme loads tested, which ranged from 50-250mg. With these findings in mind, it was decided that 100mg would be used as the enzyme load to carry out the trials in the experimental plan; this amount keeps cost manageable and did not waste the expensive enzyme (Table 4.1). Shuang et al. (2009) found that an immobilized enzyme load of 11.9% (w/w) of *R. miehei* derived enzyme was optimal to create a structured lipid using soybean oil and caprylic acid at a 5.7 molar ratio. These researchers also noted that adding more lipase then necessary would only result in a waste of resources and an increase in reaction cost (Shuang et al., 2009). It is difficult to compare past research with that presented here because not all researchers use immobilized enzymes; this would affect the mass of the enzyme load because the support is not included. There are also difficulties in comparing others' work since

different researchers use different enzymes and different reaction procedures. For instance, Osborn and Akoh (2002) used 10g of *Candida antarctica* derived immobilized enzyme in the production of a cocoa butter replacer using beef tallow (100g) and stearic acid (50g) in a 1L batch reactor while Liu et al. (2007) used approximately 100 times less (90mg) of *R. miehei*-derived immobilized enzyme in their super-critical carbon dioxide batch reactor to develop a cocoa butter analog using lard (55mg) and tristearin (30mg). Huang and Akoh (1996) tested both free and immobilized enzymes in small amounts (~20mg) to develop structured lipids from triolein (100mg) and caprylic acid ethyl ester (78.0mg). Perhaps the most important consideration is to ensure that sufficient enzyme is present for the mass of lipid to be modified. Most researchers seem to use an immobilized enzyme load that is no larger than the mass of lipid, as was done in the research being presented. It should be noted that the use of excess enzyme is unwanted due to its cost but there are also limitations as to how low a mass can be measured due to its physical properties.

Another way to determine if the appropriate amount of enzyme was being used is by examining its enzyme activity. The enzyme activity of *R. miehei* derived lipase was 86.8U/g (Fluka Analytical, 2010). As was mentioned previously this would lead to 0.002mg of stearic acid being freed from the TAG

molecule per minute. When a total cocoa butter mass of approximately 100mg was used, which is approximately 0.00012mol and 3 times the amount of ALA, or 0.00036mol, this would be equivalent to 103.6mg of stearic acid. A minimum of 6 hours was used so the enzyme would be capable of freeing 0.88mg of stearic acid in those 6 hours. Thus, the enzyme was present in an amount far in excess of that required to catalyze the release of all fatty acid molecules from the cocoa butter. However, using smaller amounts of enzyme was simply not possible due to difficulties in handling.

Another aspect to consider is the kinetics of the enzymatic reaction. The rate of the reaction depends on the amount of reactants present, cocoa butter and fatty acids. Once the amount of fatty acids being freed from the TAG molecules and those attached reach an equilibrium, the reaction will continue occurring but at a steady rate. Therefore, once at equilibrium the reaction rate will be constant regardless of the amount of enzyme present because the enzyme will continue functioning as long as there are reactants present.

### 4.4.2 Effect of molar ratio

Molar ratio was tested at 1:3, 1:6, and 1:9 (cocoa butter: fatty acid). Research in the lipase-mediated interesterification field has mainly focused on

testing molar ratios of 1:1 to 1:3. This is typically justified by the argument that since TAG molecules contain 3 fatty acid, they can only incorporate 3 new fatty acid into their glycerol backbone. Therefore, a 1:3 molar ratio would be sufficient to reach a maximum incorporation (Hamam and Shahidi, 2005). In this research, it was found that as the mole ratio increased from 1:3 to 1:9, incorporation of DA and ALA increased. For instance, when looking at ALA incorporation when time and temperature were kept constant, at 18 hours and 50˚C, ALA incorporation increased from 52.50 mole% to 66.79 mole% to 72.27 mole% at 1:3, 1:6, and 1:9, respectively (Table 4.3). Obviously, at mole ratios above 1:3 there is higher incorporation. For both DA and ALA incorporation, it was found that mole ratio was a significant variable in both first order and second order parameters. When considering second order parameters, the interaction terms, molar ratio x time and molar ratio x temperature, were both significant for ALA incorporation responses, while molar ratio x time was the only interaction term significant for DA incorporation (Equation 1 and 2). However, when considering these results, cost must also be considered. An increase in mole ratio from 1:3 to 1:9 would require using three times more fatty acid and this would be a considerable cost when ALA is being used.

Increasing the molar ratio to 1:9 results in about 20% more ALA being incorporated and this increase may not be sufficient to justify the cost.

When using RSM to develop an experimental plan, not every variable combination is tested, thus making it difficult to determine what the effect of just one variable is when looking at the results. In situations like this response surface plots are more helpful because they allow the researcher to observe how the individual variables affect the model in relation to another changing variable, while the third variable is kept constant.

### 4.4.3 Effect of time

Time was tested at 6, 18, and 30 hours. This was thought to provide a wide enough range of testing and fell within that of other research performed in this field (Table 2.1). Increases in reaction time led to significant increases in the incorporation of DA and ALA. Other researchers in this field have found similar results. Shuang et al. (2009) found that longer reaction times resulted in higher incorporation of caprylic acid into soybean oil using immobilized enzyme derived from *R. miehei*. The times tested in their experiment ranged from 16.56-31.44 hrs, and their optimum was 20.4 hrs (Shuang et al., 2009). Xu et al (1998a) also found that longer reaction times increased incorporation of

capric acid into rapeseed oil using *R. miehei*–derived immobilized enzyme. Their testing times ranged from 10–30 hrs (Xu et al., 1998a). Similar to mole ratio, it is worth considering if an approximate increase of 17% is worth waiting an additional 24 hours of reaction time. When used in a larger scale production, this additional reaction time might not be cost effective for the manufacturer. Perhaps more worrisome is that longer reaction times could also lead to the removal of the fatty acid that was initially incorporated into the TAG molecule which would eventually lead to a decrease in incorporation.

### 4.4.4 Effect of temperature

Temperature gave varying results when DA and ALA were compared. For the incorporation of DA into cocoa butter, temperature was found to be insignificant ($p>0.05$), contrasting with the results found for ALA incorporation. When choosing temperature parameters, several things had to be considered. The optimum temperature of an interesterification reaction depends on the enzyme being used, its source, whether it is immobilized or not, form of immobilization, and the reaction mixture pH (Huang and Akoh, 1996). Xu (2000) suggested that denaturation of *R. miehei* derived enzyme occurs above 70–80˚C, so temperatures above this are not advised. However, the temperature must also be above the melting range of cocoa butter (32–35˚C)

and the fatty acid to ensure they remain in solution. DA had a particularly low melting point of 32˚C so 40˚C was set as the minimum temperature to test (Sangwal, 2007). The results for DA incorporation were surprising since most other researchers found that temperature had a significant effect, with the effect especially pronounced at higher temperatures. For example, Huang and Akoh (1996), tested optimum temperatures for *R. miehei* derived enzyme at 25-65˚C for incorporation of caprylic acid ethyl ester into triolein and found that the enzyme activity was constant throughout the temperature range, but was slightly higher at higher temperatures (Huang and Akoh, 1996). Temperature can also affect substrate dissolution; an increase in temperature would make the solution less viscous which can make the reaction rate increase (Shuang et al., 2009). Liu et al. (2007) found similar results, where optimal diffusion between substrates and products was beneficial for incorporation of tristearin into lard to produce a cocoa butter analog; this was achieved at 50˚C. The researchers also found that reactions at temperatures above 50˚C were favorable but not statistically significant for the interesterification reaction using *R. miehei* derived enzyme in a supercritical carbon-dioxide system (Liu et al., 2007). This suggests that an optimal temperature can be reached for the enzymatic reaction and that temperatures above this do not provide added benefits. Also too high

a temperature can affect the enzyme stability and reactivity (Shuang et al., 2009). It is also interesting to point out that some groups have found that lipase activity, with regards to different TAG structures, changes with temperature. Specifically, it was found that as temperature was increased, the rate of long-chain fatty acids released is faster than the rate of short chain fatty acids released (Akoh et al., 2002).

### 4.4.5 Fatty acid model comparison

There are several reasons to explain the inadequacy of DA as a model for ALA incorporation into cocoa butter. The most obvious difference between these two fatty acids is chain length. DA is short chain fatty acid, composed of ten carbons. ALA is a long chain fatty acid, composed of eighteen carbons with three double bonds. These chain length characteristics make for fatty acids with very different properties. For example, at room temperature DA is a solid with melting point of 32˚C while ALA is a liquid with a much lower melting point of -11˚C (Sangwal, 2007 and Weast, 1990). In addition DA has a linear structure while ALA has a "bent" structure due to the *cis* double bonds present. Besides reaction conditions and lipase specificity, the incorporation of a fatty acid into a TAG molecule is affected by chain length and double bond number, location, and configuration (Hamam and Shahidi, 2007). Research conducted by Yang et

al. (2001) incorporating linoleic and conjugated linoleic acid into tristearin found that linoleic was more easily incorporated than conjugated linoleic acid in the same acidolysis reaction using immobilized *R. miehei* lipase. The researchers suggest that the lower incorporation of conjugated linoleic acid (28 mole%), compared to linoleic acid (50 mole%) was due to its rigid structure, which is a result of its conjugated double bonds. The rigid structure of conjugated linoleic acid could make it less accessible to the lipase active site leading to a lower incorporation (Yang et al., 2001). The results of the research presented here agree with those of Yang et al. (2001); there was a lower incorporation of DA compared to ALA and this could possibly be attributed to its more rigid structure compared to ALA (Table 4.3). As previously mentioned, DA was used in this study because it was readily available in the testing facility and inexpensive. However, stearic acid and oleic acid are other possible model fatty acids since both of these are 18C fatty acids and are present in cocoa butter.

