Development of a Predictive Model for the Enzymatic Interesterification of Edible Fats and Oils Under Solvent-Free Conditions

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DEVELOPMENT OF A PREDICTIVE MODEL FOR THE ENZYMATIC INTERESTERIFICATION OF EDIBLE FATS AND OILS UNDER SOLVENT-FREE CONDITIONS.

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Abstract

The modification of the properties of fats and oils through enzymatic interesterification processes has great potential for industrial usage. The objective of this study was to investigate the process of enzymatic batch interesterification of edible fats and oils, under solvent-free conditions, and the development mathematical equations through modeling studies for predicting the outcomes of interesterification given specified reaction conditions. These modeling studies would provide industry with tools for optimising the enzymatic interesterification process and assist in producing tailor-made fats with specific physical properties.

The solvent-free enzymatic interesterification process was examined using a combination of existing and newly developed techniques for assessing and monitoring changes in the chemical and physical properties. Model equations were developed to predict the outcomes of a batch interesterification reaction over time based on the initial reaction conditions of water and enzyme content.

An appropriate fat system for studying the interesterification process was established after initial investigations into the selection of a suitable immobilised lipase and natural fat. The majority of the interesterifications in this study were carried out using the commercially available immobilised 1,3-specific lipase, Lipozyme IM, as the enzymatic catalyst and cocoa butter as the natural fat system. The interesterification process was monitored by taking samples throughout the reaction, then analysing them for solid fat content, lipid class levels and triacylglycerol composition. The initial water content and the amount of immobilised enzyme in the reaction mixture were the main reaction parameters studied in this thesis. These parameters were shown to affect the rate and extent of 1,3-specific and random interesterification as well as the levels of by-products at hydrolysis equilibrium.

It was found that an hydrolysis equilibrium is established between the lipid classes of free fatty acids (FFA), diacylglycerols (DG) and triacylglycerols (TG) in a relatively short time after the start of the reaction, and well before the interesterification equilibrium. Typically, the hydrolysis equilibrium was established within 2 hours,

whereas the interesterification equilibrium was often not established within 24 hours. The level of by-products in the end-product is important, as they affect the yield of interesterified triacylglycerols and may also influence the physical properties of the product. The initial water content of the reaction system was found to have a major influence on the level of by-products, with increasing water content increasing them in a linear relationship. The relationships between the initial reaction conditions and the levels of FFA, DG and TG were formalised into linear equations, based on the outcomes of several interesterifications, using the data from 2 hours of reaction onwards. These equations were tested for their ability to predict the lipid class levels for interesterified cocoa butter, cottonseed oil and a blend of the two. The results showed that the lipid classes could be predicted reasonably well for cocoa butter and the other fat systems tested.

The asymmetric fatty acid distribution of cocoa butter allowed the changes in the triacylglycerol composition over time due to interesterification to be monitored with distinctions made between 1,3-specific and random interesterification. This distinction was important because it was found that although the immobilised lipase used was characterised as 1,3-specific by the manufacturer, longer reaction times led to a randomised product. A new method was developed that provided a triacylglycerol reaction profile of the interesterification process, by comparing the sample triacylglycerol compositions to calculated fully interesterified triacylglycerol composition. This comparison calculation generated triacylglycerol reaction profile 'distance' values, a measure of the similarity of the two triacylglycerol compositions. When the distances were plotted, the rate and degree of 1,3-specific and random interesterification could be monitored over time in a graphical form. Therefore, the triacylglycerol reaction profile method was a good tool for determining the stage and completeness of the enzymatic interesterification process.

It was found that the solid fat melting profiles could be related to the stage of the interesterification process. The solid fat profiles for 1,3-specific and random interesterified cocoa butter were characteristic and distinct from each other and the initial cocoa butter. The randomised product had a much flatter curve than that of the 1,3-specific interesterified product, where the original cocoa butter had a sharp

melting curve. The effect of lipid by-products on the physical properties was minor in comparison to the changes due to interesterification.

The relationship between the initial reaction conditions and the triacylglycerol reaction profiles was quantified using standard non-linear equations of the type: linear divided by linear (LDL) for random interesterification and quadratic divided by linear (QDL) for 1,3-specific interesterification. The equations were developed initially on an individual basis for interesterification under specific conditions, incorporating terms for the reaction time only. The individual equations were then combined to form overall equations incorporating terms for the initial enzyme and water contents as well as reaction time. These overall equations were assessed for their ability to predict the 1,3-specific and random triacylglycerol reaction profiles of cocoa butter interesterified under specified conditions. It was found that the overall triacylglycerol reaction profile equations could be used to predict the effect of altering the enzyme or water content on the interesterification reaction, however, they were not robust enough to accurately predict the measured triacylglycerol reaction profile distance values. The triacylglycerol reaction profile equations and analytical methodology need to be tested and refined further before adoption or application to different fat systems could be recommended.

Declaration

I hereby declare that all of the work contained within this thesis was carried out at Food Science Australia, formerly known as the Australian Food Industry Science Centre, during my candidature as a PhD student. To the best of my knowledge no part of this thesis has been submitted in part or in full for any other degree or diploma at any other University. Moreover, I declare that no material contained within this thesis has been written or published by any other person, excepting where due reference has been made to individuals in the text.

Sandra Ainsworth
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Abbreviations

AMF	anhydrous milkfat
BAUN	Batch Acidolysis Units Novo
BIU	Batch interesterification activity
BSTFA	(N,O)-bis(trimethyl silyl)-trifluoroacetamide
DG	diacylglycerol
FFA	free fatty acid
FID	flame ionisation detector
g	gram
GC	gas chromatography
h	hour
HPLC	high performance liquid chromatography
LDL	linear divided by linear
Lipozyme	Lipozyme IM
MG	monoacylglycerol
MUFA	monounsaturated fatty acid
NMR	nuclear magnetic resonance
Novozym	Novozym 435
O	oleic acid
OPM	oscillations per minute
P	palmitic acid
PUFA	polyunsaturated fatty acid
QDL	quadratic divided by linear
QDQ	quadratic divided by quadratic
S	stearic acid
SFC	solid fat content
sn	stereospecific numbering
Std	standard
TG	triacylglycerol
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
Trt	treatment
Wt	weight

Chapter 1

Introduction

1.1 Fats and oils

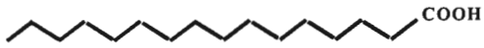
Edible fats and oils have been used in the preparation and processing of foods since ancient times (Bockisch, 1998). Fat, in its many forms, is an integral part of the human diet and is included in the daily intake of foods in most parts of the world. The importance of the fats and oils industry is not confined to the food industry since many industrial applications are found for fat products and fat by-products including: soaps, detergents, surfactants, paints, varnishes, resins and lacquers (Johnson, 1998).

Fats and oils consist mainly of triacylglycerols (commonly termed triglycerides), which are tri-esters of glycerol and fatty acids. A fatty acid is a lipid that consists of a hydrocarbon chain with a carboxylic acid end group. Saturated fatty acids have no double bonds and all the carbon atoms of the chain are saturated with hydrogen. Unsaturated fatty acids contain at least one double bond between two adjacent carbons of the chain. Monounsaturated fatty acids contain only one double bond in the hydrocarbon chain, whereas polyunsaturated fatty acids contain two or more double bonds (Hoffmann, 1989).

The IUPAC – IUB stereospecific numbering (abbreviation *sn*) convention is the system used for the nomenclature of triacylglycerols. In this system, each of the carbon atoms in the glycerol molecule is numbered 1-, 2-, and 3-. The position of each fatty acid in glycerol backbone is reflected in the number representing it. The *sn* convention recognises the fact that the two primary groups of glycerol are not identical, therefore the fatty acids at positions 1 and 3 should not be interchangeable. However, if there is no center of asymmetry, or the asymmetry is not known, the nomenclature 1,2,3-triacylglycerol is used without the insertion of the term "*sn*-". The structures of several fatty acids and a triacylglycerol are given in Figures 1.1 and 1.2.

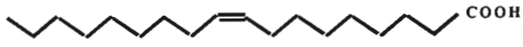
Figure 1.1. Structures of several common fatty acids. The notation C16:0 for palmitic acid refers to the 16 carbons in the fatty acid chain, while the zero indicates no double bonds in the carbon chain.

A. Palmitic acid (P), C16:0 (saturated):



B. Stearic acid (S), C18:0 (saturated):

C. Oleic acid (O), C18:1 (monounsaturated):



D. Linoleic acid, (L), C18:2 (polyunsaturated):

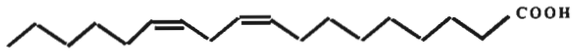
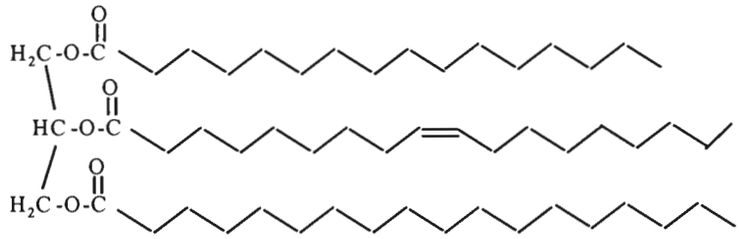
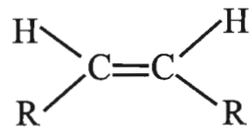


Figure 1.2 The structure of a triacylglycerol (POS), *sn*-1-palmitoyl-2-oleoyl-3-stearoyl-glycerol.

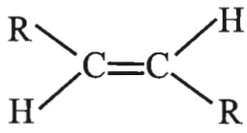


There are also two types of double bond, depending on the orientation of the carbon chains. The more common naturally occurring type is the *cis* bond, while the less common type in nature is the *trans* bond, which in terms of nutritional and melting properties, behaves more like a saturated fatty acid (Stauffer, 1996). The two types of double bond are given in Figure 1.3.

Figure 1.3. The structures of the configurations of a double bond in the chain of a fatty acid, comparing *cis* and *trans*.



cis bond



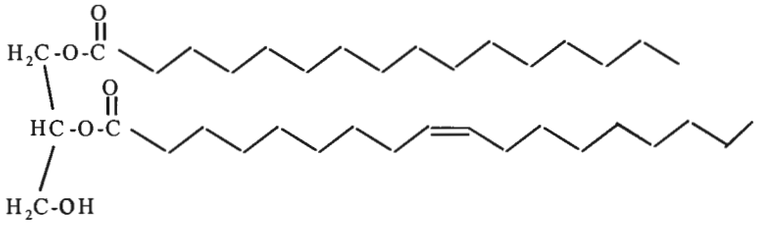
trans bond

Other components that may be present in the fat in minor amounts include: monoacylglycerols, diacylglycerols, free fatty acids, cholesterol, phosphoacylglycerols, alcohols, sterols, tocopherols (antioxidants), waxes, pigments, hydrocarbons, glucosides and the fat-soluble vitamins A, D, E and K (Hoffmann, 1989). The structures of the partial acylglycerols monoacylglycerols and diacylglycerols are given in Figure 1.4.

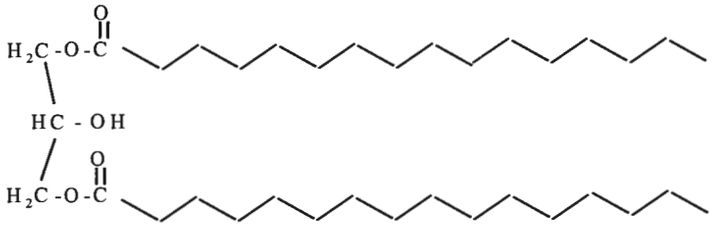
Most fat sources have a characteristic fatty acid composition, which is distributed positionally in a particular way, creating a distinctive triacylglycerol composition and unique functional properties. Fats can be characterised by their physical properties, particularly their melting behaviour at various temperatures. The physical properties of a fat depend on: the amount of unsatured fatty acids, with unsaturated fatty acids having lower melting points than saturated fatty acids of the same chain length (Mathews and van Holde, 1990); the length of the carbon chains, with longer carbon chains having higher melting points (Hoffmann, 1989); the positional distribution of the fatty acids within the triacylglycerols (Bockisch, 1998); and the contribution of each different triacylglycerol type to the overall composition (Kawahara, 1993).

Figure 1.4. The structures of several diacylglycerols and monoacylglycerols.

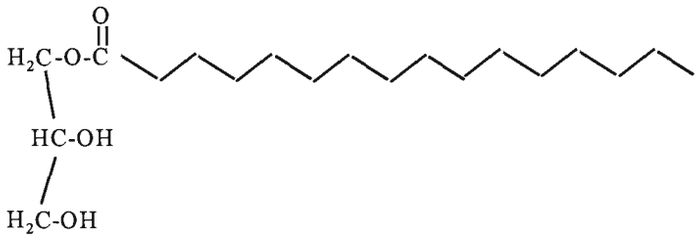
A. *sn*-1-palmitoyl-2-oleoyl-glycerol, a 1,2-diacylglycerol.



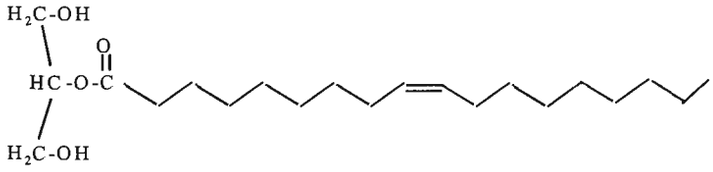
B. *sn*-1-palmitoyl-3-stearoyl-glycerol, a 1,3-diacylglycerol.



C. *sn*-1-monopalmitoyl-glycerol, a 1-monoacylglycerol.



D. *sn*-2-monooleoyl-glycerol, a 2-monoacylglycerol.



The pressure is increasing on producers and processors to provide consumers with products that are beneficial to health, yet functional and made by 'natural means'. Environmental and health concerns mean that manufacturers are moving away from the use of chemicals and solvents in their processes and enzymes are becoming increasingly accepted as an alternative. The trend is towards lowering the amount of fat in the diet, particularly saturated fat (Erickson and Frey, 1994). The development of fat-substitutes and fat-replacers has led to an extensive range of low-fat food products being available. It is believed that the position of a fatty acid within the triacylglycerol is important in digestion, with fatty acids at the 2-position absorbed more easily into the body (O'Carroll, 1995). The chemical composition of fats and oils, with respect to the positional distribution of particular fatty acids, will be more important in the future and more research into ways to control this positional distribution is needed.

1.2 Processing of fats and oils

Most fats and oils undergo a number of refining and/or modification processes to meet various quality and stability requirements that can relate to physical or chemical characteristics (Johnson, 1998). The quality of a fat or oil is a measure of its acceptability in its present state, while stability relates to its resistance to future changes (Johnson, 1998). Refining processes remove contaminants and off-flavours, they range from the simplest, which is water washing, to extensive treatments like alkali refining, bleaching and deodorising (Stauffer, 1996).

Modification processes alter certain functional characteristics, mainly physical properties, of the fat or oil, as well as increasing stability (Stauffer, 1996). These processes can create a fat with desired properties that can allow cheaper sources of fats to be utilised in wider applications (Bockisch, 1998). Some processes, such as fractionation and blending, do not change the basic molecular structure of the fat, while other processes such as hydrogenation and interesterification, are more complex involving structural alterations of fat components (Bockisch, 1998). There are also various biotechnology alternatives such as genetic engineering, DNA technology, traditional plant breeding programs and tissue culture (James, 1985; Sharp, 1986; Yamane, 1987; Kawahara, 1993), which are aimed at altering various aspects of the oil prior to extraction and processing.

Hydrogenation changes the physical characteristics of the starting fat, typically an oil. A metal catalyst, usually nickel, and hydrogen gas are used to add hydrogen across the carbon-carbon double bonds of unsaturated fatty acids, converting them to saturated fatty acids (Stauffer, 1996). This changes the melting point of the fat to a higher temperature, resulting in an hydrogenated product that is usually solid at room temperature. The oxidative stability is also increased as there are less sites (double bonds) for oxidation to occur (Bockisch, 1998).

When partial hydrogenation is carried out, a certain amount of *trans* isomers of unsaturated fatty acids are formed. Controversy has arisen over the health effects of *trans* fatty acids since it is generally accepted that *trans* fatty acids can be grouped together with saturated fatty acids, from a cardiac health point of view (Bockisch, 1998). Interesterification and blending, particularly using combinations of saturated and unsaturated fats, where the saturated fatty acids are derived from fractionation or full hydrogenation, are often used as alternatives to partial hydrogenation for the solidification of fats and oils to produce '*trans*-free' margarines. This is one product where the enzymatic interesterification process may find application, by producing desired changes in the physical properties of a fat product and where predictive modelling studies may be useful in determining the appropriate conditions to use.

1.3 Interesterification

Interesterification is a process that rearranges the positional distribution of the fatty acids among the triacylglycerols, by cleaving fatty acids from the triacylglycerols (hydrolysis) and subsequently re-attaching them (esterification) (Bockisch, 1998). Traditionally, chemical catalysts are used for interesterification, however lipase enzymes have proven to be successful in small-scale research studies thus providing great potential for future interesterification applications (Malcata et al, 1990).

During the interesterification process, fatty acids can change their position within a triacylglycerol, as well as move from one triacylglycerol to another. This leads to a change in the triacylglycerol composition, without a change in the overall fatty acid composition, resulting in changes in the melting and crystallisation properties (Bockisch, 1998). Interesterification of a single fat is not common; usually blends are interesterified to achieve greater effects on the properties, adding value to the starting blend. Improvements in the physical properties of a blend are the main reason behind most interesterifications, however the relationship between the original triacylglycerol composition and that of the final product to the physical properties is not widely understood. More studies relating the positional distribution of fatty acids to the physical properties are needed in order to understand what specific changes are required to achieve a particular melting property and how to achieve that change.

There are a number of fatty acid exchange reactions, with different substrates. The following terms and their relevant equations have been taken from the literature (Gandhi, 1997; Yamane, 1987).

Hydrolysis: ($TG + H_2O \rightarrow DG + FFA$)

$RCOOR' + H_2O \rightarrow RCOOH + R'OH$

Esterification: ($DG + FFA \rightarrow TG + H_2O$)

$RCOOH + R'OH \rightarrow RCOOR' + H_2O$

Transesterifications:

Acidolysis: ($TG_1 + FFA_1 \rightarrow TG_2 + FFA_2$)

$R_1COOR' + R_2COOH \rightarrow R_2COOR' + R_1COOH$

Alcoholysis: ($TG_1 + Alcohol_1 \rightarrow TG_2 + Alcohol_2$)

$RCOOR_1' + R_2OH \rightarrow RCOOR_2 + R_1'OH$

Interesterification: ($TG_1 + TG_2 \rightarrow TG_3 + TG_4$)

$RCOOR' + R"COOR^* \rightarrow RCOOR^* + R"COOR'$

Throughout this thesis, the term interesterification will refer specifically to fatty acid exchanges between and within triacylglycerols, as either single sources of fats or fat blends were examined with no additional sources of fatty acids. Transesterification is a term that could refer to interesterification, acidolysis or alcoholysis, but more commonly describes the exchange between a fat or triacylglycerol source and a fatty acid source (acidolysis). Transesterification can be carried out to create specific triacylglycerols or to enrich a fat with a particular fatty acid (Akoh and Moussata, 1998).