RSM has become a popular technique to determine optimum conditions in the synthesis of structured lipid, yielding a maximum incorporation. It must be noted that maximum incorporation is not necessarily accomplished by reaching 100%; a maximum is reached when the highest incorporation is obtained and is followed by a continuous drop in incorporation. The highest

incorporation for ALA was 77.4±1.3 mole%. However, this level of incorporation is not necessarily desired because of the physical characteristics of cocoa butter. By adding ALA, the melting point of cocoa butter would be depressed, and its crystalline structure would change. This would lead to a lipid that no longer behaves like cocoa butter, which would be unwanted when used in a chocolate or confectionary product. So, when RSM was used in this research the goal was to observe how the variables related to each other, rather than establishing optimal conditions for maximum incorporation.

Although it was not done in this study, the aqueous phase, or bottom layer of the neutralization step in the enzymatic interesterification reaction, could have been collected. By doing so, the fatty acid incorporation could be confirmed by identifying the fatty acids present and then quantifying them. The amount of either DA or ALA incorporated could be confirmed by determining the amount remaining in the aqueous phase; as well, the fatty acids freed from the cocoa butter TAG molecule could also be identified. This could be suggested for those continuing research in this field.

### 4.4.6 By-products of interesterification

Another factor that must be considered in the interesterification reaction are the products obtained. Ideally TAG would be the only product of the reaction but considering the HPLC data obtained, 1, 2-DAG and 1, 3-DAG were also present. Therefore, the reaction must be performed in a way that maximum TAG is obtained with minimal DAG by-products, to reduce oxidative instability while also achieving a desired level of incorporation. The response surfaces for %TAG (Figure 4.9 and 4.10) show that the maximum amount of TAG is produced at the lowest time, temperature, and molar ratio. However, there was substantial 1, 2-DAG and 1, 3-DAG in the final product. DAG molecules are an unavoidable intermediate during structured lipid TAG formation. The presence of 1, 3-DAG indicates that acyl migration is occurring because *R. miehei* is a sn-1, 3 specific lipase so only 1, 2-DAG would be expected. Acyl migration can take place between the fatty acid in the *sn*-2 position and that in the *sn*-3 or *sn*-1, or the opposite can occur. Akoh et al. (2002) suggested that 1, 2-DAG is not thermodynamically stable and acyl migration is constantly occurring so that the more stable 1, 3-DAG can persist. They also found that 1, 2-DAG is converted into 1, 3-DAG at a rate of 1:1.5, until equilibrium is reached. Migration rates depend on fatty acid chain lengths, as well as the

saturation of the fatty acid, with unsaturated fatty acid migrating more quickly (Akoh et al., 2002). The average 1, 2-DAG : 1, 3-DAG ratio for the research presented here was 1:7.0, so there was approximately four times the amount of 1, 3-DAG present in all the ALA incorporation cocoa butter samples, which is slightly more than that predicted by Akoh et al. (2002).

Many factors have been found to influence acyl migration. Strong acids have been shown to cause acyl migration in DAG and there was always excess fatty acid present in the reaction media; however, fatty acids are weak acids and their effects on acyl migration are most likely small. When solvents are compared, nonpolar solvents can cause higher rates of acyl migration versus polar solvents or nonpolar solvents to which water has been added. Thus, using hexane, a non-polar solvent, may lead to greater amounts of acyl migration. The enzyme support of some immobilized enzymes can also influence acyl migration. This is why absolute specificity of an enzyme cannot be claimed. Time and temperature also influence acyl migration, and are related factors. Time relates to temperature in that at higher temperatures less time is needed to reach equilibrium (Xu et al., 1998a). However, high temperatures also induce more acyl migration. This effect is clear in the results obtained in this experiment, where higher temperatures, 50-60°C, produced relatively more 1,

3-DAG than experiments performed at 40°C. Xu et al (1998b) have also found a similar effect. Finally, substrate mole ratio, despite having a significant effect on incorporation, has been found to have only a minor effect on acyl migration (Xu et al., 1998b). Similar results were found in the current work where mole ratio had an effect on incorporation but was insignificant in TAG production ($p>0.05$).

## 4.5 CONCLUSION

In this study, the effects of mole ratio, time and temperature on ALA and DA incorporation into cocoa butter were studied. RSM was used to observe the relationship between the variables being tested, instead of its traditional use to determine conditions for maximum incorporation. The maximum incorporation for the two fatty acids was different with DA at 68.2±1.1 mole% and ALA at 77.3±1.3 mole%; both were obtained using the maximum levels of the variable parameters being tested, 1:9 mole ratio, 30 hours, and 60°C. The lowest incorporation was obtained when testing parameters were 1:3 mole ratio, 6 hours, and 40°C; results were again different for the two fatty acids at 45.8±1.4 mole% and 41.6±0.6 mole % for DA and ALA, respectively. These variable parameters are also the lowest of those tested. These results indicate that DA was not an adequate model fatty acid for the incorporation of ALA into cocoa

butter. The different levels of incorporation point to this, but it was also found that temperature had no effect on DA incorporation while it was significant for ALA incorporation along with mole ratio and time. The products of enzymatic interesterification were TAG, 1, 3-DAG and, 1, 2-DAG. The maximum %TAG produced was 90.2±1.6; this was obtained using a 1:3 mole ratio, 6 hours, and 40°C. A maximum amount of TAG with minimum DAG by-products is wanted. Acyl migration occurred during enzymatic interesterification, resulting in the presence of 1, 3-DAG in a ratio of 1 : 7.0, 1, 2-DAG : 1, 3-DAG. The reader should remember that a maximum fatty acid incorporation is not desired because this would result in a lipid that no longer has crystalline properties similar to cocoa butter, which would be undesirable in the chocolate and confectionary industry. These properties will be discussed in the following chapter.

# CHAPTER 5 CRYSTALLINE BEHAVIOR OF UNMODIFIED AND MODIFIED COCOA BUTTER

## 5.1 INTRODUCTION

Cocoa butter exhibits polymorphism which is the existence of several crystalline forms in the same material. Cocoa butter will crystallize into different forms depending on its processing and storage conditions (Afoakwa et al., 2009). Cocoa butter exhibits six crystalline forms numbered I to VI as described by Willie and Lutton in 1966 and the ideal form for chocolate preparation is form V. These crystals melt between 32-34˚C, and provide the product with good snap, contractibility, gloss, and melt-in-mouth feel (Beckett, 2008). Tempering is used to induce the formation of stable polymorphs of crystals using controlled pre-crystallization. Proper tempering is a combination of shear, temperature, and time. In chocolate manufacturing tempering is extremely important because it influences the quality of the final product (Afoakwa et al., 2009).

The more stable crystalline forms can be obtained through phase transitions. Phase transition involves an irreversible change from a less stable form to a more stable form and depends on time and temperature (Afoakwa et al., 2009). Differential scanning calorimetry (DSC) has been used to study the

crystalline structure of cocoa butter and its phase transitions. DSC allows for the crystalline forms and transitions to be identified using their melting points (Le Révérend et al., 2009). DSC is also commonly used because the technique is simple and can be used in industrial laboratories for routine quality control, and since it provides information about phase transitions it can be used in place of more sophisticated analysis methods (Fessas et al., 2005). Some researchers have gone so far as to say that other methods such as solid fat content using pulsed nuclear magnetic resonance do not give additional information when compared to DSC techniques (Le Révérend et al., 2009).

The aim of this study was to compare unmodified cocoa butter to modified cocoa butter with ALA incorporated using a lipase after static tempering. Samples were prepared with two incorporation amounts to mimic dark chocolate and milk chocolate products. Extent of ALA incorporation was confirmed with GC-FID and TAG content was also monitored. DSC analysis was used to examine the crystalline structure of the modified and unmodified samples.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Modified cocoa butter sample preparation

In order to manufacture cocoa butter samples that contained the required amount of ALA to meet omega-3 label claims, first the amount of cocoa butter in a milk chocolate and dark chocolate had to be determined. The amounts of ALA needed to meet labeling claims were then determined. This was accomplished using the calculation presented in the Appendix; cocoa butter in milk chocolate must be 3.75 wt% of the total product and 6.30 wt% for a dark chocolate. The amount of ALA needed in the product to meet the 0.3g per serving specifications was then found to be 21 wt% and 11.9 wt% for milk and dark chocolate, respectively. Once the amount of ALA incorporation needed to meet labeling claims was determined Equation 2 was used to determine the reaction parameters (mole ratio, time, temperature) needed to accomplish the desired ALA incorporation. Samples of modified cocoa butter were then prepared according to the procedure described in the previous chapter. Samples were prepared as either "pure" or "blend". "Pure" samples are those in which ALA was incorporated to meet the omega-3 label claims directly, using the parameters obtained from Equation 2. "Blend" samples were created by preparing ALA incorporated cocoa butter of a higher concentration than needed

(about 40 mole%), after which the samples were diluted with unmodified cocoa butter to meet omega-3 label claims. Sample FAMEs were then prepared according to the procedure described in the previous chapter and FAMEs were analyzed by GC-FID to confirm that the desired ALA incorporation was achieved.

### 5.2.2 High Performance Liquid Chromatography (HPLC) – Normal phase

HPLC analysis was conducted as described in the previous chapter.

### 5.2.3 Differential Scanning Calorimetry (DSC)

DSC on a TA Instruments DSC-Q100 series with a refrigerated cooling system (New Castle, Delaware, USA) was used to determine the melting profile of cocoa butter samples. Samples (7-10mg) were loaded into aluminum pans and hermetically sealed with an aluminum lid using a sample press. The sealed pans were then left to sit at 22.9±0.5°C for 4 weeks, with an empty pan serving as reference. Thermograms were generated starting at 20°C, then decreasing to -30°C, and finally increasing to 80°C, all at a rate of 5°C/min. The DSC monitored changes in sample heat flow and expressed these results as heat flow (W/g) vs. temperature (°C).

### 5.2.4 Physical property determination

Samples were placed in 4mL glass vials under $N_2$ for 4 weeks at 22.9±0.5˚C; photographs were then taken to observe the modified cocoa butter physical properties.

## 5.3 RESULTS

### 5.3.1 Fatty acid composition of "pure" and "blend" modified cocoa butter samples

According to the calculations performed in the Appendix to determine the amount of cocoa butter per serving size of milk chocolate and dark chocolate, a 38g serving of milk chocolate must contain at least 1.45g cocoa butter and a 40g serving of dark chocolate at least 2.52g. In order to meet omega-3 labeling claims 21 wt% and 11.9 wt% ALA incorporation was needed, per serving for milk and dark chocolate, respectively. GC-FID analysis shows that for samples created aiming for 21wt% incorporation (milk chocolate), "pure" and "blend" samples were 19.3±1.1 wt% and 21.6±0.2 wt%, respectively (Table 5.1). For samples with an 11.9wt% incorporation goal, 14.3±0.4 wt% for "pure" and 13.9±0.7 wt% for "blend" were obtained (Table 5.1).

Table 5.1 Incorporation of ALA into cocoa butter as either "Pure" or "Blends" for milk chocolate and dark chocolate products, presented as average wt% ± standard deviation, n=3.

| Sample | Molar ratio (Cocoa butter : ALA) | Time (hrs) | Temperature (˚C) | Average (wt %) | St Dev (wt%) |
|---|---|---|---|---|---|
| 21% "Pure" | 1 : 1.75 | 1 | 30 | 19.3 | ±1.1 |
| 21% "Blend" | 1 : 3 | 6 | 40 | 21.6 | ±0.2 |
| 11.9% "Pure" | 1 : 1.25 | 1 | 30 | 14.3 | ±0.4 |
| 11.9% "Blend" | 1 : 3 | 6 | 40 | 13.9 | ±0.7 |

### 5.3.2 Determination of interesterification by-products in "pure" and "blend" modified cocoa butter samples

HPLC analysis of "pure" and "blend" samples was performed. The highest %TAG was achieved for the 11.9% "blend" sample, 97.3±1.0 %TAG (Table 5.2). The lowest %1, 3-DAG and 1, 2-DAG were also found in the same sample. Overall the %TAG produced in all four products were quite similar and the %1, 2-DAG : %1, 3-DAG was 1 : 1.7.

Table 5.2 Interesterification product identification of "pure" and "blend" cocoa butter samples using HPLC (Average % ± standard deviation, n=3).

| Sample | Molar ratio (Cocoa butter :ALA) | Time (hrs) | Temperature (˚C) | %TAG | %1,3-DAG | %1,2-DAG | %1,2-DAG : %1,3-DAG |
|---|---|---|---|---|---|---|---|
| 21% "Pure" | 1 : 1.75 | 1 | 30 | 93.7 ±0.7 | 4.1 ±0.4 | 2.2 ±0.4 | 1 : 1.9 |
| 21% "Blend" | 1 : 3 | 6 | 40 | 95.3 ±1.6 | 3.3 ±1.6 | 1.4 ±0.2 | 1 : 2.3 |
| 11.9% "Pure" | 1 : 1.25 | 1 | 30 | 95.5 ±0.8 | 2.7 ±0.4 | 1.8 ±0.4 | 1 : 1.5 |
| 11.9% "Blend" | 1 : 3 | 6 | 40 | 97.3 ±1.0 | 1.7 ±0.5 | 1.0 ±0.6 | 1 : 1.7 |

### 5.3.3 Analysis of thermal properties

Figure 5.1 shows the melting curve of unmodified cocoa butter. The thermogram shows one distinct peak at about 32°C for the two samples, with a smaller peak at 22-24°C, making these samples quite similar. The thermogram also shows several smaller peaks ranging from 0-5˚C which demonstrate crystallization at lower temperatures followed by melting of those crystals as

temperature increased. The melting of the crystals is seen where the thermogram heat flow decreases (0.00 to -2.0).

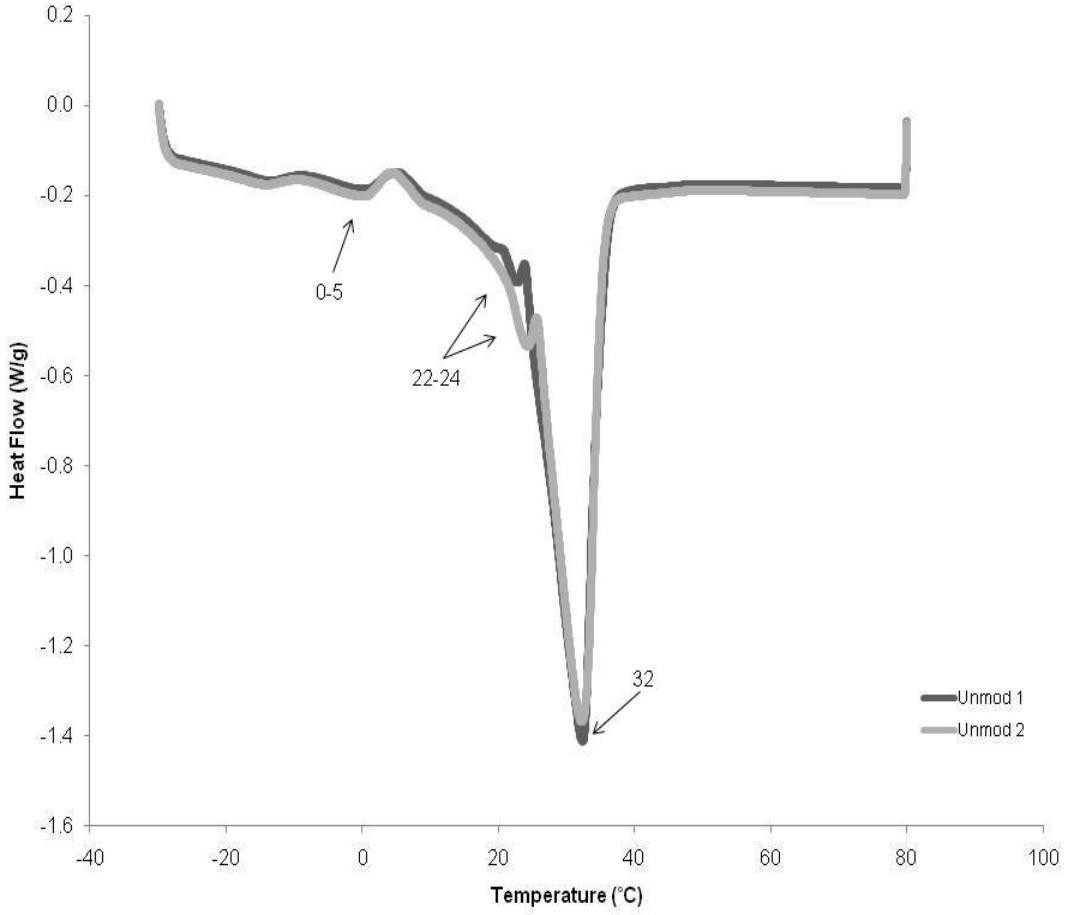


Figure 5.1 Characteristic melting profile obtained by DSC of two unmodified cocoa butter samples.

For 11.9 wt% ALA incorporation samples, the "pure" sample (Figure 5.2) had a sharp melting peak at 31.2°C with a smaller peak at 23.2°C. There were

also smaller crystallization and melting peaks before the two predominant peaks at 7.1˚C and 15.7˚C. For the “blend” sample (Figure 5.2), there was a sharp melting peak at 32.6ºC with a smaller peak at 22.3ºC. As with the 11.9 wt% “pure” sample, there were also smaller crystallization and melting peaks before the two predominant peaks at 8.9˚C and 18.5˚C.