The specific area of solvent-free enzymatic interesterification of edible fats and oils using an immobilised lipase and examining the chemical and physical property changes is the main focus of this research. In chapter 2, a more detailed review of the literature relating to lipase catalysed interesterification reactions is presented, however, some of the literature cited may refer to a transesterification reaction, as the fundamental processes are similar and several experimental conditions can affect both reactions in similar ways.

Chemical interesterification can be catalysed by alkali catalysts such as sodium and potassium methoxide as well as sodium hydroxide (Schmidt, 1996). Metallic catalysts such as a sodium and potassium alloy are more common in industry today, although the catalysts require an additional reaction step before the reaction they catalyse (Bockisch, 1998). Interesterification is normally carried out in a batch reactor, although continuous processes are increasingly utilised (Rousseau and Marangoni, 1997). Chemical interesterification generally results in a random rearrangement of the fatty acids across all three positions of the triacylglycerol (Willis and Marangoni, 1997). Directed interesterification can be achieved however, through temperature control, where the higher melting glycerides formed are

crystallised and removed, disturbing the reaction equilibrium causing more of them to be produced (Bockisch, 1998). Regio-selectivity, where rearrangement of fatty acids on positions 1 and 3 is favoured, is also possible to a limited extent (Konishi et al, 1993).

Chemical interesterification has been investigated, using various sources of hard and soft fats, to produce plastic fats used in margarine. Examples include: completely hydrogenated soybean oil with soybean oil (List et al, 1977); fully hardened soybean oil with nine different vegetable oils in a 1:1 ratio (Zeitoun et al, 1993); tallow with soybean oil (Lo and Handel, 1983); tallow with corn oil (Chacon and Handel, 1985) and olive oil with tristearin (Gavriilidou and Boskou, 1991).

Lipase enzymes can also catalyse the interesterification process. Enzymatic reactors have not been reported in commercial scale production to date, largely due to the comparatively high process and catalyst costs. Production of tailor-made fats with improved functionality or by 'natural' and 'clean-green' processing techniques, as well as cheaper, more effective lipase catalysts could increase opportunities for lipase applications (Rozendaal and Macrae, 1997). In a very recent development, a new cost-effective immobilised lipase has become available commercially, Lipozyme TL IM, which could have implications regarding future industrial scale applications (Christensen et al, 2001). For enzymatic interesterification to have any industrial relevance in the future, toxic and expensive solvents should be avoided (Kim and Rhee, 1991). Therefore, to be commercially feasible, large-scale continuous, solvent-free immobilised lipase reactors should be developed (Marangoni and Rousseau, 1995; Malcata et al, 1990; Willis and Marangoni, 1997). It is recommended however, that initial trials be carried out in batch reactors prior to starting column operations (O'Carroll, 1995).

The development of mathematical models to predict the outcome of a lipase catalysed interesterification would greatly assist manufacturers in achieving both the desired chemical and physical property requirements of a tailor-made fat. Such mathematical tools and modelling studies are lacking in the literature and are investigated in this thesis.

Chapter 2

Literature Review

2.1 Lipase enzymes

Lipases are enzymes that catalyse the reversible hydrolysis of triglycerides and are widely distributed in animals, plants and microbes (Weete, 1997). Lipases for enzymatic interesterification are normally recovered from microorganisms that excrete lipases into their growth medium (Rozendaal and Macrae, 1997). Lipases are highly soluble in water, however, they have a high affinity for hydrophobic surfaces, so are particularly active at an oil:water interface (Weete, 1997). While lipases have found applications in the detergent and food industries, they are also of special interest in the processing of edible fats and oils (Gandhi, 1997).

Lipases can be divided into classes based on their specificity. Non-specific or random lipases have no preference for the position of a fatty acid, hence they can randomise the distribution of fatty acids among all three positions, creating products similar to those made by chemical interesterification (Macrae, 1983; Sil Roy and Bhattacharyya, 1993). For example, non-specific lipases can be isolated from *Candida* species, particularly *C. cylindracea* (Ergan et al, 1991; Kalo et al, 1986a; Thomas et al, 1988). Lipases can also be regio- or positionally-specific, such as 1,3-specific lipases which act preferentially on the fatty acids on positions 1 and 3 of the triglyceride molecule (Macrae, 1983). A number of 1,3-specific lipases have been isolated and investigated including lipases from *Rhizopus arrhizus* (Marangoni et al, 1993) and *Mucor miehei* (Huge-Jensen et al, 1988; Ison et al, 1994). There are also lipases that can react selectively with particular fatty acids, regardless of their position on the triglyceride, such as the lipase from *Geotrichum candidum,* which is specific towards long chain fatty acids containing a *cis* double bond in the 9-position (Macrae, 1983).

Lipases can catalyse ester-exchange reactions between a variety of substrates, including triglycerides, fatty acids and fatty acid esters. This range of substrates as well as the various specificities and types of lipases, particularly 1,3-specific lipases, allows for some unique products to be produced through lipase reactions, which can

be an advantage over chemical catalysts. Studies detailing the interesterification behaviour of 1,3-specific lipases in solvent-free natural fat systems, where the changes in both the chemical and physical properties are monitored are lacking in the literature. The ability to control and predict the outcomes of the enzymatic interesterification process would provide potential benefits to manufacturers.

Lipases are available in the free form, which can then be immobilised onto a carrier. Enzymes are usually immobilised to facilitate their removal from the reaction mixture as this aids in their re-use (Malcata et al, 1990). One of the most important factors to consider when immobilising an enzyme is the retention of its activity. The immobilisation of a lipase onto a carrier or an inert support can be difficult, as the lipase must be free to move to the interface or loss of activity will result (Wisdom et al, 1987). When immobilised on Celite, a diatomaceous earth, lipase retained 85-90% of its activity (Goderis et al, 1987).

Immobilisation techniques are based on the attachment of the lipase to a carrier or support by physical or chemical methods. Chemical methods involve covalent bonds being formed between the enzyme and support, while physical methods are based on adsorption or entrapment. Supports available for immobilisation include Celite, Duolite, kieselguhr, clay and Sepherosil (Brady et al, 1988; Malcata et al, 1990; Marangoni et al, 1993). The selection of support material and enzyme immobilisation process are important in determining the effectiveness of the reaction system (Marangoni et al, 1993; Ison et al, 1994).

There are also a number of immobilised lipases available commercially. There is no standard procedure for the immobilisation of a lipase enzyme to a carrier. Screening trials would need to be carried out in order to examine the issues involved more closely and obtain a suitable enzymatic catalyst for further work.

The nutritional aspects of various fats can also be studied and enhanced utilising lipase reactions (Kawahara, 1993). For example, a human milk fat analogue can be produced through interesterification of milkfat with long-chain polyunsaturated fatty acids, this resembles the fatty acid composition and positional distribution of human milk, hence it is highly suitable for infant formulas (Christensen et al, 1993).

Nutritionally beneficial long chain omega-3 polyunsaturated fatty acids, found in fish and fish oil, can be incorporated into various products such as vegetable oil, through enzymatic interesterification (Li and Ward, 1993). Although enzymatic interesterification technology has been successfully applied on a laboratory and pilot-scale, prohibitive costs have limited industrial usage. Further research continues to add to knowledge and applications of this technology, particularly in the area of solvent-free enzymatic interesterification (Bockisch, 1998).

2.2 Reaction media

Enzymatic interesterification can be carried out with an organic solvent as the reaction medium. The main solvent used for interesterification of fats and oils is hexane as it is approved for use in the food industry (Rozendaal and Macrae, 1997). Initially, lipases were immobilised by adsorption onto diatomaceous earth carriers where organic solvents prevented the lipase from becoming free from the carrier (Macrae et al, 1993). Organic solvents convert a two-phase (aqueous and fat) system into a single-phase system facilitating contact between the lipase and the fat substrate (Van der Padt et al, 1990). The viscosity of the reaction medium is lower when using organic solvents, an advantage when using a continuous reactor. Organic solvents may also allow the use of low reaction temperatures (30-50°C) without the higher melting fats crystallising, which can help to prolong the catalytic life of the lipase (Bockisch, 1998). The type of solvent used can have an effect on the activity of the lipase, the efficiency of the interesterification and the degree of acyl migration (Carta et al, 1991; Valivety, 1991).

A reverse micelle system is one type of organic solvent system that can be used for lipase catalysed interesterification, where the lipase and fat substrate are in contact through the interface of micelles (Willis and Marangoni, 1997). In this system the lipase is at the core of the micelle, which has been described as a nanometer-sized water droplet dispersed in organic media with surfactants stabilising the interface (Quinlan and Moore, 1993). Reverse micelle systems can involve free lipases (Marangoni et al, 1993) as well as immobilised lipases (Mojovic et al, 1993).

The water content during enzymatic interesterification is critical in interesterification reactions systems, with or without the addition of solvents. Gorman and Dordick

(1992) suggested that the amount of enzyme-bound water controls the activity of the enzyme, owing to the fact that organic solvents cause a disruption to this bound water, resulting in enzyme deactivation. Lower activities in polar organic solvents compared with non-polar could be due to an immediate loss in enzymatic hydration, thus increasing the solubility of water in polar solvents (Gorman and Dordick, 1992; Malcata et al, 1990). It was found that as the concentration of solvent used increased, the rate of interesterification decreased, hence the purity of the interesterified products decreased, with solvent-free systems being superior in terms of interesterification activity (Bloomer et al, 1990). Defining the relationship between the water content of the reaction system and the outcomes of the enzymatic interesterification process by a mathematical model would provide manufacturers with information needed to optimise their production in terms of purity of interesterified product and minimise reaction time.

Organic solvent systems can be costly in the production process, as not only can the solvents themselves be expensive, the solvent removal process can add to the overall processing costs (Kalo et al, 1990). Solvent-free reaction systems offer several advantages including a 'cleaner, greener' image, no solvent extraction costs and increases in reactant concentrations which may mean higher conversion rates (Malcata et al, 1990; Ison et al, 1988). Some processing may still be required to remove by-products which adds costs, it may also affect the quality of the product. This quality aspect is particularly important when using fats like butterfat, where further processing could remove the fine butter flavour (Kalo et al, 1990).

In solvent-free systems, the fat acts as both a substrate and a dispersant (Bornaz et al, 1994). Higher temperatures of around 70°C are often necessary for solvent-free reactions to ensure that the reactants and products are molten and facilitate contact with the lipase (Rozendaal and Macrae, 1997). This may place a limitation on the type of lipase that can be used, however, there are a number of lipases that are active at these higher temperatures from sources such as *Mucor miehei* (Novo Industri, 1986b), and *Candida antartica* (Rozendaal and Macrae, 1997).

The viscosity of the reaction medium is often higher in a solvent-free system, which may impact on the choice of reactor design. A solvent-free continuous system was found to be impractical, due to large pressure drops experienced from using a carrier with a small particle size, diatomaceous earth, as well as the increased viscosity of the reaction medium (Ison et al, 1988). This problem however, may be overcome by using a lipase immobilised onto a carrier of a sufficiently high particle size, around 0.3 - 0.6mm (Forssell et al, 1993). The choice of lipase and carrier combination will ultimately influence the reactor design.

2.3 Reactor design considerations

There are several types of reactors including fixed-bed continuous, stirred tank batch, membrane, and fluidised-bed continuous (Willis and Marangoni, 1997). Important factors that affect the interesterification process also influence reactor design, including the mechanical properties and size of the support particles (Rozendaal and Macrae, 1997). For example, due to the particle size and nature of the carrier, Lipozyme, a commercially available immobilised lipase, is best suited for fixed-bed or gently stirred batch reactors (Novo Industri, 1986b). In general, for large scale processing, mainly stirred tank batch and fixed-bed continuous reactors are used (Quinlan and Moore, 1993).

In a continuous reactor, the immobilised lipase is usually contained within a column and the substrate stream is pumped through. The advantages of a continuous reactor include: high productivity (Rozendaal and Macrae, 1997); high throughput with the largest amount of product per unit catalyst (Posorske et al, 1988); low cost, higher yields and ease of operation (Willis and Marangoni, 1997). Mass transfer limitations can occur (Ison et al 1988, 1994), and so a compromise between pressure drop and activity must be reached (Novo Industri, 1986b). Continuous reactors require large reactor volumes and very small flow rates for reasonable degrees of interesterification, constraints which make bench-scale investigations impractical (Malcata et al, 1990). The costs of operation will depend largely on the lifetime of the catalyst (Forssell et al, 1993), although for commercial feasibility, continuous large-scale solvent-free immobilised enzyme reactors will have to be used (Marangoni and Rousseau, 1995).

Technical feasibility can be demonstrated with a batch reactor prior to scaling-up to continuous process (Posorske et al, 1988; Malcata et al, 1990). Advantages of batch reactors include: no pumping, systems can be small, sampling is carried out on a time scale instead of volume over time (Malcata et al, 1990); lipase can be easily removed by filtration, sedimentation, or centrifugation (Malcata et al, 1990; O'Carroll, 1995); easy to set up and operate (Willis and Marangoni, 1997). Disadvantages include: larger system or longer reaction time is required to achieve degrees of conversion equivalent to those of other systems, also side reactions can be significant (Van der Padt et al, 1990); the enzyme may not be fully recovered depending on procedure (Rozendaal and Macrae, 1997). Furthermore, process considerations that include damage to the lipase through mechanical action of stirring and handling can deactivate the lipase (Malcata et al, 1990; Mukesh et al, 1993; Rozendaal and Macrae, 1997).

A number of studies have been carried out under solvent-free conditions using immobilised lipases and several are summarised in Table 2.3.1. The majority of these utilise a commercial immobilised lipase and a batch reactor system. Although several of these studies examined the interesterification of fat blends, they concentrated on changes in either the physical or compositional properties, not both.

2.4 Process considerations

Whatever the selected reactor design, the process should be optimised to minimise costs and maximise yields. Control of the water content of the system is extremely important in achieving high interesterification activity, without the side products of hydrolysis (Willis and Marangoni, 1997; Yee at al, 1997). Acyl migration can decrease yields of 1,3-specific interesterified products, as the fatty acids move among all the available positions within the triglyceride, randomising the distribution of the fatty acids. Optimisation of the interesterification process includes minimising hydrolysis and acyl migration (Forssell et al, 1993). Process considerations include reaction parameters such as water content, reaction temperature, substrate concentrations, lipase concentration as well as factors dependent on reactor type: reaction time and flow rates (Posorske et al, 1988).

Table 2.3.1 Various examples from the literature of lipase reactions carried out under solvent-free conditions.

Substrates	Reaction type	Lipase	Reactor design	Reference
Tallow fractions with liquid oils	Interesterification	Lipozyme	batch	Bhuttacharyya et al, 2000
Butterfat, and with high melting triglycerides	Interesterification	Lipozyme	batch	Bornaz et al, 1994
Fully hydrogenated cottonseed oil with olive oil	Interesterification	Lipozyme	batch	Chang et al, 1990
Palm olein with stearic acid	Transesterification	Lipozyme	batch	Chong et al, 1992
Tallow with sunflower oil	Interesterification	Lipozyme	batch	Foglia et al, 1993
Tallow with rapeseed oil	Interesterification	Lipozyme	batch	Forssell et al, 1992
Rapeseed oil with lauric acid	Transesterification	Lipozyme	continuous	Forssell et al, 1994
Olive oil with lauric acid	Transesterification	*Rhizomucor miehei*	continuous	Ison et al, 1994
Glycerol with capric acid	Triglyceride synthesis	Lipozyme	batch	Kim and Rhee, 1991
Canola with palm oil	Interesterification	*Rhizopus delemar*	batch and continuous	Kurashige et al, 1993
Palm stearin with sunflower oil	Interesterification	several	batch	Lai et al, 1998
Palm stearin with AMF	Interesterification	Lipozyme *Pseudomonas* sp.	batch	Lai et al, 2000a
Rapeseed oil with hydrogenation palm stearin	Interesterification	Lipozyme	batch	Ledochowska and Datta, 1998
Cottonseed oil with fully hardened soybean oil	Interesterification	Lipozyme *C. antartica*	batch	Mohamed et al, 1993
Canola oil with lauric acid, trilaurin and fully hydrogenated high erucic acid rapeseed oil	Interesterification Transesterification	several	batch	Thomas et al, 1988
Palm stearin with coconut oil	Interesterification	Lipozyme	batch	Zhang et al, 2000

The water content controls the equilibrium between hydrolysis and ester synthesis (Kalo et al, 1990; Willis and Marangoni, 1997). High water contents (>1% overall) favour hydrolysis, resulting in a high amount of by-products, while not enough water (<0.01% overall) results in no activity (Goderis et al, 1987; Yee et al, 1997). The optimum amount of water required can depend on the nature of the support material and will be different for different enzymes (Lee and Akoh, 1996).

During interesterification, an hydrolysis equilibrium is established between triglycerides, water, diglycerides and free fatty acids (Macrae, 1983). In batch reactors, the immobilised lipase catalyst is usually hydrated before use. In a continuous reactor, there is an initial period where water is removed from a hydrated catalyst through the production of diglycerides and free fatty acids until a steady state

is reached (Mohamed et al, 1993; Macrae, 1983). Hydration of the feedstream is often necessary due to the water stripping effect (Novo Industri, 1986b; Posorske et al, 1988).

Other factors can complicate lipase reactions. The 1,3-positional specificity of certain lipases supposedly ensures that the reaction only takes place at these positions (Akoh and Moussata, 1998). It has been found however that at the end of the reaction, particularly after prolonged reaction times, random products have been obtained (Rozendaal and Macrae, 1997; Safari and Kermasha, 1994; Willis and Marangoni, 1997). Ghazali et al (1995) suggested that the positional specificity of the enzyme was probably a secondary factor in determining the properties of the end-products of reaction, compared with acyl migration due to long reaction times. It should be noted that acyl migration is not an issue where the intention is to randomise the fatty acid distribution.

When studying the specificity of a lipase, only a limited extent of hydrolysis (<20%) should be carried out, to avoid complications arising from acyl migration or enzymatic hydrolysis of the partial glyceride products that can lead to errors in the observation (Villeneuve and Foglia, 1997). It is also difficult to distinguish between the specificity of the enzyme due to selectivity and preference for particular fatty acid, and the consequence of a particular fatty acid distribution and composition. In addition, the specificity of lipase on a triglyceride analogue may not be the same as for natural triglyceride substrates (Villeneuve and Foglia, 1997).