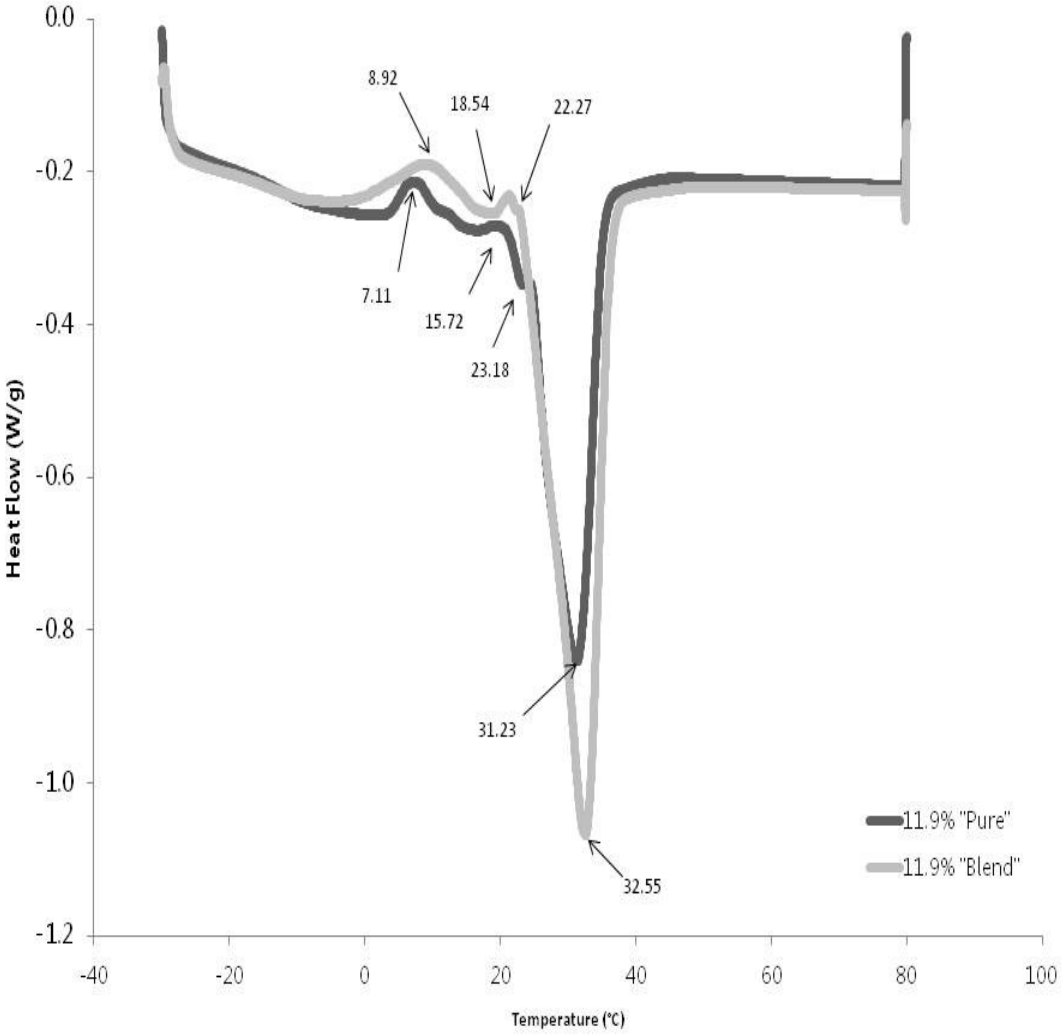


Figure 5.2 Thermogram demonstrating the melting profile obtained by DSC of 11.9 wt% "pure" and "blend" modified cocoa butter samples.

The two samples with 21 wt% ALA incorporation, prepared as "pure" and "blend" were not as similar as those shown above. The 21 wt% "pure" sample had a large melting peak at 33.29°C with a smaller peak at 23.51°C (Figure 5.3). As with previously described samples it too had several smaller peaks at 9.1˚C

and 20.0˚C where crystallization took place followed by decreases in heat flow where melting of these crystals occurred. For the 21 wt% “blend” the larger peak was composed of two crystalline types one at 29.9ºC and another at 32.5ºC accompanied by a smaller melting peak at 23.9ºC (Figure 5.3). This thermogram also demonstrated several smaller melting and crystallization peaks, as did all others, at 7.9˚C and 20.2˚C.

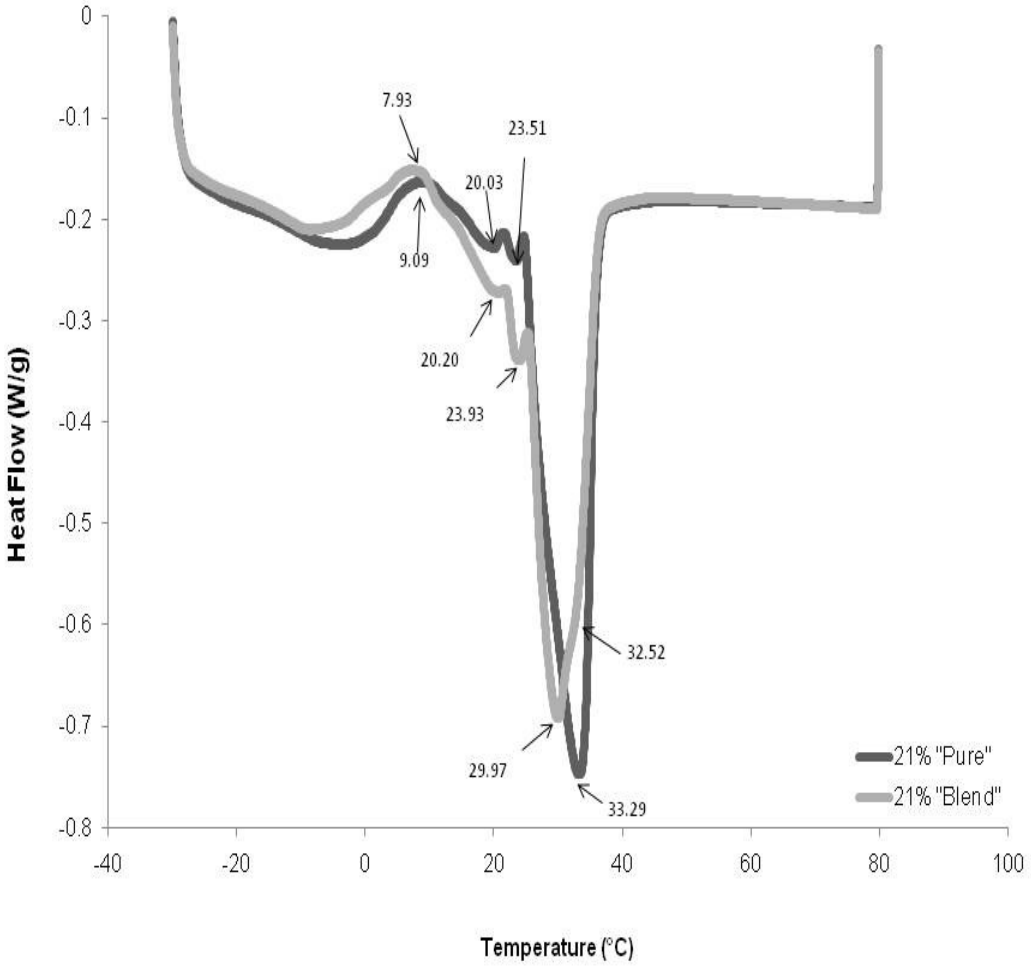


Figure 5.3 Thermogram demonstrating the melting profile obtained by DSC of a 21 wt% “pure” and “blend” modified cocoa butter samples.

### 5.3.4 Analysis of physical properties

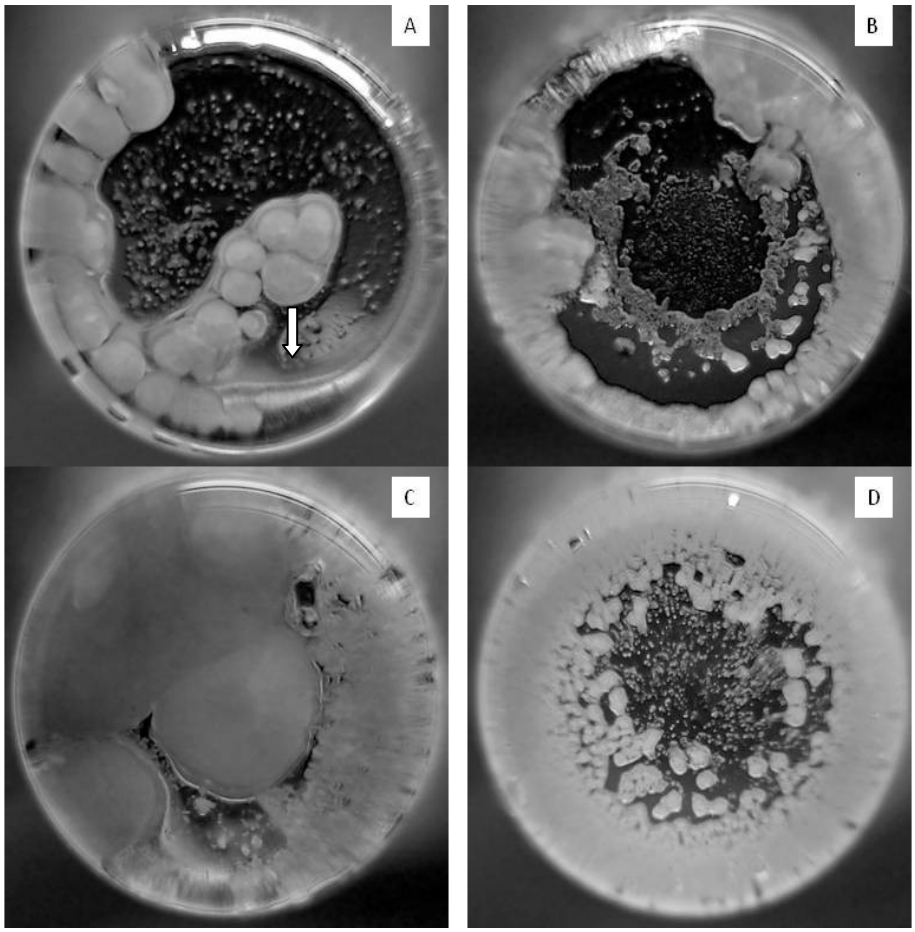


Figure 5.4 Photographs depicting physical properties of cocoa butter crystallization a) 21 wt% "Blend" (arrow points to liquid) b) 21 wt% "Pure" c) 11.9 wt% "Blend" d) 11.9 wt% "Pure". (Photographs by Damian Lidgard)

Samples of the same modified cocoa butter in DSC pans were placed in glass vials under $N_2$, under the same storage conditions and their photographs

(Figure 5.4a-d) were taken after 4 weeks. Visual analysis of these showed that there was still some liquid material (Figure 5.4a arrow) at the end of the allotted time period; this is especially noticeable in Figure 5.4a. It was also noticed that larger crystal aggregates seemed to form in the "blend" (Figure 5.4a and c) samples when compared to the "pure" samples (Figure 5.4b and d).

## 5.4 DISCUSSION

### 5.4.1 Fatty acid composition of "pure" and "blend" modified cocoa butter samples

RSM predicted model accuracy was evaluated by performing independent experiments at the conditions that were calculated to yield an 11.9 wt% and 21 wt% ALA incorporation into cocoa butter, using Equation 2. Liu et al (2007) conducted research in which urea complexation of tuna oil was performed to concentrate DHA and EPA in oil. Their variables, urea : fatty acid mole ratio, temperature, and time were optimized using RSM; the model was then verified and their predicted values for lipid recovery and DHA and EPA content were 23.74% and 89.38%, respectively. Their actual values were 25.10% lipid recovery and 85.02% DHA and EPA content. These results show that the predicted values were reasonably close to the actual values obtained, which resemble the results obtained here (Liu et al., 2007). When using a model equation, as was done

here, the first step can be considered an extrapolation because the equation gives a theoretical incorporation yield for variable levels not included in the original design; actually conducting the experiment and gathering real results showed that the equation model could be applied. Using the equation to determine the theoretical incorporation of an experiment suggested that temperature played the largest role in obtaining the desired incorporation, followed by time and finally mole ratio. When using short reaction times careful consideration must be given to the point at which to begin timing because the sample does not immediately reach reaction temperature when placed in the incubator. To deal with this complication a simple experiment was carried out to determine time required for a sample mixture to reach 30°C and it was found that 15 minutes was required. The incubation times were therefore increased by that amount of time to compensate for time spent reaching temperature.

When preparing the 21 wt% "pure" sample an initial run was performed where 21.3±0.9 wt% ALA incorporation was obtained. This experiment was then repeated in order to obtain a higher yield of modified cocoa butter and the incorporation obtained was 19.3±1.1 wt% (Table 5.1). These results demonstrate that the amount of incorporation varies slightly when comparing two runs of the same experiment, but this variation is likely to be insignificant.

### 5.4.2 Interesterification by-products in "pure" and "blend" modified cocoa butter samples

As was stated in the previous chapter, maximum %TAG is desired with minimal DAG by-products. When the results for the "pure" and "blend" samples were compared, the 11.9 wt% "blend" resulted in the highest %TAG production with the least %1, 2-DAG and %1, 3-DAG (Table 5.2). The 11.9 wt% "blend" experiments also resulted in the highest %TAG of all the experiments performed during the research study. In general, all the experiments ran for "pure" and "blend" samples had higher amounts of %TAG than those described in the previous chapter. This can likely be attributed to reaction conditions; for "pure" samples lower molar ratios, reaction times, and temperatures were used than for any experiment in the previous chapter. "Blend" samples, prepared by diluting high ALA incorporation samples with unmodified cocoa butter, included modified cocoa butter that had been prepared through the use of high molar ratios, reaction times, and temperatures. Despite the more extreme reaction conditions, these "blend" samples had higher %TAG than the "pure" samples. This is likely because of the higher amount of unmodified cocoa butter in the samples when compared to the "pure" samples. According to Chaiseri and Dimick (1995) unmodified cocoa butter contains 96.8-98.0% TAG and other

simple lipids account for 2.9-3.2%, of which 40.4-57.4% is DAG, suggesting that all products would contain at least approximately 1.6% DAG from the unmodified cocoa butter (Chaiseri and Dimick, 1995). "Blend" samples contain a large proportion of unmodified cocoa butter so it would be expected then that the DAG contribution from that would dominate. Milder reaction conditions and the dilution effect also led to lower levels of acyl migration, for the "pure" and "blend" trials. The overall ratio of %1, 2-DAG : %1, 3-DAG was 1 : 1.7, which is similar to the results found by Akoh et al. (2002) of 1 : 1.5, and less than what was found in the previous chapter, 1 : 7.0 (Akoh et al., 2002). It was surprising to find that "pure" samples resulted in higher DAG by-product ratios because these reactions use lower temperatures, shorter times, and lower mole ratios which should result in less acyl migration. These results suggest that acyl migration was still occurring, although DAG formation in general seemed to be at a much lower rate than that found in the experiments presented in the previous chapter.

### 5.4.3 Thermal properties

DSC analysis was performed to determine if the modified cocoa butter samples were similar to unmodified cocoa butter after holding at the same temperature (22.9±0.5˚C) for 4 weeks to mimic static tempering. Static

tempering is not regularly used in chocolate and confectionary manufacturing. The results showed that the DSC thermograms of modified cocoa butter were similar to those of unmodified cocoa butter but not exactly the same. The polymorphic forms were determined from the peak melting temperatures and identified using published melting ranges.

Unmodified cocoa butter samples demonstrated a large peak at 32.3±0.1˚C which corresponds to crystalline form V (32-34˚C). This crystalline form is one of the most stable forms of cocoa butter and is the preferred crystalline form in chocolate production. This is the main form present in properly tempered chocolate and is what gives the product its snap, glossy appearance, and resistance to bloom (Beckett, 2008). The other peak found in unmodified cocoa butter samples was at 23.5±1.0˚C which would correspond to form II (22-24˚C), a more unstable crystalline form (Beckett, 2008). Form II is needed to obtain form V via the II to IV to V polymorphic transition because cocoa butter does not directly crystallize into form V from the melt (Pérez-Martínez et al., 2005). After four weeks at 22.9±0.5˚C only form V was expected to be present which would be similar to results obtained by Marangoni and McGauley (2003). Those investigators stored unmodified cocoa butter samples at 22˚C for 28 days and obtained a peak melting temperature of

32.7˚C indicative of form V crystallization (Marangoni and McGauley, 2003). This suggests that the samples may have needed more time to crystallize into form V and that slight variations in temperature might play a role. It is also important to consider that the results being presented were obtained after static tempering and it is known that shearing is important for a timely formation of form V crystals.

Another variable to consider is that the melting of cocoa butter does not solely depend on the polymorphic forms present in a sample but also on the composition of the lipid material. Cocoa butter is composed of different TAG molecules and each of these TAG molecules will crystallize at a different temperature and at a different rate. TAG molecules composed of mostly saturated fatty acid (SSS) will melt at higher temperatures, compared to those with more unsaturated fatty acids (SOO) which will be mostly liquid at room temperature. Combined, the presence of various TAG molecules in cocoa butter gives this lipid a melting range instead of a set melting point (Beckett, 2008). This would also explain some of the differences found between modified and unmodified cocoa butter samples. ALA, being an unsaturated fatty acid, is more likely to cause higher amounts of liquid to be present in modified cocoa butter samples.

Thermograms for 11.9 wt% ALA incorporation were similar. The "pure" sample, like the unmodified cocoa butter, had a split peak with a peak at 31.2˚C and a second at 23.2˚C, corresponding to form II. For this sample, the peak at 31.2˚C is likely a combination of form IV and V because its melting temperature falls between the temperatures of form IV (26-28˚C) and form V (32-34˚C) (Beckett, 2008 and Marangoni and McGauley, 2003). Marangoni and McGauley (2003) also found that these split peaks could be due to both crystalline forms being present or as a result of artifactual heat-induced transformation of form II to IV while in the DSC (Marangoni and McGauley, 2003). Thermograms for the 11.9 wt% ALA incorporation "blend" sample also had two main peaks with one at 32.6˚C and another at 22.3˚C, corresponding to form V and II, respectively. There was also a smaller peak 18.5˚C, which could be attributed to the presence of a combination of form I (16-18˚C) and II crystals. Chaiseri and Dimick (1995) classified peaks as low-melting (25.4-29.2˚C) and high-melting (35.6-45.5˚C), with those in the low-melting range composed mostly of form IV. This group also stated that broad peaks in the low-melting range are likely composed of a combination of form IV and V crystals (Chaiseri and Dimick, 1995). This suggest that the crystalline forms present in the "pure" and "blend" samples of 11.9 wt% ALA incorporation were mainly composed of a combination

of form IV and V. Since form V is the desired crystalline form in properly tempered chocolate, these findings stress the importance of properly tempering chocolate products to obtain form V crystals (Beckett, 2008).

Samples for 21 wt% ALA incorporation also produced thermograms similar to those obtained for unmodified cocoa butter. The thermogram for the "pure" samples had a peak at 33.3˚C and another at 23.5˚C. These peaks suggest that the sample contained form V and II (Beckett, 2008). The sample would be considered a combination of low and high melting range polymorphs if Chaiseri and Dimick (1995) classifications are used. The researchers state that in static tempering, as was performed in this study, seed crystal development is slow and crystallization of high melting components is favored, specifically the crystallization of saturated TAG molecules. However, with tempered samples that use a combination of temperature, time, and shear, seed crystal formation occurs rapidly with more crystallization occurring early and resulting in crystals that melt at lower temperatures (Chaiseri and Dimick, 1995). The "blend" sample was different because it contained 3 peaks at 32.5˚C, 29.9˚C, and 23.9˚C. Marangoni and McGauley (2003) reported thermograms with peaks at 27.3˚C and 31.8˚C and stated that the sample was experiencing polymorphic transformation from form IV to V at different rates,

which is what appears to be occurring in the "blend" sample (Marangoni and McGauley 2003). This could be due to the addition of ALA and would explain the results obtained for both "blend" samples since these are a mixture of modified and unmodified cocoa butters. The peak at 23.9˚C also shows the presence of form II crystals which would also lead to form V crystals being formed (Pérez-Martínez et al., 2005).