2.5 Monitoring of interesterification reactions

The end-point of interesterification, or interesterification equilibrium, can depend on the purpose of the interesterification. Interesterification equilibrium does not necessarily occur at the same time as hydrolysis equilibrium (Safari et al, 1993). It can be monitored by measuring changes over time in the physical properties of the interesterified fat, or by following changes in the triglyceride composition or fatty acid distribution (Marangoni and Rousseau, 1995; Mohamed et al, 1994).

Changes in the physical properties of the interesterified fat can be monitored using a variety of methods. The technique employed depends on the nature of the fat and its

intended use. The solid fat content, by pulsed NMR, provides information regarding the proportion of liquid and solid fat in a sample over a range of temperatures (Zeitoun et al, 1993). The solids content in a fat is, however, not necessarily an indication of its hardness (de Mann et al, 1989). Hardness evaluation is carried out using a cone penetrometer technique where the fats resistance to penetration is measured.

The melting and crystallisation behaviour can also be studied using differential scanning calorimetry (DSC), where samples can be heated and cooled at different rates then changes in energy absorbed or given out are measured (Seriburi and Akoh, 1998). Detailed crystal structure studies may be carried out by x-ray diffraction methods to determine the packing arrangement of the triglyceride crystals (Ledochowska, 1999a). The physical properties of the interesterified fats could also be affected by the presence of the minor lipids by-products of the reaction (Tietz and Hartel, 2000).

Changes in the triglyceride composition are often difficult to monitor, particularly if the substrates are complex fats with a mixture of triglyceride types. The analysis can be simplified by analysing for a specific peak in a chromatogram that indicates changes in a particular triglyceride or triglyceride group (Chang et al, 1990; Ghazali et al, 1995). The analysis of the fatty acid composition at the 2-position also provides information regarding the specificity of the reaction products and the degree of random interesterification (Dutta et al, 1978). At interesterification equilibrium, a constant triglyceride composition is reached, and the fatty acid distribution will remain unchanged. Samples taken during the reaction could be analysed for triglyceride composition to estimate when interesterification equilibrium has occurred. For most mixtures of oils and fats however, the complexity of the triglyceride composition does not allow simple kinetic models for the calculation of reaction rates (Rozendaal and Macrae, 1997). Optimisation and modelling studies will be useful in the future for designing and predicting the behaviour of large-scale reactors under a variety of conditions (Rozendaal and Macrae, 1997).

2.6 Aims of the research

The progress in the development of the interesterification process and the application of using immobilised lipase enzymes under solvent-free conditions has been described in the literature review. The work in this thesis will expand knowledge in this specific area through the achievement of the following aims:

1) To examine the process of solvent-free interesterification of edible fats and oils and select an appropriate immobilised enzyme and fat system for further investigation.

2) To develop a novel process for monitoring interesterification reactions using a natural fat system.

3) To investigate the effect of varying the initial reaction conditions on the interesterification process by analysing the chemical and physical properties of the reaction products over time.

4) To develop model equations to predict the outcomes of the interesterification process from the input of the initial reaction conditions and time of reaction.

5) To assess the ability of the developed equations to predict the outcomes of the interesterification under given initial reaction conditions.

Chapter 3

Methodology

3.1 Enzymatic interesterification

The basic batch reaction conditions for solvent-free enzymatic interesterification used in the work for this thesis were based on several literature sources (Chong et al, 1992; Bornaz et al, 1994). The conditions include a batch size of 60-80 grams, an enzyme preparation level of 5-10% w/w fat, an initial water content of 0.5-1.0%, a reaction temperature of 60°C and samples being taken at 0, 2, 8, 24 and 48 hours.

Procedure:

A weighed amount of molten fat substrate was placed into a 250 mL stoppered, flat-bottomed, round QuickFit flask, and either refrigerated overnight (4°C) for use the next day, or placed immediately in a shaking incubator (Paton Industries, Model 461) at 60°C operated at 120 oscillations per min. The substrate fat mixtures were equilibrated at the reaction temperature before addition of immobilised lipase. The lipase preparation was pre-weighed and added directly to the flask. In cases where additional water was added to the reaction mixture, it was added to the molten fat, just before the lipase preparation. Samples were taken after stopping the shaking motion of the incubator and allowing the immobilised lipase to settle to the bottom of the flask. Samples were withdrawn using heated transfer pipettes to avoid fat crystallisation on the pipette. The samples were placed into closed glass vials and heat-treated by placing in an oven at 100°C for 15 minutes to denature any residual lipase. The samples were cooled and stored in a freezer (-18°C) until ready for analysis.

3.2 Enzyme Immobilisation

The procedure for immobilising a lipase onto a carrier was based on an adsorption procedure described by Bloomer et al, 1990. Variations of this method examined in section 4.2 included; the amount of buffer solution used; adding a filtering step before drying; and comparing oven-drying to freeze-drying of immobilised lipase.

Procedure:

An amount (1g) of free lipase was dissolved in various amounts of buffer solution (sodium phosphate, pH 7.0) in a beaker. A weighed amount (10g) of carrier (Hyflo-supercel, Merck) was then added to the lipase solution and mixed thoroughly. When a filtering step was included before drying to remove excess buffer, the samples were vacuum-filtered through Whatman #1 filter paper. Two different methods of drying the immobilised lipases were used. Freeze-dried samples were placed in a Dynavac Freeze-Dryer (Model FD-5) at –45°C and 10^{-3} Torr for 72 h. Oven-dried samples were placed in a vacuum oven dryer (Heraeus Instruments, Vacu-therm) at room temperature (21°C) and 100 mbar pressure overnight (15 h). The immobilised lipases were then stored in a refrigerator (4°C) until required for further analysis.

3.3 Hydrolysis activity assay

The hydrolysis activity assay for lipase was based on a method described by Pronk et al, 1988, with a number of modifications. Olive oil was used instead of tributyrin, acacia gum at 0.4% (w/v) was substituted for arabic gum at 0.1%, and the reaction vessel contents were held at 37°C and pH 7.

Procedure:

An emulsion of 5 mL of olive oil (commercial variety –Vetta, Meadow Lea Foods, Product of Spain) in 250 mL of a 2mM maleic acid (SIGMA) solution was prepared by homogenising using an ultraturrax (Ultra-turrax, T45, IKA) at 2000 rpm for 5 minutes while slowly adding 1g of emulsifier (Acacia gum, Merck). Twenty-five mL of this emulsion was then added to a water-jacketed reaction vessel, stirred with a magnetic stirrer and equilibrated to pH 7.0 at 37°C. A Metrohm automatic titrator with a chart recorder was used to maintain the pH at 7.0. A weighed sample of lipase was introduced to the reaction vessel to begin the titration. The rate of addition of a standardised 0.01M sodium hydroxide (NaOH) solution required to keep the pH constant at 7.0 was recorded using the plotter. The slope of the line, the concentration of the NaOH solution and the amount of lipase added were used to calculate the hydrolysis activity. The hydrolysis

activity results were calculated in terms of the amount of free fatty acids released per minute (U = μmol FFA/min) per gram of lipase preparation (U/g).

Calculations:

$$A_h = \frac{C_{OH}.s.1000}{m_e}$$

where A_h = hydrolysis activity (U/g = μmole FFA.min^{-1}.g^{-1})

C_{OH} = concentration of NaOH solution (M)

s = slope of recorded line (mL/min)

m_e = amount of lipase added (g)

3.4 Interesterification activity assay

The interesterification activity assay for immobilised lipases in a solvent-free system was based on a standard method from the suppliers of Lipozyme (Novo Industri A/S, 1986a). The amounts of immobilised lipase and substrates used were scaled down to 1/6 of the recommended amounts.

Procedure:

The rate of interesterification between an equimolar mixture of triglyceride (triolein, OOO) and free fatty acid (palmitic acid, P) was analysed by assessing the amount of palmitic acid incorporated over time for a given amount of lipase. Triolein (0.50 g, SIGMA) and palmitic acid (0.145g, SIGMA) were placed in a closed 4 mL glass vial, and equilibrated to 60°C in a shaking incubator (Paton Industries, Model 461) operated at 120 oscillations per minute. The reaction was started by addition of 0.05g equivalent dry weight of lipase preparation (10% wt/wt triglyceride). Samples were taken at various intervals and analysed for triglyceride composition using the GC method described in section 3.10. From the amounts of different triglycerides (OOO, OOP, POP, PPP) present in the samples, the amount of palmitic acid incorporated into triolein was calculated:

Equation for the calculation of the amount of palmitic acid incorporated into triolein when a 1,3-specific lipase is used.

$$P_{inc} = \frac{\%POO + 2.\%POP}{3} \quad \text{.....3.3.1}$$

Equation for the calculation of the amount of palmitic acid incorporated into triolein when a non-specific lipase is used.

$$P_{inc} = \frac{\%POO + 2.\%POP + 3.\%PPP}{6} \quad \text{.....3.3.2}$$

The extent of the reaction at each sampling time was then calculated as a percentage of the equilibrium P_{inc}. The theoretical equilibrium percentage of palmitic acid incorporated into triolein at equilibrium ($P_{inc,\,eqm}$) can be calculated and is 24.5% for non-specific and 21.8% for 1,3-specific interesterification. According to the Novo standard method, the most accurate batch interesterification activity is calculated using the data from the sample taken at a reaction time where the amount of palmitic acid incorporated (P_{inc}) is in the range of 40-60% of equilibrium conversion. Interesterification activity is expressed in terms of Batch Interesterification Units per gram of catalyst (BIU/g), where one unit is the equivalent of one μmole of palmitic acid incorporated into triglyceride per minute.

Equation for calculation of the batch interesterification activity.

$$BIU/g = \frac{P_{inc,eqm} \cdot \ln\left(\frac{P_{inc,eqm}}{\left(P_{inc,eqm} - P_{inc}\right)}\right) \cdot M}{t \cdot W} \quad \text{.....3.3.3}$$

where: BIU/g = batch interesterification activity per gram of catalyst

$P_{inc,eqm}$ = amount of palmitic acid incorporated at equilibrium (%)

P_{inc} = amount of palmitic acid incorporated at time **t** (%)

M = μmole of triglyceride in reaction

t = reaction time (min)

W = amount of dry lipase preparation (g).

3.5 Determination of water content

The procedure to determine the water content of the lipase preparations and the substrate fats was based on the Karl Fischer method for moisture in oils and fats using an automatic potentiometric titration (AOAC Official method 984.20).

Procedure:

A Metrohm automatic titration system consisting of a Multi-Dosimat 655, Impulsomat 614 and pH Meter 632 was used to carry out the titration under the exclusion of atmospheric moisture. The combined and pyridine-free Karl Fischer reagent (Merck) was standardised prior to sample analysis. Particulate samples (lipase and preparations) were suspended in methanol (HPLC grade, Merck), while fat samples were dissolved in a chloroform (HPLC grade, Merck) / methanol (60/40) solution. The water equivalent of the Karl Fischer reagent was determined by titration of a water standard, either 5 mg/mL in methanol (Merck) or sodium tartrate dihydrate (Sigma). Solvent was added to the reaction vessel, allowed to equilibrate, and pre-titrated to end-point. The end-point was reached when there was an excess of iodine ions and the voltage reading remained below 70 mV for a specified time delay (25 seconds). Sample was then introduced and dissolved or suspended in the appropriate solvent system and titrated. The unknown water content of the sample was calculated using the weight of sample added, the titration volume and the water equivalent of the Karl Fischer reagent.

Calculations:

$$W = \frac{V.T.100}{m}$$

where W = water content (% w/w)

V = volume of reagent used for sample (mL)

m = weight of sample (mg)

T = water equivalent of reagent (mg/mL)

3.6 Determination of free fatty acid content.

The determination of free fatty acid method was based on the Standards Association of Australia method AS 2300.8.4 (1983) that involves dissolving the fat in an ethanol solution then titrating with a standardised sodium hydroxide solution.

Procedure:

A weighed sample of molten fat was added to ethanol (95 percent, Merck) and heated on a hotplate until the fat has dissolved or the mixture boiled. Phenolphthalein indicator (10g/L in 95 percent ethanol) was then added and the mixture immediately titrated with standardised 0.01 mol/L sodium hydroxide solution until a faint pink color persists for 30 seconds. Results for the percentage by mass of free fatty acid in the sample is given as percentage oleic acid.

Calculations:

$$\%\text{FFA (as oleic acid)} = \frac{T * M * 28.2}{W}$$

where T = titration volume (mL)

M = molarity of NaOH

W = weight of sample (g)

3.7 Solid fat content

The solid fat content (SFC) was measured by pulsed nuclear magnetic resonance (NMR) and was based on AOCS official method Cd-16b-93.

Procedure:

The samples were analysed using a Bruker Minispec PC120 NMR and flat thin walled 180 x 10 mm NMR tubes supplied by Bruker (Australia) Pty. Ltd. The NMR was calibrated using three different percentage solid fat standards (0, 31.0 and 74.7%) before measuring the percent solid fat content of the samples. The fat samples were melted, mixed and poured into NMR tubes to a minimum level of 2.5 cm from the bottom. The samples were then tempered according to the type of fat and held at each measuring

temperature for 30 minutes before analysing. The tempering procedures for milkfat and cocoa butter are given below. The Bruker Minispex PC120 automatically calculated the % solid fat in the sample. Samples were analysed in duplicate and the results averaged. The acceptable variation in the duplicate analysis was 0.2%. A difference greater than 0.2% between samples can be considered to be a true difference. A reference fat was also analysed on the day of analysis. Results were typically presented as a plot of % SFC versus measuring temperature to generate a melting profile.

Tempering procedure for milkfat:

1.Melt fat and pour into NMR tubes.

2.Melt and temper fat in tubes at 60°C for 30 min in a water bath or for 45 min in an incubator oven.

3. Hold tubes at 0°C for 90 min in an ice/water bath

4. Take % solid fat reading for each tube.

5. Stabilise fat for 35 min at each higher measuring temperature before taking % solid fat readings.

5. Measure % solid fat at increasing temperature intervals including 5, 10, 15, 20, 25, 30, 35 and 40°C.

Tempering procedure for cocoa butter and cocoa butter / milkfat blends (Petterson, 1986)

1. Melt fat (and mix fats if applicable) and pour into NMR tubes.

2. Melt fat in tubes at 80°C by holding in water bath for 10 min.

3. Temper fat at 60°C by holding in oven for 20 min.

4. Hold fat at 0°C for 150 min in an ice/water bath.

5. Keep pure cocoa butter at 26.5°C and cocoa butter/milkfat blends at 19°C for 40 h.

6. Hold fat at 0°C for 90 min in an ice/water bath.

7. Measure the % solid fat at temperatures of interest after stabilising the samples at each measuring temperature for 35 min. For cocoa butter measuring temperatures typically include: 20, 25, 27.5, 30, 32.5, 35, 37.5 and 40°C.

3.8 Determination of fatty acid composition

This method is based on the method by Bannon et al (1985). Fatty acids are hydrolysed from the triglycerides, converted to fatty acid methyl esters and analysed using a gas chromatograph (GC). Fatty acids are separated on the basis of chain length and degree of saturation. Results are calculated from response factors and are presented in terms of weight % unless otherwise specified. The reported results are the mean of two injections per sample. Standard deviations of less than 2% were considered acceptable. Refer to Appendix 1 for calculations and a typical chromatogram.

Procedure:

Sample preparation

A sample of melted fat (0.2 mL) was mixed with 3.8 mL hexane (Merck, HPLC grade) in a 5 ml glass vial with a teflon-lined screw cap lid. To form the methyl esters, 100 µL of 2M KOH in methanol (Merck, Analr grade) was added. The mixture was shaken vigorously by hand for one minute and allowed to react for five minutes. The reaction was terminated by adding 100µL of 2M HCl – methyl orange solution and the mixture allowed to separate for a further 30 minutes. Two layers were formed and the top solvent layer was analysed by gas chromatography (1µL injection volume).

Standard preparation

The milkfat standard used was a milkfat standard reference mixture from the Bureau of European Communities CRM164 and was prepared in the same way as for samples. See Appendix 3.8 for details of the fatty acid composition of the reference mixture, a chromatogram of the standard and calculations.

GC conditions

The samples were analysed using a Varian 3400 gas chromatograph installed with a split/splitless injector, a Varian 8100 autosampler and flame ionisation detector (FID). The column used was a 25m x 0.25µm polyamide fused silica capillary column (25QC2/BPX70 0.25, SGE, Australia). The injection port was held at 240°C, the detector was held at 280°C and an oven temperature program was used (50°C for 2 min, increased

to 160°C at 10°C per min, then increased to 180°C at 2°C per min, and finally increased to 240°C at 10°C per min with no hold). The injector was operated in split mode with a split flow ratio of 100:1. Helium was used as a carrier gas at 74.7 cm/s (2.2 mL/min) at 26 psi and the FID gases were Hydrogen (30 mL/min), Air (290 mL/min) and the make-up gas Helium (28 mL/min). Peaks were integrated using Varian Star Integration Software (Version 4, Varian Associated Inc., USA).

3.9 Determination of 2-positional fatty acid composition

The determination of the fatty acid composition at the middle (sn-2) position of the triglycerides involved using a 1,3-specific lipase to hydrolyse the fatty acids acids at the end (sn-1 and sn-3) positions to produce 2-monoglycerides. These 2-monoglycerides were then isolated by thin layer chromatography (TLC) and analysed for fatty acid composition (as in section3.8). The method described here is based on Dutta et al (1978) with some modification, it utilises an on-plate hydrolysis technique where the 1,3-specific hydrolysis, and subsequent separation of the 2-monoglycerides occurs on the same TLC plate. Modifications include; the lipase solution being made up in water (instead of buffer) and using 2',7'-dichlorofluorescein solution (instead of iodine vapour) to visualise the bands (Pan and Hammond, 1983) and separation of lipid classes using hexane:diethyl ether:formic acid, 80:20:2 (Christie, 1989).

Procedure:

Silica Gel G TLC plates (Alltech), 0.5 mm thick, 20 x 20 cm, were activated in an oven at 110°C for 1.5 hours and stored in a desiccator. The plates were pre-developed to the top of the gel using diethyl ether to remove any contaminates and a narrow band was scraped off. The plates were then air-dried ready for immediate use.

A solution of 1,3-specific Porcine pancreas lipase (133.3 U/mg protein, 16.0 U/mg solid) from ICN Biomedicals Inc. was prepared (100mg lipase in 1 mL de-ionised water). Lipase solution (0.1 mL) was applied as evenly as possible in a band 5 cm long, 2 cm from the side and bottom of the plate. Triglyceride solution (0.01 mL of a 1:1 fat:hexane mixture, approximately 5 mg) was applied over the lipase band and the plate placed

coated side down in a 40°C oven, over a dish of water to prevent over-drying of the reaction zone, for 3 minutes. The reaction was stopped by exposing the plate to hydrogen chloride vapour for 1 min in a closed TLC chamber containing concentrated hydrochloric acid in several small beakers. The acid fumes were removed from the plate using a cold hairdryer for 30 seconds. The lipid material was removed from the reaction zone by developing the plate three times to 2 cm above the reaction zone with diethyl ether. The plate was then dried and developed up to 14 cm further with a hexane:diethyl ether:formic acid (80:20:2, v/v) solution to resolve the reaction products and unreacted triglycerides. The plate was dried and sprayed with 0.1% 2',7'-dichlorofluorescein (SIGMA, #6665) in methanol solution, to expose the glyceride bands which were visualised under UV light. The order of elution of the mixture of lipid classes was: cholesterol esters, triglycerides, free fatty acids, cholesterol, diglycerides, monoglycerides and phospholipids.

The 2-monoglyceride band was scraped off and extracted three times with 2 mL aliquots of diethyl ether. The collected ether was evaporated under nitrogen and the glycerides re-dissolved in hexane (1 mL). The samples were then analysed for fatty acid composition using the procedure described in section 3.8, with an adjustment of the split flow ratio to 20:1 to allow for the diluted sample.

3.10 Determination of triglyceride composition

This method for triglyceride composition was developed based on Christie (1989). Triglycerides were separated on the basis of carbon number only. Results were calculated from relative response factors and are presented in terms of relative weight %, unless otherwise specified. The reported results are the mean of two injections per sample. Standard deviations of less than 3% were considered acceptable. . Refer to Appendix 2 for calculations and typical chromatograms.

Note: This triglyceride composition method was modified to include a lipid class analysis, with details in section 3.11 and 3.12. The particular triglyceride method used will be referred to in the experimental procedures of each chapter.

Sample preparation

Fat samples were heated at 60°C for 45 minutes or until melted. The molten fat (2 μL) was mixed with 998 μL of nonane (Merck, Analr) in a 2 mL autosampler vial with a screw top lid and septa. This prepared 0.2% v/v sample solution was ready for direct injection into the GC. Standards were prepared in the same way, with standard triglycerides made up to desired concentration in nonane.

GC conditions

The samples were analysed using a Varian 3400 gas chromatograph installed with a Septum Equipped Programmable Injector port (SPI), a Varian 8100 Autosampler and Flame Ionisation Detector (FID). Injection volume was 0.5μL. The column used was a HT-SimDist WCOT Ultimetal capillary column 5m x 0.53mm i.d. with a film thickness 0.17μm (Chrompack, The Netherlands). The detector temperature was held at 380°C, the injector port was operated in on-column mode with a temperature program (80°C initially, increased to 380°C at 250°C per min and held for 15 minutes) and a column oven temperature program was used (200°C initially, held for 1 minute, then increased to 300°C at 10°C per min, then to 380°C at 16°C per minute with no hold). Helium was used as a carrier gas at 136.0 cm/s (18 mL/min) at 5 psi and the FID gases were hydrogen (30 mL/min), and air (300 mL/min). Peaks were integrated using Varian Star Integration Software (Version 4, Varian Associated Inc., USA).

3.11 Determination of lipid classes and triglyceride composition (1)

This method for the determination of lipid classes and triglyceride composition was based on Siew and Ng (1994) for the sample preparation, with a modification of the amounts of sample, solvent, and derivatising agents used, while the separation and analysis utilises the same column as for triglyceride analysis (section 3.10), with a modified temperature program. The triglycerides were not derivatised, eluting as a separate class with peak separation as in section 3.10. Results were calculated from relative response factors and are presented in terms of weight %, unless otherwise specified. The reported results are the mean values from two duplicate injections. Standard deviations of less than 3% were considered acceptable. Refer to Appendix 3 for calculations and typical chromatograms.

Sample preparation

Fat samples were heated at 60°C for 45 minutes or until melted. A weighed amount of the fat sample (2-5mg) was placed into a 2mL glass autosampler vial. The fat was dissolved in nonane (0.7 mL) before the derivatisation agents (N,O)-bis(trimethyl silyl) trifluoroacetamide (BSTFA, 0.2mL) and trimethylchlorosilane (TMCS, 0.1mL) were added. The mixture was shaken vigorously by hand for one minute before being placed in an oven and heated at 80°C for 45 minutes. The sample was cooled to room temperature before direct injection on the GC. Duplicate injections were performed for each sample.

A standard stock solution containing a range of lipid classes and triglycerides was prepared in nonane. Lipid class standards were derivatised in the same way, the vial contained lipid class standard solution (0.2mL), BSTFA (0.2mL), TMCS (0.1mL) and nonane (0.5mL). The standards used included oleic acid, mono-olein, di-olein and triglycerides of PPO (T50), POS (T52), SSO (T54).

GC conditions

The samples were analysed using a Varian 3400 gas chromatograph installed with a Septum Equipped Programmable Injector (SPI), a Varian 8100 Autosampler and Flame Ionisation Detector (FID). Injection volume was 0.5μL. The column used was a Chrompack HT-SimDist WCOT Ultimetal capillary column (5m x 0.53mm i.d.) with a film thickness 0.17μm. The detector temperature was held at 380°C, the injector port operated in on-column mode with a temperature program (80°C initially, increased to 380°C at 250°C per min and held for 15 minutes) and an oven temperature program was used (200°C initially, held for 1 minute, then increased to 300°C at 10°C per min, then to 380°C at 16°C per minute with no hold). Helium was used as a carrier gas at 136.0 cm/s (18 mL/min) at 5 psi and the FID gases were Hydrogen (30 mL/min), Air (300 mL/min). Peaks were integrated using Varian Star Integration Software (Version 4, Varian Associated Inc., USA).

3.12 Determination of lipid classes and triglyceride composition (2)

The lipid class and triglyceride method (section 3.11) was modified to include an internal standard, based on AOCS Official Method Cd 11b-91 and using tricaprylin (T24) as the internal standard. Results were calculated from response factors and are presented in terms of weight %, unless otherwise specified. The results are reported as the mean of two duplicate injections. Standard deviations of less than 3% were considered acceptable. Refer to Appendix 4 for calculations and typical chromatograms.

Sample preparation

A stock solution of tricaprylin (SIGMA) was prepared in nonane to give a response in the same range as the lipid class standards and samples. Sample and standard preparation was carried out as in section 3.11, except for substitution of 0.1mL nonane with 0.1mL internal standard solution and the samples weight was recorded.

GC conditions

As in section 3.11.

Chapter 4

Initial interesterification and lipase studies

The work in this chapter addressed the initial part of the first aim of this research as outlined in section 2.6, which was to examine the process of solvent-free interesterification of edible fats and oils. As a result of the screening studies in this chapter, an appropriate immobilised enzyme was selected and issues with standard activity assays were identified. In the first section, the interesterification process using two commercially available immobilised lipases, Lipozyme IM and Novozym 435, was investigated. In the second section, a series of immobilisations were carried out using Lipase AY 30, with celite as the carrier, then the hydrolysis activities of the prepared immobilised lipases were measured. In the third and final section, the interesterification activities of the prepared and commercial immobilised lipases were measured and compared.

4.1 Interesterification of a milkfat and canola oil blend

The aim of this section was to investigate the enzymatic interesterification of a blend of two typical fats under solvent-free conditions, where the interesterification was between two triglyceride sources. Lipozyme IM and Novozym 435, both from Novo Nordisk A/S, Denmark, were used to interesterify a blend of anhydrous milkfat (AMF) and canola oil (1:1) in a batch reactor under solvent-free conditions. Lipozyme IM lipase is derived from a selected strain of *Mucor miehei,* from which the gene-coding for lipase has been transferred to a host organism (*Aspergillus oryzae*). It is immobilised on a macroporous anion-exchange resin. The positional specificity of Lipozyme IM is reported to be 1,3-specific (Novo Nordisk, 1992a). The stated interesterification activity expressed in terms of Batch Acidolysis Units Novo for this batch of Lipozyme IM was 7.7 BAUN/g. The typical protein content for this product according to the manufacturer is 6%.

Novozym 435 lipase is also produced by a host organism (*Aspergillus oryzae*) using the gene coding for lipase from a selected strain of *Candida antartica*. It is immobilised on a macroporous acrylic resin. The typical protein content for this product according to the manufacturer is 21%. The positional specificity of Novozym 435 is generally considered to be non-specific, but has been reported to vary depending on the reactants (Novo Nordisk,

1992b). No activity data was provided for this sample batch of Novozym. From this point on throughout the thesis, Novozym 435 will be referred to as Novozym and Lipozyme IM as Lipozyme.

A batch reactor was used for the interesterifications, consisting of a stoppered flask placed in a shaking incubator, which was used to achieve both temperature control and to facilitate continuous contact between the immobilised lipase and the fat substrate. The gentle action of the shaking incubator avoided any possible effects of a mechanical stirring system, which is not recommended for immobilised lipases (Xu et al, 2000). A reaction temperature of 60°C was selected based on the recommendations by the supplier. Samples were taken throughout the interesterification reaction and were analysed for certain chemical and physical properties to monitor changes over time.

Experimental Procedures:

A 1:1 blend (w/w) was prepared from AMF (Victorian supplier, Western district) and canola oil (high oleic variety, Victorian supplier, Northern district). The blend (100g) was interesterified according to the procedure described in section 3.1, using Lipozyme, 7.7 BAUN/g, (5g) and Novozym (5g) as supplied. Samples were taken throughout the reaction at 0, 1, 3, 5, 24 and 48 hours. Selected samples were analysed for free fatty acid content (section 3.6) and overall fatty acid composition (section 3.8). All samples were analysed for solid fat content (section 3.7 using tempering procedure for milkfat), however for clarity, not all solid fat content results are shown for each interesterification as some melting profiles were very similar.

Results:

Figure 4.1.1 Free fatty acid content results for selected samples taken during the interesterification of a blend of AMF and canola oil (1:1) using either Lipozyme or Novozym as the immobilised lipase catalyst (5%) and a reaction temperature of 60°C.
Standard deviations: FFA ± 0.1%

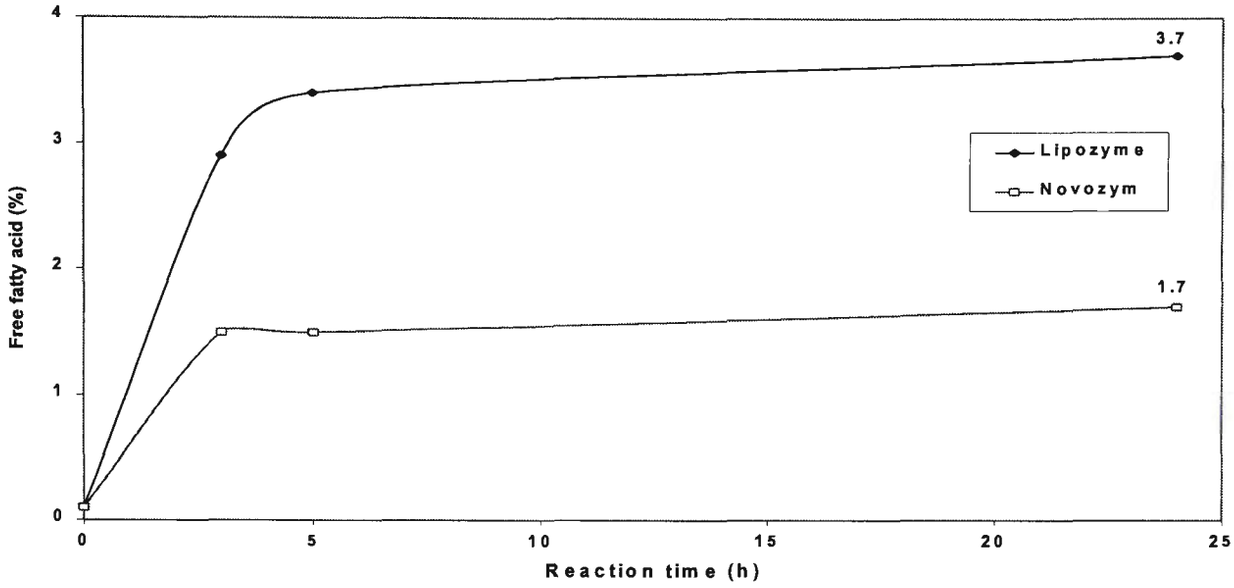


Table 4.1.1 Comparative fatty acid composition (wt%) of milkfat, canola oil, the calculated blend data, the actual blend of milkfat and canola oil (1:1), and samples taken after 3 h of interesterification of the blend using Lipozyme or Novozym as the immobilised lipase catalyst (5%) at a reaction temperature of 60°C.

Fatty Acid	AMF	Canola oil	Blend AMF:canola oil (1:1)		Lipozyme	Novozym
			Calculated	Actual	3 h	3 h
C4:0	3.98	0.00	1.99	2.20	2.07	2.27
C6:0	2.36	0.00	1.18	1.31	1.18	1.33
C8:0	1.40	0.00	1.20	0.78	0.68	0.76
C10:0	3.04	0.00	1.52	1.61	1.49	1.58
C10:1	0.28	0.00	0.14	0.15	0.13	0.14
C12:0	3.36	0.00	1.68	1.73	1.63	1.62
C14:0	10.66	0.00	5.33	5.37	5.20	5.12
C14:1	0.76	0.00	0.38	0.38	0.37	0.36
C15:0ai	0.53	0.00	0.27	0.26	0.26	0.25
C15:0	1.01	0.00	0.51	0.50	0.49	0.49
C16:0	0.19	0.00	0.10	0.10	0.09	0.09
C16:0	27.45	3.61	15.53	15.38	15.31	16.05
C16:1	1.37	0.17	0.77	0.76	0.75	0.75
C17:0iso	0.51	0.00	0.26	0.23	0.23	0.24
C17:0ai	0.37	0.07	0.22	0.19	0.18	0.17
C17:0	0.59	0.08	0.34	0.44	0.41	0.42
C17:1	0.25	0.00	0.13	0.42	0.40	0.42
C18:0	12.08	2.04	7.06	6.75	6.82	7.06
C18:1	20.01	68.75	44.38	45.60	46.93	46.56
C18:2	1.37	14.63	8.00	7.92	8.25	8.03
C18:3	0.76	6.27	3.52	3.45	3.58	3.51
C20:0	0.09	0.93	0.51	0.50	0.48	0.49
Unknown	7.59	3.44	5.52	3.99	3.07	2.29

Figure 4.1.2 Solid fat content of samples taken during the interesterification of a blend of AMF and canola oil (1:1) using Lipozyme as a catalyst at 5% under solvent-free conditions and a reaction temperature of 60°C.

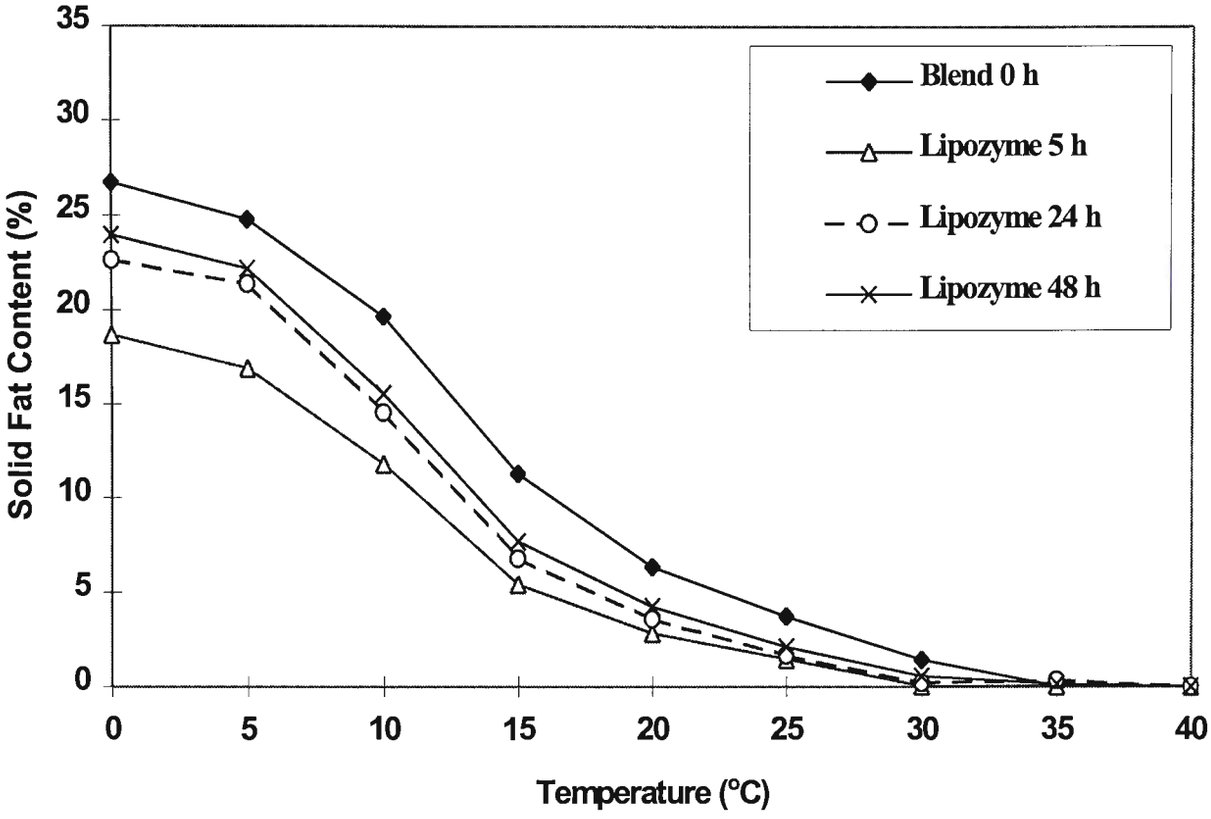
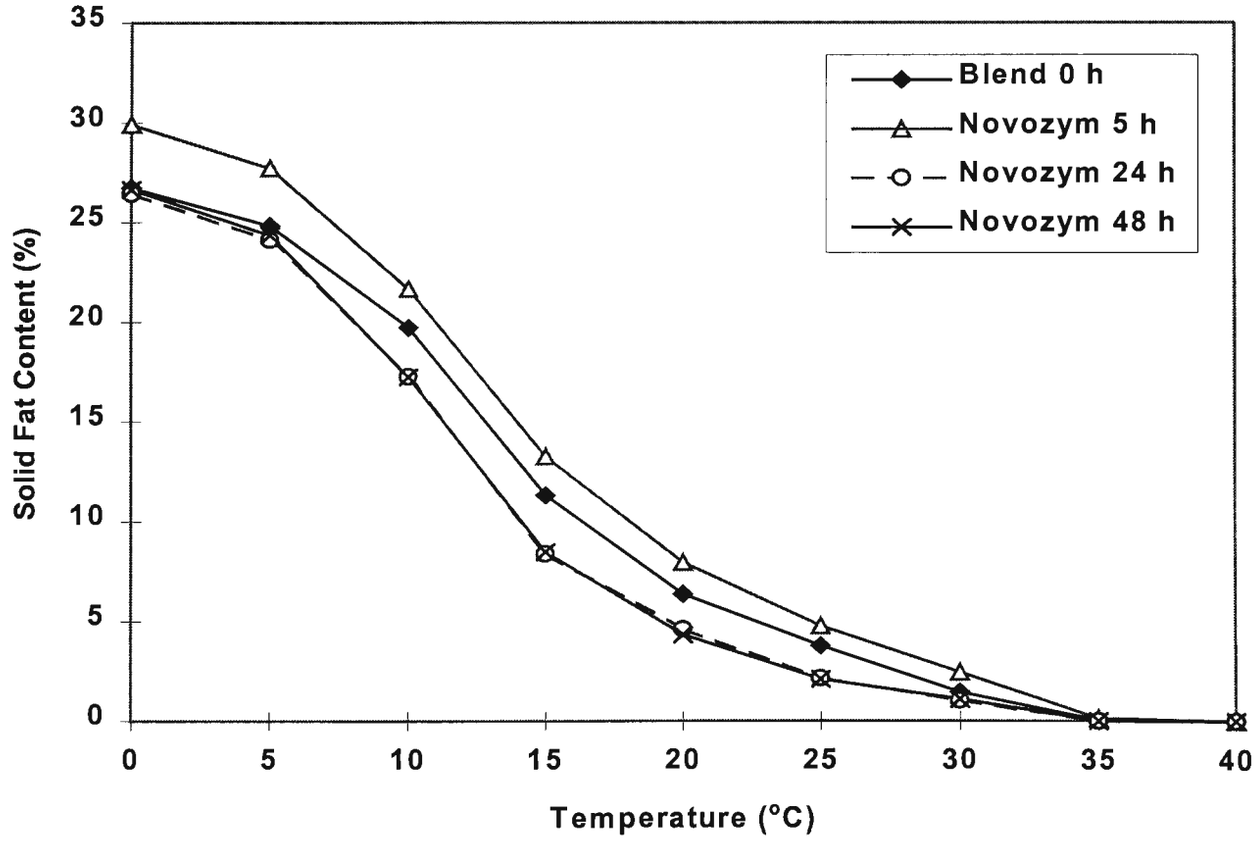


Figure 4.1.3 Solid fat content of samples taken during the interesterification of a blend of AMF and canola oil (1:1) using Novozym as a catalyst at 5% under solvent-free conditions and a reaction temperature of 60°C.