The smaller peaks seen in all the thermograms are due to unstable crystals forming due to rapid cooling of the samples when the temperature in the DSC changed from 20˚C to -30˚C. At 20˚C, some small amount of liquid cocoa butter would still exist which would crystallize with cooling. During reheating, these unstable crystals melt at low temperatures with a crystallization peak immediately after that which suggests a recrystallization of the melt into a more stable crystalline form or a transition from an unstable from to a more stable form. With increasing temperature, these crystals in turn, melt (Le Révérend et al., 2009). Photographs taken of the modified cocoa butter samples showed that there was some liquid material remaining; these results, together with DSC findings, suggest that more than one crystalline form is present. In this study, the melting properties of cocoa butter were expected to change substantially when ALA was incorporated compared to unmodified cocoa butter.

These changes were expected to be greater in the samples with higher incorporation because more ALA was present in the TAG molecule, resulting in less POP, POS, and SOS TAG molecules remaining. Changes in the melting profile, seen in the thermograms, were more evident at lower temperatures; this was to be expected because ALA (-11.3˚C) has a much lower melting point than P (63˚C), O (16.3˚C), or S (71-72˚C), and this would explain the smaller peaks at lower temperatures described above (Weast, 1990). At higher melting temperatures there are fewer differences between unmodified and modified cocoa butter and this is probably due to larger amounts of unmodified TAG structures remaining, which is more noticeable in the "blend" samples.

When considering which sample, "pure" vs. "blend", resulted in the best final product, ease of manufacture must also be considered. It must be determined if it is easier to produce a "pure" sample which has a lower incorporation of ALA in many TAG molecules or a "blend" sample that contains fewer modified TAG molecules that incorporate several ALA molecules in each modified TAG. If a manufacturer is making both milk and dark chocolate products, "blends" might be easier to make because one modified cocoa butter sample with high incorporation can be made, divided, and diluted to result in incorporations needed for both milk and dark chocolate product. Making

"blends" can also result in a more consistent product because a batch can be made, checked for incorporation, and adjusted accordingly. However, reaction time is always important in industry so making a "pure" incorporation product might be more beneficial because the reaction only takes an hour. Such a short reaction time may offset any losses due to inconsistency in the product. Lastly, it would be critical to determine the behavior of this enzyme-catalyzed system with scale-up from the test-tube level. For large scale production, a continuous flow bioreactor would likely be necessary and at least one study found that reproducibility was an issue with those designs (Hamam and Budge, 2010).

Considering that various crystalline forms and liquid material were found during sample analysis, it can be concluded that these modified cocoa butters might not be of practical use in making a chocolate bar. However, chocolate can also be used in other areas where form V crystallization is not necessary. Chocolate coatings on ice cream are popular and do not use normally tempered chocolate. If tempered chocolate were used the resulting coating would not have a pleasant melt-in-mouth feel because storage at freezer temperature (-18˚C) would result in all the fat becoming solid and taking a long time to melt. To bypass this problem, ice cream manufacturers add the chocolate in an untempered state so that it solidifies in form I or II, making the coating softer

and allowing it to melt at about 16°C (Beckett, 2008). This application of chocolate would be practical using the ALA incorporated cocoa butter. However, the issue then becomes having enough chocolate coating on the ice cream bar to meet labeling claims.

In order to confirm the results of DSC analysis powder X-ray diffraction (XRD) should be conducted along with solid fat content (SFC) analysis. Due to knowledge and resource limitations, this analysis was unfortunately not possible. However, Le Révérend et al. (2009) have stated that methods like SFC using pulsed nuclear magnetic resonance do not give additional information compared to DSC, in the industrial setting (Le Révérend et al., 2009). Also, DSC is considered a simple method that can be used in industrial laboratories in place of more sophisticated analysis (Fessas et al., 2005).

However, a second look at the Canadian Department of Justice, Food and Drug Regulations, revealed that the previously stated values for "pure" and "blend" modified cocoa butter samples were miscalculated and in reality instead of 11.9wt% and 21wt% for dark and milk chocolate, respectively, the values were actually quite lower. The law states that a milk chocolate product should contain no less than 25% total cocoa solids and 15% cocoa butter, while a dark chocolate product should contain no less than 35% total cocoa solids and 18%

cocoa butter, although the exact wording used does not make this clear (Canadian Department of Justice, 2010b), leading to the confusion encountered here. Therefore, modified cocoa butter samples should have had 4.3wt% and 5.2wt% ALA incorporated per serving for dark chocolate and milk chocolate, respectively. The calculations for these values are included in the appendix. This means that the "pure" and "blend" modified cocoa butter samples previously discussed were in reality 3-4 times more concentrated than that needed to meet minimum omega-3 fatty acid labeling claims. This, however, is not necessarily a negative outcome. DSC thermograms revealed that modified and unmodified cocoa butter samples were quite similar. At very low incorporation levels, such as those needed to meet labeling claims, these would be even more similar. Another factor to consider is that, in industry, a minimum amount of omega-3 fatty acid incorporation is not necessarily desired. Most manufacturers aim for higher incorporation levels because this assures that their products meet labeling claims and consumers usually look for products that give them higher omega-3 fatty acid amounts. So, although this misinterpretation occurred, it does not take away from the fact that the modified and unmodified cocoa butters were similar.

## 5.5 CONCLUSION

In this study, modified cocoa butter samples were prepared to comply with omega-3 fatty acid labeling claims for a milk chocolate and dark chocolate product. For a dark chocolate product, 11.9 wt% ALA incorporation was needed and, using the model equation developed with RSM of the experiments discussed in the previous chapter, a molar ratio of 1 : 1.25, time of 1 hour, and temperature of 30˚C were used to manufacture samples for the "pure" dark chocolate sample. These variables resulted in a 14.3±0.4 wt% incorporation. Samples for 11.9 wt% ALA incorporation were also made as a "blend" by manufacturing a higher incorporation sample at about 40 mole% and diluting it with unmodified cocoa butter. This resulted in a 13.9±0.7 wt% incorporation. For a milk chocolate product 21 wt% ALA incorporation was needed and the variable parameters used to obtain this incorporation were 1 : 1.75 mole ratio, 1 hour, and 30˚C; these resulted in a 19.3±1.1 wt% incorporation. The same variable parameters used to make the modified cocoa butter for the dark chocolate "blend" were also used to make the milk chocolate "blend"; with dilution with unmodified cocoa butter, this resulted in 21.6±0.2 wt% incorporation. Percent TAG levels ranged from a low of 93.7±0.7 to a high of 97.3±1.0. DSC thermograms revealed that modified and unmodified cocoa

butter samples were quite similar at temperatures above 20˚C. Most samples seemed to contain a combination of unstable form II and stable form V or a combination of forms II, IV, and V. The sample with the most similar thermogram to unmodified cocoa butter was that for the 21 wt% ALA incorporation “pure”.

# CHAPTER 6 CONCLUSION

The effects of temperature, reaction time, and molar concentration on the incorporation of DA and ALA into cocoa butter were compared using an immobilized enzyme derived from *R. miehei*. RSM was used to determine the relationship between these variables and their effects on the amount of DA and ALA incorporation. The variables tested did not have equivalent effects on the incorporation of the two fatty acids. Most notably temperature did not have a significant effect on DA incorporation while all three variables tested were significant for ALA incorporation. This finding led to the conclusion that DA was not an adequate model fatty acid for the incorporation of ALA into cocoa butter. HPLC analysis revealed that the products of the enzymatic interesterification were TAG, 1, 2-DAG, and 1, 3-DAG. The presence of 1, 3-DAG also indicates that acyl migration occurred during the reaction. Amounts of 1, 3-DAG increased as time and temperature increased.

Samples were prepared as either “pure” or “blend” to comply with omega-3 labeling claims for milk and dark chocolates. Dark chocolate samples required 11.9 wt% ALA incorporation while milk chocolate samples required 21 wt% ALA incorporation. The model equation obtained with RSM was used to determine the variable parameters needed to produce the “pure” samples for

both milk and dark chocolate products. "Blend" samples were prepared by diluting higher incorporation samples, about 40 mole%, with unmodified cocoa butter.

DSC analysis revealed that the statically tempered "pure" and "blend" samples were composed of a combination of unstable form II polymorphs and more stable form V polymorphs or a combination of forms II, IV, and V. Physical analysis of the samples revealed that these also contained some liquid material. The presence of the liquid material lead to the conclusion that the ALA incorporated cocoa butter might be better suited for ice cream coatings instead of chocolate products at room temperature.

In future research it will be of benefit to test the actual minimum omega-3 fatty acid incorporation needed for dark and milk chocolate products, 4.2wt% and 5.7wt%. It would also be helpful to conduct scale-up testing from the test-tube level. It would also be beneficial to conduct powdered XRD and SFC to determine if there are other similarities between modified and unmodified cocoa butter. It would also be useful to perform oxidative stability testing should in future research. Finally, it would be advantageous to make a chocolate product from the modified cocoa butter to determine how the other chocolate ingredients interact and the physical properties of the final product.