Discussion:

The overall fatty acid composition was analysed for the AMF, canola oil, the starting blend and samples that were taken after 3 hours of reaction with Lipozyme and Novozym. The actual fatty acid composition of the starting blend was typical of a 1:1 mixture of AMF and canola oil, as seen by comparison to the calculated fatty acid composition for the blend (Table 4.1.1). After 3 hours of reaction with both Lipozyme and Novozym, the overall fatty acid composition was still similar to that of the starting blend. This was to be expected, as the process of interesterification only re-arranges the position of fatty acids on the triglycerides, not the type of fatty acids (Bockisch, 1998). If, however, any particular fatty acid was being selectively removed, this would have been noticed, as the fatty acid composition method does not include free fatty acids.

During interesterification, it was expected that the free fatty acid (FFA) level would rise due to the initial hydrolysis reaction, and that a hydrolysis equilibrium would be established between the triglycerides, free fatty acids and diglycerides (Macrae, 1983). In this preliminary study, only the free fatty acid levels were analysed. The FFA content of the starting blend was low (0.1%) and increased over time for both Lipozyme and Novozym, as shown in Figure 4.1.1. For Novozym, the FFA level reached equilibrium in less than 3 hours, as the levels of FFA did not change considerably from 3 hours (1.5%) to 24 hours (1.7%). For Lipozyme, the FFA level reached equilibrium after 5 hours, where the FFA levels did not change considerably from 5 hours (3.4%) to 24 hours (3.7%). After 24 hours, the Lipozyme treated sample had a higher FFA content (3.7%) than the Novozym treated sample (1.7%).

Differences in the equilibrium levels of FFA between the two different lipase treated samples at 24 hours could be related to different lipase activities, or the initial water contents of the immobilised lipases. The water contents of the immobilised lipases and the reaction systems were not measured in these experiments. The amount of water present initially in the reaction system has been shown to effect the degree of hydrolysis and hence the level of FFA at equilibrium (Forssell et al, 1992; Foglia et at, 1993; Kalo et al, 1988b). No extra water was added to either enzyme preparation. The estimated water contents given by the manufacturer were 2-3% for Lipozyme and 1-2% for Novozym, suggesting that Lipozyme could have produced a slightly higher FFA content due to a higher initial water content. It has been

shown that Novozym requires less water than Lipozyme for catalysis, the hydrolysis reaction, when used for transesterification, with this difference attributed to the type of carrier (Lee and Akoh, 1996).

As the interesterification reaction proceeded, it was expected that the physical properties would change due to the distribution of the fatty acids among the triglycerides re-arranging. Solid fat content analysis by NMR was used to investigate how the melting properties of the interesterified samples changed over time. Melting profiles were obtained by plotting the solid fat contents over the range of measuring temperatures for the samples taken throughout the interesterification reaction using Lipozyme and Novozym (refer to Figures 4.1.2 and 4.1.3). The solid fat content results are reported as the average of two samples that have been analysed. For this method, the average standard deviation for each reading is less than 0.2% SFC. A difference greater than 0.2% between samples can be considered to be a real difference. This analysis is important for distinguishing differences in the physical properties of the fats, as the melting curves can be very different for different interesterified fats, even though the melting points may be similar, and this can be related to the triglyceride composition of the fats (Mohamed et al, 1994).

Lipozyme lowered the solid fat content of the starting blend at all measuring temperatures, with the greatest effect being achieved in the early stages up to 5 hours of reaction. After 5 hours, the solid fat contents increased, but were still lower than the starting blend. The melting profiles for samples taken at 24 and 48 hours were similar, indicating an equilibrium with respect to changes in the physical properties was close to being reached. A reduction in the solid fat content of a similar blend was found for a butterfat:sunflower oil (1:1) blend interesterified by Lipozyme under solvent-free conditions, where the solid fat content at 10°C was reduced from 26.7% to 18.5% (Foglia et al, 1993).

Interestingly, the Novozym sample taken at 5 hours of reaction had a higher solid fat content that the original blend at all measuring temperatures, while samples taken after this time had lower solid fat contents than the original untreated blend. The melting profiles for samples taken at 24 and 48 hours were almost identical, indicating an equilibrium with respect to changes in the physical properties had been reached by 24 hours. This initial increase in solid

fat contents for Novozym may be attributed to its random specificity as it has been suggested that random interesterification may result in higher levels of high melting triglycerides than 1,3-specific interesterification (Mohamed et al, 1993). The presence of the minor lipids may have also impacted on the physical properties of the interesterified products, in particular the diglycerides, which were not analysed in this initial study.

After 48 hours of reaction, the solid fat curves for both Novozym and Lipozyme interesterified samples were similar at measuring temperatures greater than 10°C, where interesterification had reduced the solid fat content of the blend. The similarity of the melting properties of the two end-products suggests that their triglyceride compositions may also be similar, despite the expected difference in the positional specificity of the two lipases and therefore different interesterified triglyceride composition. The positional specificity of the immobilised lipases may not be as expected under solvent-free conditions, or was only applicable to the early stages of reaction where the reaction products had quite different melting profiles.

In studies of the enzymatic interesterification of butterfat, it was found that four commercial 1,3-specific lipases, including Lipozyme, could exhibit, under certain conditions different positional specificity (Safari and Kermasha, 1994). In other studies, it was found that the interesterified products did not reflect the expected 1,3-specificity of *A. niger* and *M. miehei* (Kalo et al, 1988b) and *H. lanuginosa* and *M. miehei* (Goh et al, 1993) under conditions of specific solvents and solvent concentrations. Lai et al (2000b) also found no clear correlation between enzyme positional specificity and formed products after interesterification of a blend of tristearin and sunflower oil by different lipases.

Acyl-migration, where 1,2-diglycerides are isomerised to 1,3-diglycerides, is thought to play a major role in randomising interesterified products where a 1,3-specific interesterified composition is expected (Goh et al, 1993). This isomerisation has been mentioned in many studies as a process that competes with interesterification for the partial glyceride products (Lortie et al, 1993; Forssell et al, 1993; Xu et al, 1998a). Ergan et al (1990) suggested that while the hydrolysis reaction catalysed by Lipozyme is 1,3-specific, the reverse reaction may not be so, leading to randomisation and suggest that spontaneous isomerisation from positions 1 and 3 to position 2 is not responsible. Whether the process responsible for the eventual

randomisation of reaction products is acyl migration or a change in the positional specificity of the lipase enzyme is the subject of on-going investigations. In this initial study, the interesterification process was not studied in such detail as to make any conclusions regarding the specificity of the interesterified products from Lipozyme compared to those using Novozym.

Overall, these initial investigations have indicated using several simple techniques, that as expected, the hydrolysis equilibrium was reached before the interesterification equilibrium, as further changes to the physical properties occurred after the level of free fatty acids was relatively stable. More detailed sample information would be required to make more specific conclusions regarding the interesterification process. This section has provided an insight into the enzymatic interesterification of fats using commercially available lipases.

4.2 Lipase immobilisation and hydrolysis activity studies

Although the commercial immobilised lipases used in the previous section appeared to be suitable for further study, investigations were carried out into the appropriateness of producing a particular immobilised enzyme from a free lipase for the interesterification studies. The aim of the work in this section was to investigate an immobilisation procedure for the fixing of a lipase onto a carrier through adsorption. The procedure to immobilise an enzyme onto a carrier is as important as the choice of enzyme and carrier to the ultimate effectiveness of the immobilised lipase (Ison et al, 1994; Marangoni et al, 1993).

A type of Celite, Hyflo supercel (BDH, Poole, UK), was selected as the carrier to use as it has been found to be an effective support material for batch reactors (Wisdom et al, 1984; Kalo et al, 1990). Lipase AY 30 (Amano Pharmaceutical Co., Ltd, Japan), a non-specific lipase derived from *Candida rugosa,* was selected for study due to its acceptability as a food grade lipase and availability. The certified activity of the sample of lipase supplied was 32700 units/g, where one unit is defined as the lipolytic activity to liberate 1 μequivalent of acids per minute under standard assay conditions at pH 7.7 at 37°C.

A number of variations of a basic immobilisation method were studied. Each method involved adsorbing lipase (1g) onto a carrier (10g). The immobilised lipases were prepared in

duplicate, with one series freeze-dried and the other oven-dried. The prepared immobilised lipases were analysed for water content and hydrolysis activity. Although hydrolysis activities should not be taken as indicators of interesterification activities, the assay provides a comparison for lipase activities of the immobilised preparations (Thomas et al, 1988). An assay for measuring the interesterification activity of several immobilised lipases was also examined and is reported in the following section.

Experimental Procedures:

The basic method used to immobilise the lipase onto a carrier, is described in section 3.2. Four variations were assessed, differing in the amount of phosphate buffer used and the inclusion of a filtering step, as outlined in Table 4.2.1. The water contents of the free and immobilised lipase preparations were analysed according to the procedure described in section 3.5, using a Karl Fischer reagent with the water equivalent of 5.18 mg/mL for the oven-dried samples and 5.08 mg/mL for the freeze-dried samples. The hydrolysis activities of the free and immobilised lipase preparations were analysed according to the procedure described in section 3.3, using a standardised 0.010M NaOH solution.

Table 4.2.1 Method variations for the immobilisation of *C. rugosa* lipase onto a celite carrier.

Method	Lipase (g)	Buffer (mL)	Carrier (g)	Filtered
M1	1.0	10	10	No
M2	1.0	20	10	No
M3	1.0	30	10	No
M4	1.0	40	10	Yes

Results:

Table 4.2.2 Water contents and hydrolysis activities (U = μmole FFA/min) of prepared immobilised *C. rugosa* lipase.

Sample	Water Content* (%)	Hydrolysis Activity U/g	Adjusted for water content U/g
Free *C. rugosa* lipase	-	1190	1190
Oven-dried			
M1	15.3	94.0	111.0
M2	38.9	65.3	106.8
M3	43.2	62.3, 63.4	110.6, 111.6
M4	50.2	19.6	39.0
Freeze-dried			
M1	1.3	54.7	55.4
M2	0.5	241.0	242.2
M3	0.6	376.3	378.6
M4	0.3	10.9	10.9

*Standard deviation of 0.1% water

Discussion:

The hydrolysis activity method required the immobilised lipase to be added to an emulsion of olive oil in an aqueous solution, where any free fatty acids produced were detected through a change in the pH of the reaction medium. The rate of hydrolysis was calculated from the rate of addition of an alkali solution to maintain a constant pH. The protein contents of the free lipase and the immobilised lipase preparations were not determined; therefore the activity results are per gram of lipase preparation. These screening trials were a comparative study, where each method started with the same amount of free lipase. The lack of a protein analysis did not impact on the conclusions from this section. The hydrolysis activity for the free *C. rugosa* lipase was 1190 U/g. In the immobilised lipase preparations, the lipase was added to the carrier at a ratio of 1:10. The theoretical activity of 100% retained, active lipase was calculated to be 108.2 U/g. The variation in the method was quite small as can be estimated from the duplicate analysis results for oven-dried M3 sample, with results of 62.3 and 63.4 U/g.

The water contents for the oven-dried series were quite high for all of the methods tried, suggesting the oven-drying procedure was not optimised. The first three immobilisation methods, where the slurried solutions were not filtered, had very similar hydrolysis activities on a per gram of dry weight basis (M1;111.0, M2;106.8, M3;110.6 U/g). These values were very close to the calculated theoretical hydrolysis activity of 108.2 U/g. This could indicate that the lipase behaved as a free enzyme and was not bound to the carrier. Lipase, being a water-soluble enzyme, when placed in the substrate emulsion could have washed off, and if undamaged, returned to the active free form. Under typical interesterification reaction conditions, the aqueous phase is very small (< 1% overall) and any lipase that is associated with a carrier particle is likely to stay adsorbed rather than enter the fat phase in free form (Wisdom et al, 1984). Method 4, which included a filtration step, had a lower hydrolysis activity (39.0 U/g), which suggests that filtering removed most of the enzyme prior to the oven-drying process.

The freeze-dried immobilised lipases had very low moisture contents for all four methods (<1.5%). The hydrolysis activities increased with increasing buffer content for the unfiltered methods (M1; 54.7, M2; 241.0, M3; 376.3 U/g). Method 4, which included a filtration step,

had the lowest hydrolysis activity (10.9 U/g) compared to the unfiltered methods, again suggesting that filtering removed most of the enzyme. Using more buffer solution to create a slurry appears to increase the lipase activity on the carrier with freeze-drying. This may have enabled the lipase to contact the carrier in more places, or the slower drying process allowed the lipase to create a stronger adsorption bond to the carrier, which allows for higher activity.

It would appear that only a small proportion of the enzyme, as assessed by the activity results, is absorbed on the carrier in the liquid and that it is dependent upon the drying process. These investigations have drawn attention to some of the issues associated with the immobilisation of an enzyme to a carrier. There are many immobilisation methods available, each with different success rates and involving a variety of carriers and enzymes. This thesis, however, is not focussed on investigating immobilisation techniques and for future work a reliable, reproducible immobilised lipase will be required in bulk. Therefore, for reasons of convenience and consistent performance, a commercially available enzyme was selected for most of the studies in chapter 5 and onwards.

4.3 Interesterification activity studies

The aim of the work in this section was to investigate an interesterification activity assay that was provided by the manufacturer of Lipozyme (Novo Industri, 1986a). It involves monitoring the incorporation of palmitic acid into triolein over time under solvent-free conditions, by analysing samples taken during the interesterification for triglyceride composition using gas chromatography. This activity method was used to determine and compare the interesterification activities of three immobilised lipases at different water contents. Lipozyme, 7.7 BAUN/g, Novozym and an immobilised *C. rugosa* lipase (same batch as prepared in the previous section using method 2 and freeze-drying) were the lipases studied.

Experimental Procedures:

The initial water contents of the immobilised lipases were taken from the labelling on the packaging for the commercial lipases and previous analysis information for the immobilised *C. rugosa* and were 4% for Lipozyme and 2% for Novozym, while the *C. rugosa* preparation contained no water. The immobilised lipases were hydrated to levels of 6, 8,10 and 12 % water by adding an exact amount of water to a weighed sample of catalyst, then leaving to

absorb overnight. The Batch Interesterification Activity assay of the prepared immobilised lipase samples was carried out as described in section 3.4.

Results:

Table 4.3.1 The percentage of equilibrium palmitic acid (% eqm P_{inc}) incorporated into triglyceride and the Batch Interesterification activity (BIU/g) of samples taken over time of interesterification of palmitic acid with triolein using Novozym (10% wt/wt triglyceride) at various water contents and a reaction temperature of 60°C.

Novozym		**Reaction Time**				
		0.5 h	**1 h**	**6 h**	**24 h**	**48 h**
2% water	**% eqm P_{inc}**	3.1	15.6	**50.5**	*	*
	BIU/g	2.6	7.4	**5.5**	*	*
6% water	**% eqm P_{inc}**	2.9	2.1	*	8.1	*
	BIU/g	2.6	1.0	*	0.2	*
8% water	**% eqm P_{inc}**	1.0	1.0	*	3.6	*
	BIU/g	0.9	0.5	*	0.1	*
10% water	**% eqm P_{inc}**	*	1.0	*	5.1	24.8
	BIU/g	*	0.5	*	0.1	0.3
12% water	**% eqm P_{inc}**	*	0.8	*	5.0	8.4
	BIU/g	*	0.3	*	0.1	0.1

*not assessed

Table 4.3.2 The percentage of equilibrium palmitic acid (% eqm P_{inc}) incorporated into triglyceride and the Batch Interesterification activity (BIU/g) of samples taken over time of interesterification of palmitic acid with triolein using Lipozyme (10% wt/wt triglyceride) at various water contents and a reaction temperature of 60°C.