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# APPENDIX I

## OMEGA-3 LABELING CLAIM CALCULATIONS FOR MILK AND DARK CHOCOLATE – ACTUAL INCORPORATION NEEDED FOR LABELING CLAIMS

Milk chocolate –should contain no less than 25% total cocoa solids of which no less than 15% is cocoa butter

A sample chocolate was chosen (Côte d'Or Milk chocolate) which had a 38g serving size and contained 12g of fat per serving

0.15*38g = 5.7g cocoa butter

To meet omega-3 labeling claims 0.3g per serving size is needed

(0.3g/5.7g)*100 = 5.3wt% ALA per serving

Dark chocolate – should contain no less than 35% total cocoa solids of which no less than 18% is cocoa butter

A sample chocolate was chosen (Camino Dark chocolate) which had a 40g serving size and 15g of fat per serving

0.18 * 40g = 7.2g cocoa butter

To meet omega-3 labeling claims 0.3g per serving size is needed

(0.3g/7.2g)*100 = 4.2wt% ALA per serving

## OMEGA-3 LABELING CLAIM CALCULATIONS FOR MILK AND DARK CHOCOLATE – MISINTERPRETATION OF INCORPORATION NEEDED FOR LABELING CLAIMS

Canadian Department of Justice Food and Drug Regulations:

Milk chocolate –should contain no less than 25% total cocoa solids of which no less than 15% is cocoa butter

A sample chocolate was chosen (Côte d'Or Milk chocolate) which had a 38g serving size and contained 12g of fat per serving

0.25*38g = 9.5g cocoa solids

9.5g cocoa solids * 0.15 = 1.45g cocoa butter per 38g serving

To meet omega-3 labeling claims 0.3g per serving size is needed

(0.3g/1.45g)*100 = 21wt% ALA per serving

Dark chocolate – should contain no less than 35% total cocoa solids of which no less than 18% is cocoa butter

A sample chocolate was chosen (Camino Dark chocolate) which had a 40g serving size and 15g of fat per serving

0.35 * 40g = 14g cocoa solids

14g cocoa solids * 0.18 = 2.52g cocoa butter per 40g serving

To meet omega-3 labeling claims 0.3g per serving size is needed

(0.3g/2.52g)*100 = 11.9wt% ALA per serving

## APPENDIX II

TABLE II.1 TOTAL LIPID RECOVERED FROM ENZYMATIC INTERESTERIFICATION REACTION OF COCOA BUTTER AND ALA FOR ALL EXPERIMENTS RAN, ACCOMPANIED BY APPROXIMATE COMPONENT LOSS DUE TO THE ENZYMATIC INTERESTERIFICATION REACTION.

| Mole ratio (Cocoa butter : ALA) | Time (hrs) | Temp (°C) | Enzyme load (g) | Cocoa butter 100 mg/ mL (mL) | ALA 101.4 mg/ mL (mL) | Approx Cocoa butter (g) | Approx ALA (g) | Lipid recovered (g) | Approx Loss (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1:3 | 6 | 40 | 0.103 | 2 | 2 | 0.2 | 0.2028 | 0.0616 | 0.3412 |
| 1:3 | 6 | 40 | 0.1022 | 2 | 2 | 0.2 | 0.2028 | 0.0826 | 0.3202 |
| 1:3 | 6 | 40 | 0.1033 | 2 | 2 | 0.2 | 0.2028 | 0.0751 | 0.3277 |
| 1:9 | 6 | 40 | 0.1018 | 1 | 3 | 0.1 | 0.3043 | 0.0395 | 0.3648 |
| 1:9 | 6 | 40 | 0.1044 | 1 | 3 | 0.1 | 0.3043 | 0.0412 | 0.3631 |
| 1:9 | 6 | 40 | 0.1018 | 1 | 3 | 0.1 | 0.3043 | 0.0760 | 0.3283 |
| 1:6 | 18 | 40 | 0.1048 | 1 | 2 | 0.1 | 0.2028 | 0.0342 | 0.2686 |
| 1:6 | 18 | 40 | 0.1020 | 1 | 2 | 0.1 | 0.2028 | 0.0362 | 0.2666 |
| 1:6 | 18 | 40 | 0.1023 | 1 | 2 | 0.1 | 0.2028 | 0.0371 | 0.2657 |
| 1:3 | 30 | 40 | 0.1039 | 2 | 2 | 0.2 | 0.2028 | 0.0623 | 0.3405 |
| 1:3 | 30 | 40 | 0.1023 | 2 | 2 | 0.2 | 0.2028 | 0.0675 | 0.3353 |
| 1:3 | 30 | 40 | 0.1021 | 2 | 2 | 0.2 | 0.2028 | 0.0593 | 0.3435 |
| 1:9 | 30 | 40 | 0.1023 | 1 | 3 | 0.1 | 0.3043 | 0.0330 | 0.3713 |
| 1:9 | 30 | 40 | 0.1012 | 1 | 3 | 0.1 | 0.3043 | 0.0250 | 0.3793 |

| Mole ratio (Cocoa butter : ALA) | Time (hrs) | Temp (°C) | Enzyme load (g) | Cocoa butter 100 mg/ mL (mL) | ALA 101.4 mg/ mL (mL) | Approx Cocoa butter (g) | Approx ALA (g) | Lipid recovered (g) | Approx Loss (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1:9 | 30 | 40 | 0.1026 | 1 | 3 | 0.1 | 0.3043 | 0.0484 | 0.3559 |
| 1:6 | 6 | 50 | 0.1018 | 1 | 2 | 0.1 | 0.2028 | 0.0420 | 0.2608 |
| 1:6 | 6 | 50 | 0.1011 | 1 | 2 | 0.1 | 0.2028 | 0.0399 | 0.2629 |
| 1:6 | 6 | 50 | 0.1019 | 1 | 2 | 0.1 | 0.2028 | 0.0399 | 0.2629 |
| 1:3 | 18 | 50 | 0.1003 | 2 | 2 | 0.2 | 0.2028 | 0.0502 | 0.3526 |
| 1:3 | 18 | 50 | 0.1018 | 2 | 2 | 0.2 | 0.2028 | 0.0917 | 0.3111 |
| 1:3 | 18 | 50 | 0.1005 | 2 | 2 | 0.2 | 0.2028 | 0.0603 | 0.3425 |
| 1:6 | 18 | 50 | 0.1011 | 1 | 2 | 0.1 | 0.2028 | 0.0276 | 0.2752 |
| 1:6 | 18 | 50 | 0.1019 | 1 | 2 | 0.1 | 0.2028 | 0.0226 | 0.2802 |
| 1:6 | 18 | 50 | 0.1005 | 1 | 2 | 0.1 | 0.2028 | 0.0289 | 0.2739 |
| 1:6 | 18 | 50 | 0.1019 | 1 | 2 | 0.1 | 0.2028 | 0.0366 | 0.2662 |
| 1:6 | 18 | 50 | 0.1015 | 1 | 2 | 0.1 | 0.2028 | 0.0379 | 0.2649 |
| 1:6 | 18 | 50 | 0.1017 | 1 | 2 | 0.1 | 0.2028 | 0.0368 | 0.2660 |
| 1:6 | 18 | 50 | 0.1018 | 1 | 2 | 0.1 | 0.2028 | 0.0382 | 0.2646 |
| 1:6 | 18 | 50 | 0.1022 | 1 | 2 | 0.1 | 0.2028 | 0.0386 | 0.2642 |
| 1:6 | 18 | 50 | 0.1012 | 1 | 2 | 0.1 | 0.2028 | 0.0382 | 0.2646 |
| 1:9 | 18 | 50 | 0.1019 | 1 | 3 | 0.1 | 0.3043 | 0.0272 | 0.3771 |
| 1:9 | 18 | 50 | 0.1017 | 1 | 3 | 0.1 | 0.3043 | 0.0303 | 0.3740 |

| Mole ratio (Cocoa butter : ALA) | Time (hrs) | Temp (°C) | Enzyme load (g) | Cocoa butter 100 mg/ mL (mL) | ALA 101.4 mg/ mL (mL) | Approx Cocoa butter (g) | Approx ALA (g) | Lipid recovered (g) | Approx Loss (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1:9 | 18 | 50 | 0.1003 | 1 | 3 | 0.1 | 0.3043 | 0.0312 | 0.3731 |
| 1:6 | 30 | 50 | 0.1011 | 1 | 2 | 0.1 | 0.2028 | 0.037 | 0.2658 |
| 1:6 | 30 | 50 | 0.1004 | 1 | 2 | 0.1 | 0.2028 | 0.0356 | 0.2672 |
| 1:6 | 30 | 50 | 0.1013 | 1 | 2 | 0.1 | 0.2028 | 0.0370 | 0.2658 |
| 1:3 | 6 | 60 | 0.1005 | 2 | 2 | 0.2 | 0.2028 | 0.0979 | 0.3049 |
| 1:3 | 6 | 60 | 0.1022 | 2 | 2 | 0.2 | 0.2028 | 0.1072 | 0.2956 |
| 1:3 | 6 | 60 | 0.1015 | 2 | 2 | 0.2 | 0.2028 | 0.1104 | 0.2924 |
| 1:9 | 6 | 60 | 0.1008 | 1 | 3 | 0.1 | 0.3043 | 0.0556 | 0.3487 |
| 1:9 | 6 | 60 | 0.1013 | 1 | 3 | 0.1 | 0.3043 | 0.051 | 0.3533 |
| 1:9 | 6 | 60 | 0.1011 | 1 | 3 | 0.1 | 0.3043 | 0.0637 | 0.3406 |
| 1:6 | 18 | 60 | 0.1013 | 1 | 2 | 0.1 | 0.2028 | 0.0519 | 0.2509 |
| 1:6 | 18 | 60 | 0.1006 | 1 | 2 | 0.1 | 0.2028 | 0.0454 | 0.2574 |
| 1:6 | 18 | 60 | 0.1001 | 1 | 2 | 0.1 | 0.2028 | 0.0460 | 0.2568 |
| 1:3 | 30 | 60 | 0.1004 | 2 | 2 | 0.2 | 0.2028 | 0.0861 | 0.3167 |
| 1:3 | 30 | 60 | 0.1002 | 2 | 2 | 0.2 | 0.2028 | 0.0917 | 0.3111 |
| 1:3 | 30 | 60 | 0.1012 | 2 | 2 | 0.2 | 0.2028 | 0.1052 | 0.2976 |
| 1:9 | 30 | 60 | 0.1015 | 1 | 3 | 0.1 | 0.3043 | 0.0458 | 0.3585 |
| 1:9 | 30 | 60 | 0.1013 | 1 | 3 | 0.1 | 0.3043 | 0.0392 | 0.3651 |

| Mole ratio (Cocoa butter : ALA) | Time (hrs) | Temp (°C) | Enzyme load (g) | Cocoa butter 100 mg/ mL (mL) | ALA 101.4 mg/ mL (mL) | Approx Cocoa butter (g) | Approx ALA (g) | Lipid recovered (g) | Approx Loss (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1:9 | 30 | 60 | 0.1012 | 1 | 3 | 0.1 | 0.3043 | 0.0488 | 0.3555 |