Lipozyme		**Reaction Time**			
		0.5 h	**1 h**	**1.5 h**	**2 h**
4% water	**% eqm P_{inc}**	29.6	**54.1**	66.4	73.2
	BIU/g	25.4	**28.2**	26.4	24.0
6% water	**% eqm P_{inc}**	35.5	**62.3**	83.8	91.6
	BIU/g	31.4	**35.0**	44.0	45.4
8% water	**% eqm P_{inc}**	42.5	**70.7**	86.8	97.0
	BIU/g	37.3	**41.6**	46.2	63.3
10% water	**% eqm P_{inc}**	37.4	**65.7**	80.0	90.0
	BIU/g	32.9	**37.7**	38.0	41.3
12% water	**% eqm P_{inc}**	43.6	**69.1**	84.1	94.4
	BIU/g	37.2	**38.4**	40.3	48.5

Table 4.3.3 The percentage of equilibrium palmitic acid (% eqm P_{inc}) incorporated into triglyceride and the Batch Interesterification activity (BIU/g) of samples taken over time of

interesterification of palmitic acid with triolein using immobilised *C. rugosa* (10% wt/wt triglyceride) at various water contents and a reaction temperature of 60°C.

Immobilised		**Reaction Time**				
C. rugosa		**0.5 h**	**1 h**	**6 h**	**24 h**	**48 h**
0% water	**% eqm P_{inc}**	0.8	*	1.2	2.0	3.5
	BIU/g	0.4	*	0.1	0.03	0.03
6% water	**% eqm P_{inc}**	6.2	6.8	14.4	**46.6**	60.1
	BIU/g	2.8	1.5	1.1	**1.1**	0.8
8% water	**% eqm P_{inc}**	7.7	11.2	24.3	**60.7**	74.4
	BIU/g	3.2	2.4	1.9	**1.4**	1.1
10% water	**% eqm P_{inc}**	12.7	18.3	31.59	**62.4**	77.0
	BIU/g	5.6	4.2	2.6	**1.6**	1.3
12% water	**% eqm P_{inc}**	15.8	22.3	34.2	**69.8**	80.2
	BIU/g	7.1	5.2	2.9	**1.9**	1.4

* not assessed

Discussion:

According to the interesterification assay (Novo Industri A/S, 1986a), the most accurate batch interesterification activity is when the percentage of palmitic acid incorporated is between 40 and 60% of the palmitic acid incorporated into triglyceride at equilibrium. In Tables 4.3.1 to 4.3.3 the interesterification assay results in bold were the results estimated to be the most accurate determination of the batch interesterification activity of each immobilised lipase.

Overall, the batch interesterification activity results for Novozym were quite low (Table 4.3.1). Increasing the water content decreased the interesterification activity of Novozym. The highest interesterification activity was recorded for Novozym with no extra water added, which reached 50% eqm P_{inc} after 6 hours and had an interesterification activity of 5.5 BIU/g.

For Lipozyme, the interesterification reaction proceeded relatively fast, with all preparations reaching 50% eqm P_{inc} after only 1 hour, and the preparations with added water achieved greater than 90% conversion after 2 hours (Table 4.3.2). Using the 1 hour reaction time results, the highest interesterification activity was 41.6 BIU/g for the Lipozyme hydrated to 8% water, while the lowest was 28.2 BIU/g for the Lipozyme used as supplied at 4% water.

The results for the immobilised *C. rugosa* preparations presented in Table 4.3.3 show that although the interesterification activity increased with increasing water content, the interesterification activities were still quite low (< 2 BIU/g after 24 hours). Without

additional water, the immobilised *C. rugosa* had very little activity (<0.5BIU/g). After 48 hours of reaction, the percent of equilibrium palmitic acid incorporated was between 60 and 80 percent for the hydrated *C. rugosa* preparations. In contrast, the same result was achieved by the Lipozyme preparations in less than 2 hours. Lipozyme showed superior interesterification activity under solvent-free conditions and was chosen as the immobilised enzyme of choice in further interesterification studies.

This method of determining the interesterification activity of immobilised lipases makes a distinction between 1,3-specific and non-specific interesterification, and assumes that this property is known, as different formulas are used for the theoretical end-points of each type (refer to section 3.4). The specificity of the lipase may not be known, however, or other factors such as acyl-migration may also play a role in the triglyceride composition of the interesterified samples. Also, when using the suggested methods for triglyceride analysis, positionally different triglyceride products with the same molecular weight cannot be distinguished from each other. For example, the triglycerides of PPO and POP (P = palmitic acid, O = oleic acid) elute at the same retention time. It was found that the Lipozyme catalysed reaction products also contained PPP (tripalmitin) as a product of interesterification, indicating that some non-specific randomisation had occurred.

As this was an initial screening investigation, no studies were carried out on the repeatability of this method, therefore no estimation of the errors can be given. The interesterification assay carried out here was not representative of the interesterification of fats and oils, as it was a measure of the exchange between a triglyceride and a fatty acid, not between triglycerides. Also, the interesterification activity determined using 'standard' substrates might not be representative of the interesterification activity in a natural fat matrix. Literature methods to monitor the 'degree' and 'rate' of transesterification and interesterification include monitoring changes in the area of particular triglyceride peaks (Chang et al, 1990; Ghazali et al, 1995; Lai et al, 1998; Zhang et al, 2000). Many of these methods, however, do not allow for discrimination between 1,3-specific and random interesterification.

A method involving a more accurate determination of the specificity of the reaction products would provide further important information for assessing the enzymatic interesterification

process. Further investigations were made into assessing the activity and behaviour of an immobilised lipase in a triglyceride:triglyceride environment using natural fats and are reported in the following chapters.

4.4 Chapter conclusions

This chapter has examined several aspects of solvent-free interesterification catalysed by immobilised lipases and addressed the initial aim of this research. Firstly, a blend of milkfat and canola oil (1:1) was interesterified using the commercially available immobilised lipases of Lipozyme and Novozym from Novo Nordisk. It was demonstrated that immobilised lipases do alter the chemical and physical properties of a blend of fats through interesterification. The overall fatty acid composition remained the same, while the FFA content increased up to an equilibrium level of about 4% within a few hours. The melting profiles from the solid fat content analysis gave an indication of the extent of changes in the melting properties over the reaction time. The melting properties initially went in opposite directions and then reached similar lower solid fat contents at all temperatures, where no further changes were observed with prolonged reaction time, thus similar physical properties were reached at reaction equilibrium.

Several issues associated with the immobilisation of a lipase onto a carrier were highlighted in investigations into an appropriate immobilisation procedure. Preparations of a *C. rugosa* lipase immobilised onto a celite carrier were made and the hydrolysis activities compared. It was concluded that in order to be able to concentrate on the purpose of the study of solvent-free enzymatic interesterifications, a commercial immobilised lipase should be used because of its availability in large quantities with consistent properties. Moreover, results obtained with commercial lipases are of immediate industry relevance.

A standard method for measuring the interesterification activities of immobilised lipases involving interesterification between a triglyceride and free fatty acid was examined. Lipozyme, Novozym and a prepared immobilised *C. rugosa* lipase were assessed at a range of water contents. It was found that Lipozyme had the highest interesterification activity and reached interesterification equilibrium in the shortest time using this assay. It was also demonstrated that the water content of the immoblised lipase can affect the interesterification

activity. It was concluded, however, that using standard methods and substrates to examine the incorporation of a free fatty acid into a triglyceride and determine the behaviour of an immobilised lipase in a real fat system was of little benefit.

Based on these initial investigations, Lipozyme was selected for use in further enzymatic interesterification studies. In the next chapter, investigations into an appropriate natural fat system for studying the interesterification process, as well as a more appropriate method for assessing and monitoring the interesterification process between triglycerides is reported.

Chapter 5

Characterisation of enzymatic interesterification using a natural fat

In chapter 4, the process of solvent-free enzymatic interesterification was investigated to a limited extent, then the hydrolysis and interesterification activities of several immobilised lipases were determined. The need to investigate the enzymatic interesterification process in a way that is relevant to a real fat system was raised. The work in this chapter addressed this need, with the selection of an appropriate fat system for further investigations and the development of an effective new way of monitoring interesterification reactions using this fat system.

An appropriate natural fat source is required for further study because the behaviour of a lipase in an actual fat system may be different to that in a system using synthesised standard triacylglycerols (Villeneuve and Foglia, 1997). A natural fat would be an ideal substrate to use for the characterisation of the enzymatic interesterification process, as the results would be much more relevant to triacylglycerol:triacylglycerol interactions in a 'real' situation.

To fully characterise the interesterification process it would be important to detect changes in the melting properties, the fatty acid distribution (triacylglycerol composition) and the amounts of each lipid class by-product: free fatty acids, monoacylglycerols and diacylglycerols. Ideally, the fat source would be readily obtainable in large quantities and not too expensive. The fat should preferably be in a solid state at room temperature, so changes in the melting properties that occurred could be monitored through solid fat content analysis. Interesterified oil may remain liquid, not exhibiting noticeable changes in its physical properties at convenient measuring temperatures above $0^{o}C$.

The natural distribution of the fatty acids should not already be random, as interesterification would not produce any detectable changes. Ideally, the fatty acid distribution should be asymmetric so that 1,3-specific interesterification can be monitored. The overall fatty acid composition of the fat should not be too complex or simple. If the fatty acid and triacylglycerol compositions were too varied, the changes due to interesterification would be difficult to determine, involving lengthy and complex analyses. On the other hand, if the fatty acid composition was too simple, for example consisting only of one molecular weight group,

changes in the triacylglycerol composition would not be detected in a triacylglycerol analysis based on molecular weight.

The natural fats that fulfill most of the requirements outlined above include palm oil, cocoa butter, milkfat, tallow and cottonseed oil. Of these, the most suitable fat for further investigation is cocoa butter. Cocoa butter has a comparatively simple fatty acid and triacylglycerol composition. In addition, it is naturally asymmetric in its fatty acid distribution.

Table 5.1 Fatty acid composition of Malaysian and Brazilian cocoa butter (Lipp and Anklam, 1998)

Fatty acid name	Symbol	Malaysian cocoa butter (area %)	Brazilian cocoa butter (area %)
Palmitic acid, 16:0	P	24.9	25.1
Stearic acid, 18:0	S	37.4	33.3
Oleic acid, 18:1	O	33.5	36.5
Linoleic acid, 18:2	Li	2.6	3.5
Linolenic acid, 18:3	Ln	0.2	0.2
Arachidic acid, 20:0	A	1.2	1.2

Table 5.2 Triacylglycerol composition of Malaysian and Brazilian cocoa butter (Chaiseri and Dimick, 1989).

Triacylglycerol (symbol)	Total carbon number	Malaysian	Brazialian
PLiP	T50	0.7	0.9
POO	T52	1.2	3.9
PLiS	T52	2.8	3.7
POP	T50	18.4	17.9
SOO	T54	2.9	6.7
SLiS	T54	2.2	3.2
POS	T52	40.0	37.1
SOS	T54	31.1	26.0
SOA	T56	0.8	0.04

The major fatty acids present in cocoa butter are palmitic, stearic and oleic acids, which make up around 95% of the total fatty acids (Table 5.1). There are slight differences in the fatty acid and triacylglycerol composition, as well as melting properties, between cocoa butters from different regions, as seen in Tables 5.1 and 5.2, which compares cocoa butter from Malaysia and Brazil (Lipp and Anklam, 1998; Chaiseri and Dimick, 1989). The triacylglycerols are mainly comprised of POP (18.4%), POS (40.0%) and SOS (31.1%), where the unsaturated fatty acid, oleic acid, is in the middle position of the triacylglycerol with a

saturated fatty acid on either side (Malaysian cocoa butter data -Table 5.2). This arrangement of the fatty acids imparts unique melting properties to cocoa butter, which is solid at room temperature and fast melting at body temperature (Lipp and Anklam, 1998). It would be expected that the narrow melting range would undergo a noticeable change after interesterification as a wider variety of triacylglycerols are formed. Therefore, the changes in the physical properties could be monitored during an interesterification reaction through changes in the solid fat profile.

The asymmetric arrangement of palmitic and stearic acids among the triacylglycerols in cocoa butter is an ideal arrangement for studying 1,3-specific and random (non-specific) interesterification. The major triacylglycerols present in cocoa butter represent three different molecular weight groups (T50, T52, T54), which would be detected as three separate peaks in a triacylglycerol chromatogram. The fatty acid exchange during interesterification could be monitored by analysing the triacylglycerol composition in terms of molecular weight groups and monitoring any changes.

The results of several investigations are reported in this chapter. Firstly, Malaysian cocoa butter was interesterified using three different immobilised lipases and the triacylglycerol composition of samples taken over time were analysed (section 5.1). The theoretical triacylglycerol compositions for fully random and 1,3-specific interesterified Malaysian cocoa butter were then calculated (section 5.2). A novel triacylglycerol reaction profile method was then developed that characterises the interesterification process, by a quantitative comparison of the triacylglycerol compositions of the samples analysed in section 5.1, to the theoretical triacylglycerol compositions calculated in section 5.2. In the final section, investigations into the suitability of other natural fats to characterise the interesterification process using the developed triacylglycerol reaction profile method are reported.

5.1 Interesterification of Malaysian cocoa butter

The work in this section aimed to investigate the interesterification of Malaysian cocoa butter catalysed by Lipozyme, Novozym and immobilised *C. rugosa* under solvent-free conditions through an analysis of the triacylglycerol composition of samples taken during the reaction to monitor any changes.

Experimental Procedures:

Deodorised Malaysian cocoa butter (Mars Confectionery, Ballarat, Victoria) was interesterified according to the procedure described in 3.1, except on a smaller scale using 8 mL reaction vials. Three interesterifications of Malaysian cocoa butter (1g) were carried out using Lipozyme, 7.7 BAUN/g, (0.1151g - 10.6% dry wt/wt fat) hydrated to 8% water, Novozym (0.1170g – 11.5% dry wt/wt fat) used as supplied at 2% water and immobilised *C. rugosa,* Lipase AY Amano, the same batch as prepared in section 4.2 using method 2 and freeze-drying (0.1042g – 9.2% dry wt/wt fat) at 12% water. Samples were taken at 0, 1, 2, 4, 24, 30, 48 and 72 hours and analysed for triacylglycerol composition (section 3.10).

Results:

Table 5.1.1 Triacylglycerol composition (relative wt%) of samples taken during interesterification of Malaysian cocoa butter (1g) using three different immobilised lipases.

	Lipozyme (10.6%) hydrated to 8% water							
TG	**0 h**	**1 h**	**2 h**	**4 h**	**24 h**	**30 h**	**48 h**	**72 h**
T48	0.40	0.33	0.34	0.47	1.37	1.74	2.02	2.13
T50	19.08	18.13	17.56	16.79	15.66	15.67	15.84	16.24
T52	45.95	46.01	45.86	45.53	43.62	42.56	42.36	42.31
T54	32.44	33.42	34.17	35.09	37.30	37.91	37.59	37.27
T56	2.13	2.11	2.07	2.11	2.05	2.11	2.18	2.04
	Novozym (11.5%) as supplied at 2% water							
TG	**0 h**	**1 h**	**2 h**	**4 h**	**24 h**	**30 h**	**48 h**	**72 h**
T48	0.40	0.47	0.67	0.92	2.00	2.20	2.30	2.37
T50	19.08	18.58	18.35	17.84	16.99	16.84	16.71	16.84
T52	45.95	45.91	45.71	45.49	43.57	42.66	42.54	42.42
T54	32.44	32.90	33.13	33.62	35.38	36.25	36.39	36.33
T56	2.13	2.14	2.14	2.12	2.06	2.05	2.05	2.04
	C. *rugosa* (9.2%) hydrated to 12 % water							
TG	**0 h**	**1 h**	**2 h**	**4 h**	**24 h**	**30 h**	**48 h**	**72 h**
T48	0.40	0.63	0.83	0.87	1.42	1.47	1.63	1.75
T50	19.08	16.28	16.07	15.80	15.43	15.64	15.47	16.01
T52	45.95	44.40	44.09	44.08	42.91	42.88	42.70	42.72
T54	32.44	36.27	36.48	36.89	37.66	37.49	37.61	37.13
T56	2.13	2.41	2.52	2.37	2.57	2.52	2.59	2.38

Discussion:

The results in Table 5.1.1 show that the triacylglycerol composition of the Malaysian cocoa butter changes during interesterification when catalysed by all three immobilised lipases. Lipozyme, a 1,3-specific lipase, showed an eventual increase in the proportion of the T48 (PPP), which may indicate that randomisation was occuring, as there was very little palmitic acid in the middle position of the initial triacylglycerol composition. Apart from very small changes in the proportion of each peak, the interesterification equilibrium was reached after

30 hours. Novozym, a non-specific lipase, showed a relatively stable triacylglycerol composition also after 30 hours, suggesting an equilibrium composition was reached at this time. Surprisingly, the initial changes in the triacylglycerol composition for immobilised C.*rugosa* were quite substantial, considering the relatively low batch interesterification activity measured in the model system in section 4.3. An interesterification equilibrium was perhaps reached after 24 hours for C. *rugosa*, with the level of T48 still increasing slightly. In all cases, the proportion of T56 molecular weight group remained approximately the same.

The interpretation of the triacylglycerol results in table form is limited. It can be determined that some changes have occurred in the triacylglycerol composition and an estimation made as to when an equilibrium triacylglycerol composition has been reached. It should also be noted that the triacylglycerol composition was analysed in groups of molecular weight and therefore any rearrangements between fatty acids of the same molecular weight were not detected using this analysis. This work, however, has provided some useful information regarding the changes in the triacylglycerol composition of Malaysian cocoa butter during enzymatic interesterification.

In order to assess whether or not the interesterification has reached completion, an end-point triacylglycerol composition has to be known. This would involve calculating the theoretical triacylglycerol composition for fully random and 1,3-specific interesterified Malaysian cocoa butter. This information could be used to provide further insight as to the progress of the interesterification process at each sampling time. The next section details the calculation of the theoretical triacylglycerol compositions of interesterified Malaysian cocoa butter.

5.2 Determination of theoretical triacylglycerol compositions for completely interesterified Malaysian cocoa butter

The theoretical triacylglycerol compositions at interesterification equilibrium for random and 1,3-specific interesterification can be calculated from the overall and 2-positional fatty acid composition. The calculation for random interesterification requires only the initial overall fatty acid composition (mol%), it can be calculated using several equations and a computer program (Kalo, 1986b; Rozendaal and Macrae, 1997).

Equations for calculating the random interesterified triacylglycerol composition of a fat:

$$\%AAA = A^3/10\,000$$

$$\%AAB = 3A^2B/10\,000$$

$$\%ABC = 6ABC/10\,000$$

where A, B and C are the concentrations of fatty acids expressed as mol% and AAA, AAB and ABC are triacylglycerols composed of one, two and three fatty acids respectively.

For 1,3-specific interesterification, the initial overall fatty acid composition as well as the 2-positional fatty acid composition are required to calculate the theoretical equilibrium triacylglycerol composition. This calculation requires a special computer program to enter the fatty acid data, such as the BASIC program Tricalc (version 3, 1994) developed by Dr Ralph Timms. The fatty acid data can be entered in terms of weight or mole percent, with the calculations being carried out on the mole percent values. The *Interesterification* function calculates the theoretical triacylglycerol composition of random interesterified fats and requires the input of the overall fatty acid composition. The *Lipolysis* function calculates the theoretical triacylglycerol composition of 1,3-specific interesterified fats and requires the input of the overall fatty acid composition, as well as the 2-positional fatty acid composition. The theoretical interesterified triacylglycerol compositions are then given in terms of both weight and mole percent.

Experimental Procedures:

The overall and 2-positional fatty acid compositions of the Malaysian cocoa butter were analysed according to the procedures described in section 3.8 and 3.9. The Interesterification and Lipolysis functions of the Tricalc program were used to calculate the theoretical completed random and 1,3-specific interesterified triacylglycerol compositions for Malaysian cocoa butter. The program outputs are given in Appendix 5 .

Results:

Table 5.2.1 Overall and 2-positional fatty acid composition of Malaysian cocoa butter.

	Fatty acid (wt %)								
	C14:0	**C16:0**	**C16:1**	**C17:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**
Overall	0.07	24.40	0.21	0.22	37.32	33.80	2.57	0.17	1.23
2-MG's	0	2.71	0.39	0	2.16	87.55	7.02	0.18	0

Table 5.2.2 The calculated theoretical triacylglycerol compositions for fully 1,3-specific and random interesterified Malaysian cocoa butter using the Tricalc program.

	Malaysian Cocoa butter (relative wt%)	
TG	**1,3-specific**	**Random**
T48	0.57	1.88
T50	15.38	15.05
T52	45.35	41.66
T54	36.69	39.60
T56	2.00	1.81

Discussion:

The overall fatty acid composition of the Malaysian cocoa butter used, given in Table 5.2.1, was similar to that reported from the literature in Table 5.1 (Lipp and Anklam, 1998). The 2-positional fatty acid composition results in Table 5.1 show that most of the fatty acids in the middle position of the triacylglycerols are unsaturated, with oleic acid predominating.

The normalised theoretical triacylglycerol compositions calculated for completely 1,3-specific and random interesterification of Malaysian cocoa butter are shown in Table 5.2.2. There appeared to be some differences between the two triacylglycerol compositions, particularly for the T48, T52 and T54 groups. The higher level of T48 triacylglycerol group in the theoretical triacylglycerol composition for random interesterification is due to the formation of tripalmitin (PPP), which would not be produced in appreciable amounts during a 1,3-specific interesterification.

These calculated theoretical triacylglycerol compositions for interesterified Malaysian cocoa butter were used in further investigations as end-points to the interesterification reactions. They were compared to the actual triacylglycerol composition of samples taken during interesterification of Malaysian cocoa butter to discriminate between 1,3-specific and random interesterification.

5.3 Characterisation of enzymatic interesterification of Malaysian cocoa butter

In section 5.1, Malaysian cocoa butter was interesterified and the triacylglycerol composition of samples taken during the process were analysed. In section 5.2, theoretical triacylglycerol compositions were calculated for completely 1,3-specific and random interesterified Malaysian cocoa butter. This section aimed to use this information to develop a triacylglycerol reaction profile that quantifies the changes in the triacylglycerol compositions over time through a numerical comparison between the sample and theoretical triacylglycerol

compositions. The numerical data generated was plotted, so that changes in the specificity of the reaction products, as well as the rate and extent of the reaction, could be more easily interpreted.

The approach of comparing sample triacylglycerol compositions to calculated fully interesterified triacylglycerol compositions has been reported in the literature. Kalo et al (1988b) compared proportions of saturated and monoene triacylglycerols in untreated and interesterified butterfat using two different lipases with those calculated according to random distribution. The results were presented in a column graph showing four results for each triacylglycerol carbon number group. This graph only described what changes had occurred at a particular reaction time.

In a study by Phillips et al (1984), cocoa butter was used as a model fat along with other vegetable oils to demonstrate a triacylglycerol composition method using HPLC with FID detection. The cocoa butter was interesterified using a chemical catalyst, and the triacylglycerol composition analysed before and after the reaction. The final triacylglycerol compositon was compared to a calculated theoretically completely random triacylglycerol composition, in table form, to highlight the differences chemical interesterification had on the triacylglycerol composition of cocoa butter and to validate the method of analysis. It is important to note that cocoa butter was selected for use in this study to test the analytical method because it was a natural fat with a non-random triacylglycerol composition where randomising gives well-defined differences.

In this thesis, a method of comparing two triacylglycerol compositions was required. This could be done by determining a 'distance' value, or a numerical representation to compare the similarity of the two triacylglycerol compositions. For example, if the two triacylglycerol composition were exactly the same, the 'distance' between them would be zero. As the two triacylglycerol compositions become less similar, the 'distance' generated would become larger. There are a number of statistical equations that could be used to compare the two sets of triacylglycerol compositions, to generate a single number.

Statistical 'distances' such as the 'Mahalanobis Distance' equation were investigated (refer to equation 5.3.1 below), which is the sum of the residual differences between each triacylglycerol group divided by the expected, or theoretical, value for each triacylglycerol

group (Chakraborty and Rao, 1991). It was found that the 'distances' generated were quite small (<5) and that changes in the different triacylglycerol groups were not of equal significance but was related to the magnitude of the expected value. For example, an increase in the proportion of the T48 group was only a minor contributor to the overall triacylglycerol composition and the overall 'distance' value due to the relatively small size of the expected value. A small change in the proportion of T48 group, compared to any other triacylglycerol group, is however of great importance in quantifying differences between 1,3-specific and random interesterification and should have a relatively major influence on the distance value. This was not the case for the Mahalanobis Distance equation.

When the Mahalanobis 'distance' equation was simplified to a 'Sum of Squares of Residuals' equation, equal weighting was given to changes among all the triacylglycerol groups (refer to equation 5.3.2 below). The 'distances' generated were much higher, allowing small changes in the proportions of the minor triacylglycerol groups to be contribute equally to the distance value. Therefore the changes in the T48 peak could contribute more and enable the distinctions between 1,3-specific and random interesterified cocoa butter composition to be clearer. This calculation was carried out using the triacylglycerol composition data for the Malaysian cocoa butter interesterifications reported in section 5.1 and the theoretical triacylglycerol compositions calculated in section 5.2. A comparison of the sample triacylglycerol composition to the initial, or original, triacylglycerol composition of untreated Malaysian cocoa butter was also carried out. The triacylglycerol residual sum of squares 'distances' were then plotted over time to generate triacylglycerol reaction profiles for the enzymatic interesterifications (Figures 5.3.1-5.3.3).

Mahalanobis Distance (D^2)

$$D^2 = \sum_i \frac{(\rho_i - \pi_i)^2}{\pi_i} \qquad \text{.....5.3.1}$$

Where: ρ_i = the observed proportion of triacylglycerol group in sample

π_i = the theoretical proportion of triacylglycerol group in fully interesterified fat

Residual Sum of Squares (RSOS) Distance

$$RSOS = \sum_{i} (\rho_i - \pi_i)^2 \quad \text{.....5.3.2}$$

Where: ρ_i = the observed proportion of triacylglycerol group in sample

π_i = the theoretical proportion of triacylglycerol group in fully interesterified fat

Table 5.3.1 Triacylglycerol reaction profile data for the three interesterifications carried out in 5.1, using the residual sum of squares calculation to compare the sample triacylglycerol compositions, to the theoretical triacylglycerol compositions for completely 1,3-specific and random interesterified Malaysian cocoa butter, as well as a comparison to the original triacylglycerol composition of Malaysian cocoa butter.

	Triacylglycerol residual sum of squares distance							
	0 h	**1 h**	**2 h**	**4 h**	**24 h**	**30 h**	**48 h**	**72 h**
Lipozyme (10.6%) hydrated to 8% water								
Original	0.00	1.87	5.32	12.45	41.69	54.84	52.54	47.65
Random	88.20	69.02	55.80	40.43	9.82	4.08	5.31	7.33
1,3-Specific	32.16	18.76	11.42	4.60	4.09	10.74	12.10	12.75
Novozym (11.5%) as supplied at 2% water								
Original	0.00	0.47	1.14	3.41	21.24	33.60	36.46	36.50
Random	88.20	77.42	70.64	59.15	25.24	15.53	14.01	14.72
1,3-Specific	32.16	24.95	21.65	15.63	9.53	12.22	12.75	14.09
***C.rugosa* (9.2%) hydrated to 12% water**								
Original	0.00	25.04	29.18	34.34	51.05	48.06	52.05	43.74
Random	88.20	21.84	18.05	14.92	6.01	6.73	5.63	8.31
1,3-Specific	32.16	2.06	2.45	2.06	7.94	7.89	9.35	9.04

Figure 5.3.1 Triacylglycerol reaction profile for Malaysian cocoa butter interesterified using Lipozyme (10.6%) hydrated to 8% water as a catalyst at 60°C. Standard deviations: Original (±0.26), 1,3-specific (±0.14) and Random (±0.30).

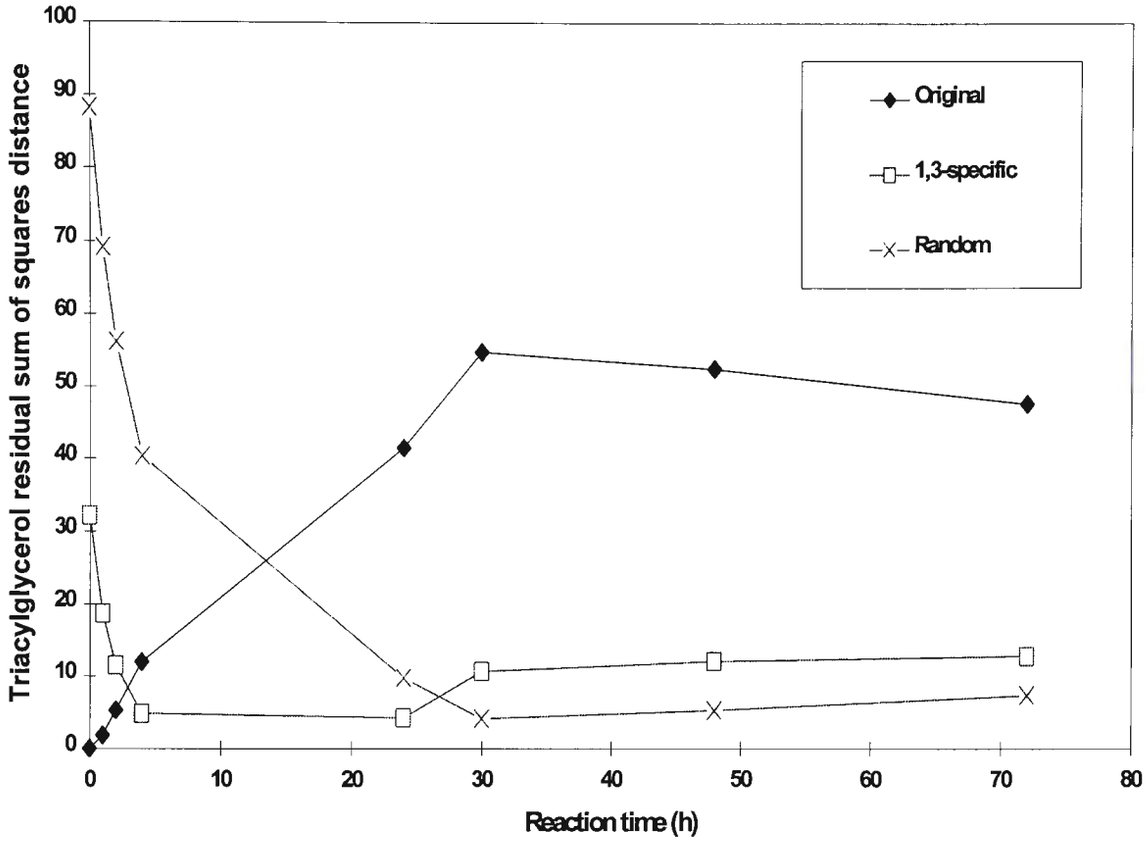


Figure 5.3.2 Triacylglycerol reaction profile for Malaysian cocoa butter interesterified using Novozym (11.5%) as supplied at 2% water as a catalyst at 60°C. Standard deviations: Original (±0.26), 1,3-specific (±0.14) and Random (±0.30).

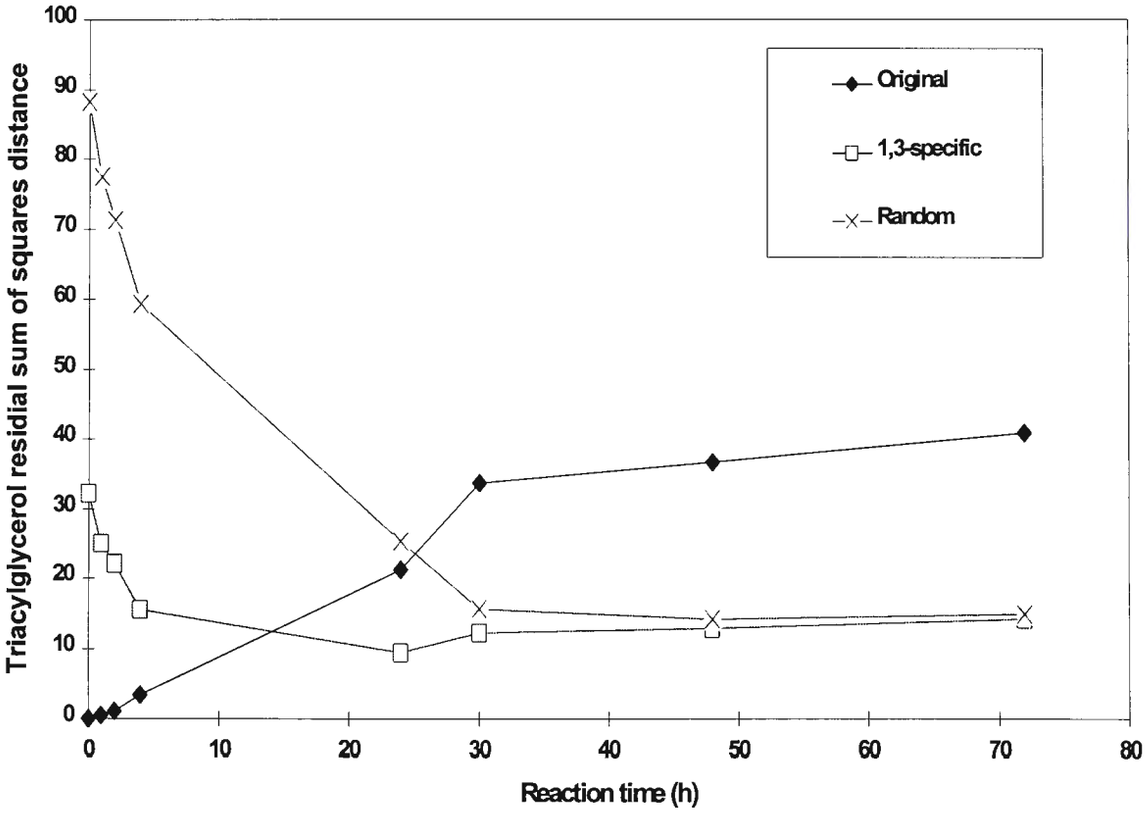
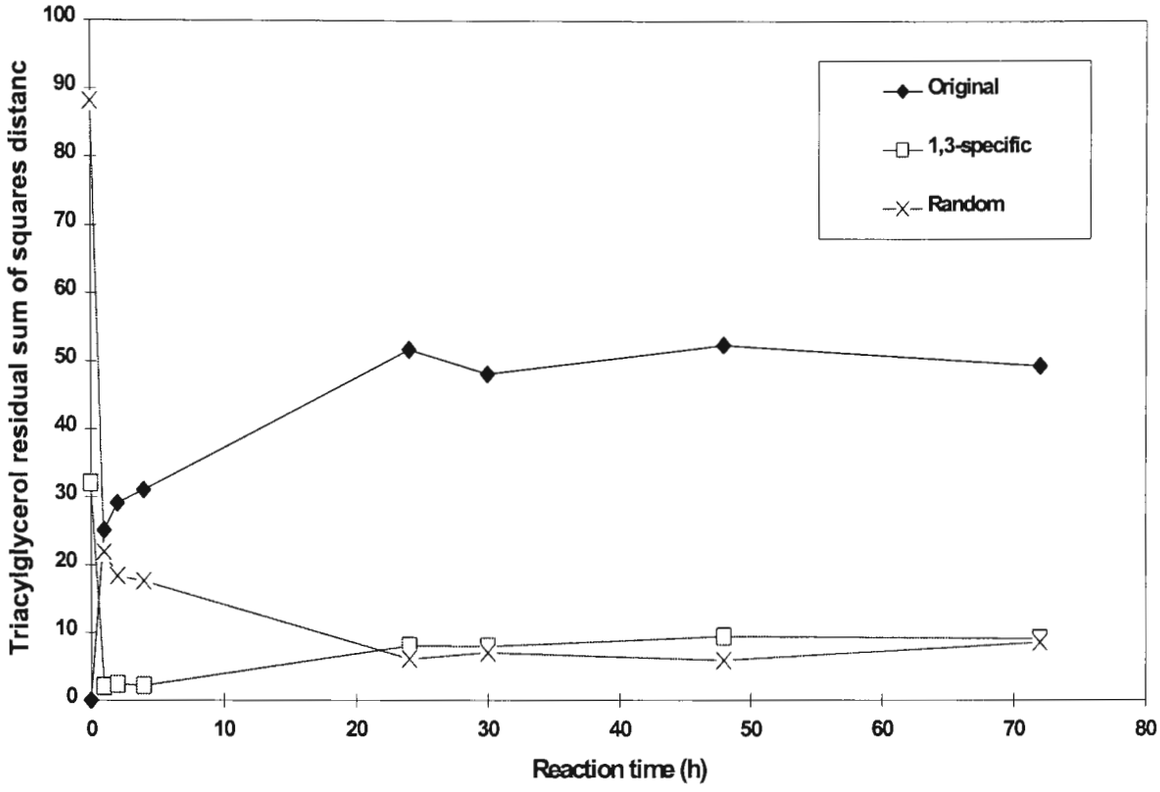


Figure 5.3.3 Triacylglycerol reaction profile for Malaysian cocoa butter interesterified using *C. rugosa* (9.2%) hydrated to 12% water as a catalyst at 60°C. Standard deviations: Original (±0.26), 1,3-specific (±0.14) and Random (±0.30).