TABLE II.2 TOTAL LIPID RECOVERED FROM ENZYMATIC INTERESTERIFICATION REACTION OF COCOA BUTTER AND DA FOR ALL EXPERIMENTS RAN, ACCOMPANIED BY APPROXIMATE COMPONENT LOSS DUE TO THE ENZYMATIC INTERESTERIFICATION REACTION.

| Mole ratio (Cocoa butter : DA) | Time (hrs) | Temp (°C) | Enzyme load (g) | Cocoa butter 100 mg/ mL (mL) | DA 62.76 mg/ mL(mL) | Lipid recovered (g) | Aprox Cocoa butter (g) | Approx DA (g) | Approx Loss (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1:3 | 6 | 40 | 0.1056 | 2 | 2 | 0.0645 | 0.2 | 0.1255 | 0.2610 |
| 1:3 | 6 | 40 | 0.1016 | 2 | 2 | 0.0672 | 0.2 | 0.1255 | 0.2583 |
| 1:3 | 6 | 40 | 0.1071 | 2 | 2 | 0.0595 | 0.2 | 0.1255 | 0.2660 |
| 1:9 | 6 | 40 | 0.1025 | 1 | 3 | 0.0336 | 0.1 | 0.1882 | 0.2546 |
| 1:9 | 6 | 40 | 0.1057 | 1 | 3 | 0.0375 | 0.1 | 0.1882 | 0.2507 |
| 1:9 | 6 | 40 | 0.1020 | 1 | 3 | 0.0340 | 0.1 | 0.1882 | 0.2542 |
| 1:6 | 18 | 40 | 0.1093 | 1 | 2 | 0.0283 | 0.1 | 0.1255 | 0.1972 |
| 1:6 | 18 | 40 | 0.1043 | 1 | 2 | 0.0326 | 0.1 | 0.1255 | 0.1929 |
| 1:6 | 18 | 40 | 0.1069 | 1 | 2 | 0.0283 | 0.1 | 0.1255 | 0.1972 |
| 1:3 | 30 | 40 | 0.1023 | 2 | 2 | 0.0499 | 0.2 | 0.1255 | 0.2756 |
| 1:3 | 30 | 40 | 0.1040 | 2 | 2 | 0.0559 | 0.2 | 0.1255 | 0.2696 |
| 1:3 | 30 | 40 | 0.1027 | 2 | 2 | 0.0553 | 0.2 | 0.1255 | 0.2702 |
| 1:9 | 30 | 40 | 0.1019 | 1 | 3 | 0.0307 | 0.1 | 0.1882 | 0.2575 |
| 1:9 | 30 | 40 | 0.1022 | 1 | 3 | 0.0305 | 0.1 | 0.1882 | 0.2577 |
| 1:9 | 30 | 40 | 0.1008 | 1 | 3 | 0.0270 | 0.1 | 0.1882 | 0.2612 |

| Mole ratio (Cocoa butter : DA) | Time (hrs) | Temp (°C) | Enzyme load (g) | Cocoa butter 100 mg/ mL (mL) | DA 62.76 mg/ mL(mL) | Lipid recovered (g) | Aprox Cocoa butter (g) | Approx DA (g) | Approx Loss (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1:6 | 6 | 50 | 0.1092 | 1 | 2 | 0.0354 | 0.1 | 0.1255 | 0.1901 |
| 1:6 | 6 | 50 | 0.1010 | 1 | 2 | 0.0376 | 0.1 | 0.1255 | 0.1879 |
| 1:6 | 6 | 50 | 0.1097 | 1 | 2 | 0.036 | 0.1 | 0.1255 | 0.1895 |
| 1:3 | 18 | 50 | 0.1083 | 2 | 2 | 0.0784 | 0.2 | 0.1255 | 0.2471 |
| 1:3 | 18 | 50 | 0.1036 | 2 | 2 | 0.0792 | 0.2 | 0.1255 | 0.2463 |
| 1:3 | 18 | 50 | 0.1006 | 2 | 2 | 0.0762 | 0.2 | 0.1255 | 0.2493 |
| 1:6 | 18 | 50 | 0.1053 | 1 | 2 | 0.0344 | 0.1 | 0.1255 | 0.1911 |
| 1:6 | 18 | 50 | 0.107 | 1 | 2 | 0.0306 | 0.1 | 0.1255 | 0.1949 |
| 1:6 | 18 | 50 | 0.1023 | 1 | 2 | 0.034 | 0.1 | 0.1255 | 0.1915 |
| 1:6 | 18 | 50 | 0.1062 | 1 | 2 | 0.0341 | 0.1 | 0.1255 | 0.1914 |
| 1:6 | 18 | 50 | 0.1066 | 1 | 2 | 0.0336 | 0.1 | 0.1255 | 0.1919 |
| 1:6 | 18 | 50 | 0.1051 | 1 | 2 | 0.0380 | 0.1 | 0.1255 | 0.1875 |
| 1:6 | 18 | 50 | 0.1016 | 1 | 2 | 0.0300 | 0.1 | 0.1255 | 0.1955 |
| 1:6 | 18 | 50 | 0.1028 | 1 | 2 | 0.0305 | 0.1 | 0.1255 | 0.1950 |
| 1:6 | 18 | 50 | 0.1065 | 1 | 2 | 0.0276 | 0.1 | 0.1255 | 0.1979 |
| 1:9 | 18 | 50 | 0.1026 | 1 | 3 | 0.0357 | 0.1 | 0.1882 | 0.2525 |
| 1:9 | 18 | 50 | 0.1061 | 1 | 3 | 0.0338 | 0.1 | 0.1882 | 0.2544 |
| 1:9 | 18 | 50 | 0.1074 | 1 | 3 | 0.0377 | 0.1 | 0.1882 | 0.2505 |

| Mole ratio (Cocoa butter : DA) | Time (hrs) | Temp (°C) | Enzyme load (g) | Cocoa butter 100 mg/ mL (mL) | DA 62.76 mg/ mL(mL) | Lipid recovered (g) | Aprox Cocoa butter (g) | Approx DA (g) | Approx Loss (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1:6 | 30 | 50 | 0.1011 | 1 | 2 | 0.0341 | 0.1 | 0.1255 | 0.1914 |
| 1:6 | 30 | 50 | 0.1002 | 1 | 2 | 0.0360 | 0.1 | 0.1255 | 0.1895 |
| 1:6 | 30 | 50 | 0.1008 | 1 | 2 | 0.0370 | 0.1 | 0.1255 | 0.1885 |
| 1:3 | 6 | 60 | 0.1047 | 2 | 2 | 0.0631 | 0.2 | 0.1255 | 0.2624 |
| 1:3 | 6 | 60 | 0.1063 | 2 | 2 | 0.0685 | 0.2 | 0.1255 | 0.2570 |
| 1:3 | 6 | 60 | 0.1037 | 2 | 2 | 0.0709 | 0.2 | 0.1255 | 0.2546 |
| 1:9 | 6 | 60 | 0.1027 | 1 | 3 | 0.034 | 0.1 | 0.1882 | 0.2542 |
| 1:9 | 6 | 60 | 0.1072 | 1 | 3 | 0.0309 | 0.1 | 0.1882 | 0.2573 |
| 1:9 | 6 | 60 | 0.1075 | 1 | 3 | 0.0329 | 0.1 | 0.1882 | 0.2553 |
| 1:6 | 18 | 60 | 0.1063 | 1 | 2 | 0.0281 | 0.1 | 0.1255 | 0.1974 |
| 1:6 | 18 | 60 | 0.1069 | 1 | 2 | 0.0258 | 0.1 | 0.1255 | 0.1997 |
| 1:6 | 18 | 60 | 0.1066 | 1 | 2 | 0.0259 | 0.1 | 0.1255 | 0.1996 |
| 1:3 | 30 | 60 | 0.1015 | 2 | 2 | 0.0767 | 0.2 | 0.1255 | 0.2488 |
| 1:3 | 30 | 60 | 0.1011 | 2 | 2 | 0.0806 | 0.2 | 0.1255 | 0.2449 |
| 1:3 | 30 | 60 | 0.1016 | 2 | 2 | 0.0746 | 0.2 | 0.1255 | 0.2509 |
| 1:9 | 30 | 60 | 0.1002 | 1 | 3 | 0.0340 | 0.1 | 0.1882 | 0.2542 |
| 1:9 | 30 | 60 | 0.1002 | 1 | 3 | 0.0381 | 0.1 | 0.1882 | 0.2501 |
| 1:9 | 30 | 60 | 0.101 | 1 | 3 | 0.0320 | 0.1 | 0.1882 | 0.2562 |