Discussion:

Triacylglycerol reaction profiles were generated using the triacylglycerol composition data for the interesterification of Malaysian cocoa butter from section 5.1. Each triacylglycerol reaction profile consists of three distinct series, representing comparisons between the sample triacylglycerol compositions and the untreated Malaysian cocoa butter triacylglycerol composition (original), as well as the theoretical triacylglycerol compositions of completed 1,3-specific and random interesterification. The starting point (zero hour) for each triacylglycerol reaction profile series provides a measure of how similar the untreated Malaysian cocoa butter is to a 1,3-specific and random interesterified product. As random interesterification would require a greater degree of rearrangements, the random triacylglycerol reaction profile has a higher initial value or 'distance'. Accordingly, the original triacylglycerol reaction profile began at zero. The standard deviations for the triacylglycerol reaction profiles are relatively small.

The triacylglycerol reaction profile for interesterification of Malaysian cocoa butter catalysed by Lipozyme is shown in Figure 5.3.1. It can be seen that as the reaction progresses, the random triacylglycerol profile approached zero and was almost there after 30 hours. This

indicates that over time, the Malaysian cocoa butter is being 'randomised', despite Lipozyme being considered to be a 1,3-specific lipase. The 1,3-specific profile approached zero in a shorter time, within 4 hours, and remained there until 24 hours. Between 24 and 30 hours, the 1,3-specific profile increased slightly, indicating a change in the triacylglycerol composition away from a 1,3-specific product. The original profile continued to increase until 30 hours, suggesting that changes in the triacylglycerol composition were occurring until this time. From all three profiles, it appeared that an interesterification equilibrium of the triacylglycerol composition was reached after 30 hours of reaction. From this triacylglycerol reaction profile, it can be seen that a 1,3-specific interesterified product would have been obtained if the reaction were stopped after 4 hours and a random interesterified product from 30 hours onwards. This triacylglycerol reaction profile also provided some information regarding the rate of the reaction, and can be used as a basis for comparing enzymatic interesterifications of Malaysian cocoa butter catalysed under different reaction conditions.

The triacylglycerol reaction profile for interesterification of Malaysian cocoa butter catalysed by Novozym is shown in Figure 5.3.2. It can be seen that the random profile approached zero over time, but the equilibrium values were slightly higher than those for Lipozyme. The 1,3-specific profile also did not approach zero as much as it did for Lipozyme. The original profile continued to increase up to 72 hours of reaction, although the increases were much lower after 30 hours. It appears that the reaction was close to interesterification equilibrium after 30 hours. The triacylglycerol reaction profile also showed that it would not be possible to obtain a 1,3-specific interesterified product using Novozym as a catalyst under these conditions.

The triacylglycerol reaction profile for interesterification of Malaysian cocoa butter catalysed by immobilised *C. rugosa* is shown in Figure 5.3.3. It can be seen that the reaction proceeded quite rapidly initially, with large changes in the profiles after 1 hour of reaction. This rapid reaction rate was indicated by the triacylglycerol composition results in section 5.1 and confirmed in this triacylglycerol reaction profile. Again, this result was unexpected due to the relative low batch interesterification activity of *C. rugosa* compared to Lipozyme analysed in section 4.3. Although a 1,3-specific interesterified product was obtained within 1 hour of reaction, it can be seen that randomisation was also occurring and continued until around 24 hours, after which an interesterification equilibrium appeared to be reached.

The calculated theoretical triacylglycerol compositions for 1,3-specific and random interesterified Malaysian cocoa butter can also be compared to each other to generate an expected triacylglycerol reaction profile distance. Using the theoretical triacylglycerol composition data given in Table 5.2.2, the reaction profile distance calculated between the 1,3-specific and random interesterified triacylglycerol compositions is 24.0. Therefore, when the triacylglycerol composition of the sample is completely 1,3-specific or random interesterified, the reaction profile values should correspond to 0 and 24.0. This information could be useful in determining the reaction time at which the interesterified cocoa butter is most 1,3-specific or random.

In the triacylglycerol reaction profile for cocoa butter interesterified using Lipozyme, shown in Figure 5.3.1, the random reaction profile reached 24.0 after around 15 hours. This could mean that a 1,3-specific product was reached at this time, however there no samples taken between 4 and 24 hours to confirm this through the 1,3-specific reaction profile being 0. This concept was examined in more detail in further interesterification studies and reported in later chapters, where the differences in the melting profiles for 1,3-specific and random interesterified cocoa butter products were also explored.

The enzymes were stored at 4°C and although no checks were made on their stability over time, some degradation may have occurred resulting in variation in results. At a storage temperature of 5°C Lipozyme retains its declared activity for 1 year (Novo Nordisk A/S, 1992a). The same batch of Lipozyme was used for several more studies and is identified by the given BAUN/g activity provided by the manufacturer. However, a fresh batch of Lipozyme was used for the modelling studies in later chapters and this is mentioned in the experimental procedures. The differences in the apparent activity of the enzymes from the interesterification activities determined in Chapter 4 are a result of the different methods used. The behaviour of a lipase towards standard triacylglyerols can differ to that of a natural fat system.

The triacylglycerol reaction profile method has allowed the interesterification process to be studied in a way that distinguishes 1,3-specific from random interesterification in the same analysis. It was found that the enzymatic interesterification process, even when using supposedly 1,3-specific lipases, eventually results in a randomised product. More importantly, the various stages in the interesterification reaction can be followed for 1,3-

specific and random interesterification. This is very important for applications where a 1,3-specific product is required and for examining and controlling the process of acyl migration.

The information gained from the 'original' triacylglycerol reaction profile, which compares the interesterified samples to the untreated cocoa butter, is limited in that the original profile shows only that the triacylglycerol composition is changing and provides only an indication of how fast these changes are occurring. As such, the 'original' triacylglycerol reaction profile results will not be examined further in any detail for cocoa butter.

One other method to distinguish between 1,3-specific and random interesterification is to analyse the fatty acids at the 2-position (Sil Roy and Bhattacharyya, 1993; Thomas et al, 1988). This is time consuming and not convenient for large number of samples. It also does not give any information regarding the rate of the reaction for 1,3-specific interesterification, as it is difficult to distinguish between no reaction and fully interesterified 1,3-specific interesterification. This aspect of using the 2-positional analysis method was raised by Ferrari et al (1997), however the other methods were also employed to confirm that extensive interesterification had taken place when using Lipozyme for interesterification.

Techniques for monitoring the enzymatic interesterification varying in their ability to assess the interesterification process. In the interesterification of palm stearin and coconut oil (75:25) using Lipozyme, the degree of interesterification was measured using changes in the ratio between certain triacylglycerol peaks obtained through HPLC analysis (Zhang et al, 2000). The two triacylglycerol peaks chosen as marker peaks, representing carbon numbers 44 and 48, were chosen as because they were present in a chemically randomised product in much different amounts to the peaks of the untreated blend. This ratio was used to define the degree of interesterification, however, it was only applicable to random interesterification. The lipase used was a 1,3-specific lipase, Lipozyme, and therefore the interesterification equilibrium found related to the eventual random interesterification, with no information as to when a 1,3-specific interesterified product was achieved.

Ghazali et al (1995) monitored the degree and rate of interesterification of palm olein through the analysis of the PPP (tripalmitin) peak in a HPLC chromatogram. They also found a randomised product was obtained with extended reaction time. The enzymatic interesterification of vegetable oils with hydrogenated cottonseed oil was monitored by the

POS peak in a HPLC chromatogram (Chang et al, 1990).

These triacylglycerol reaction profiles characterised the interesterification of Malaysian cocoa butter in a way that provided information regarding the specificity of the reaction samples over time, as well as the rate and extent of the reaction. The differences found in the triacylglycerol reaction profiles between the interesterification of Malaysian cocoa butter using different immobilised lipases could be due to, or attributed to, the different specificity of the lipases used, the initial water contents, or the levels of by-products. It has been demonstrated that this triacylglycerol reaction profile technique is a useful tool for studying and characterising the interesterification process. It would allow the process to be studied catalysed using different reaction conditions. In the next section, several alternative natural fats were assessed for their suitability to characterise the enzymatic interesterification process using the triacylglycerol reaction profile technique.

5.4 Investigation of alternative natural fats for enzymatic interesterification studies

In the previous sections, the solvent-free enzymatic interesterification process was studied and characterised using Malaysian cocoa butter as a natural fat substrate. This section reports on the potential for using several alternative natural fats to characterise the enzymatic interesterification process using the triacylglycerol reaction profile method. Although cocoa butter appeared to be very suitable for further study, in reality it is unlikely that cocoa butter will be interesterified commercially. Therefore other fats were investigated as possible alternatives that may have some industrial relevance and the applicability of the triacylglycerol method to more complex fats was examined. Three fats, namely milkfat, egg yolk lipids and cottonseed oil, were screened and assessed for their suitability to be used in further enzymatic interesterification studies.

5.4.1 Milkfat

Milkfat is a complex fat, in that it contains a large range of fatty acids, from butyric acid (C4:0) to arachidic acid (C20:0), which combine to form an even larger number of different triacylglycerols ranging in carbon number from T24 to T54 (Gunstone, 1996). Milkfat has been found to have an asymmetric fatty acid distribution, particularly with respect to the short chain fatty acids (C4:0 and C6:0) which are mainly found in position 3 of the triacylglycerols (Parodi, 1982). It was anticipated that there would be enough difference between the original milkfat triacylglycerol composition and an interesterified milkfat triacylglycerol composition

to generate a triacylglycerol reaction profile and therefore characterise the interesterification reaction.

In this preliminary investigation, only the overall fatty acid composition of milkfat was determined, due to the complexity of the triacylglycerol and fatty acid composition. Therefore, only a random triacylglycerol reaction profile was generated. It has been acknowledged in the literature that stereospecific analysis of milkfat triacylglycerols is difficult (Christie et al, 1991), although it can be done with specialised separation techniques (Kermasha et al, 1993). The milkfat was interesterified under solvent-free conditions using Lipozyme and samples were taken during the reaction.

Experimental Procedures:

The anhydrous milkfat (Victorian supplier, Western district) was analysed for overall fatty acid composition (section 3.8) and the theoretical triacylglycerol composition for completed random interesterification was calculated using the Interesterification function of the Tricalc program (Version 3, 1994). The anhydrous milkfat (80g) was interesterified according to the procedure described in section 3.1 at a reaction temperature of 60°C using Lipozyme, 7.7 BAUN/g, (8.64g – 9.9% dry wt/wt fat) pre-hydrated to 8% water. Samples were taken at 0, 2, 4, 8, 24, 30 and 48 hours and analysed for triacylglycerol composition (section 3.10), after the triacylglycerols were isolated by TLC using the lipid class separation technique described in section 3.9. Triacylglycerol reaction profile values were then calculated (section 5.3).

Results:

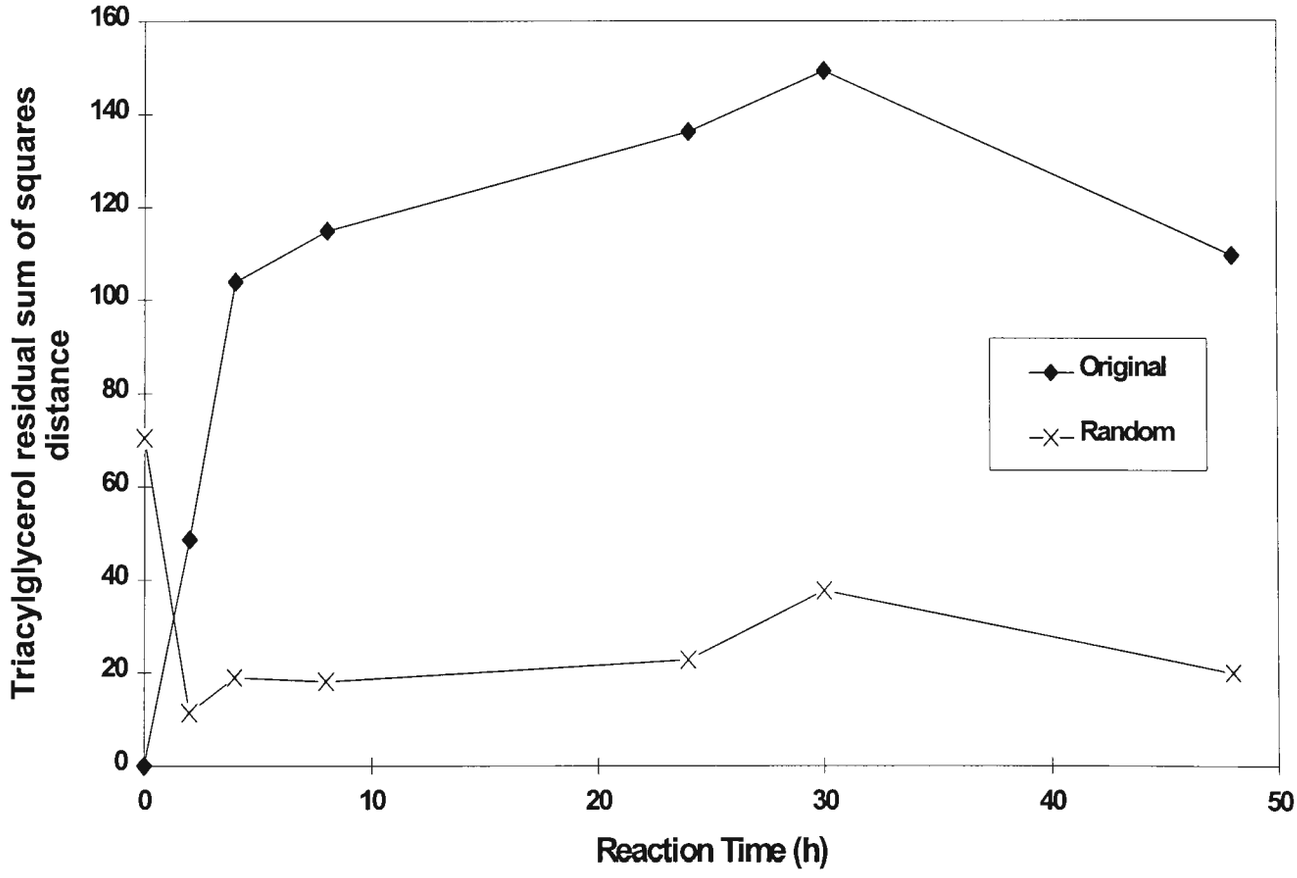
Table 5.4.1.1 The overall fatty acid composition of milkfat (wt%).

Fatty acid	%	Fatty acid	%	Fatty acid	%
C4:0	4.03	**C15:0ai**	0.52	**C17:1**	0.35
C6:0	2.24	**C15:0**	1.01	**C18:0total**	12.22
C8:0	1.29	**C16:0iso**	0.20	**C18:1**	23.23
C10:0	2.83	**C16:0total**	26.72	**C18:2ω6cis**	1.43
C10:1ω1	0.25	**C16:1**	1.35	**C18:3ω3cis**	0.78
C12:0	3.17	**C17:0iso**	0.51	**C18:2 (cla)**	1.18
C14:0	10.10	**C17:0ai**	0.37	**C20:0**	0.10
C14:1ω5	0.93	**C17:0**	0.59		

Table 5.4.1.2 The triacylglycerol composition of samples taken during the interesterification of anhydrous milkfat using Lipozyme (9.9% dry wt/wt fat) hydrated to 8% water and the theoretical triacylglycerol composition of fully random interesterified milkfat.

	Triacylglycerol composition (relative wt%)							
TG	**0 h**	**2 h**	**4 h**	**8 h**	**24 h**	**30 h**	**48 h**	**Calculated Random**
T26	0.09	0.44	0.46	0.58	0.48	0.36	0.41	1.74
T28	0.42	0.78	0.80	0.76	0.85	0.75	0.87	1.60
T30	0.90	1.49	1.36	1.36	1.32	1.11	1.39	1.71
T32	1.88	1.96	1.74	1.72	1.55	1.48	1.69	2.48
T34	4.22	3.19	2.70	2.61	2.75	2.18	2.44	3.91
T36	8.73	7.04	5.41	5.46	5.14	4.53	5.08	6.78
T38	13.15	9.98	8.39	7.96	7.52	7.02	7.87	9.15
T40	11.83	9.16	8.12	8.00	8.07	7.81	8.57	8.63
T42	7.54	6.13	6.07	6.37	6.47	6.56	6.70	6.16
T44	6.39	6.42	6.84	7.33	7.30	7.61	7.42	6.76
T46	6.81	8.48	9.35	9.98	9.90	10.39	9.99	9.08
T48	8.22	11.39	12.69	13.16	13.07	13.62	13.00	12.15
T50	11.24	14.43	15.82	15.69	15.66	16.19	15.36	14.16
T52	11.47	12.65	13.33	12.84	13.16	13.52	12.73	11.12
T54	7.10	6.48	6.48	6.17	6.57	6.88	6.48	4.55

Figure 5.4.1.1 The triacylglycerol reaction profile for the interesterification of milkfat using Lipozyme (9.9% dry wt/wt fat) hydrated to 8% water.



Discussion:

When milkfat is interesterified, as when cocoa butter is interesterified, partial glycerides by-products are formed. The diacylglycerols and monoacylglycerols that are present will co-elute with triacylglycerols of the same molecular weight (carbon number) during the triacylglycerol analysis. For example, the diacylglycerol OP (oleic:palmitic), with a carbon number of 34, would co-elute with the triacylglycerol BMP (butyric:myristic:palmitic), which also has a carbon number of 34. Therefore, in order to obtain an accurate triacylglycerol composition, the triacylglycerols were isolated by thin layer chromatography (TLC) prior to analysis by gas chromatography.

The triacylglycerol reaction profile generated followed similar trends to those generated by the interesterification of Malaysian cocoa butter. The interesterification reaction reached equilibrium after 5-8 hours and was almost completely random. Although the interesterification process could be characterised using milkfat, considerable time was involved in isolating triacylglycerols from the by-products before GC analysis. In addition, an accurate 1,3-specific interesterified triacylglycerol composition was not obtained, which is required for studies relating to the specificity of the overall interesterification process. These factors limited the use of milkfat as a suitable substrate fat, therefore milkfat was not used in subsequent interesterifications.

5.4.2 Egg yolk lipids

The triacylglycerol composition of egg yolk lipids is reported to be asymmetric between positions 1 and 3 (Couch and Saloma, 1973). In this section, the lipid content in egg yolk powder was first isolated by extracting with hexane. The recovered lipid portion was analysed for overall and 2-positional fatty acid composition and the theoretical 1,3-specific and random triacylglycerol compositions were calculated. A small scale enzymatic interesterification was carried out and samples taken over time were analysed for triacylglycerol composition and a triacylglycerol reaction profile was generated.

Experimental procedures:

Egg yolk lipids were extracted from egg yolk powder (Farm Pride, Keysborough, Victoria) using hexane (analytical grade). Egg yolk powder (20g) and hexane (200mL) were placed in a flat-bottomed round stoppered flask and stirred for at least 4 hours at room temperature (22°C). The mixture was then filtered and the hexane removed on a rotary evaporator. The

amount of lipid recovered was approximately 6g.

The egg yolk extract was analysed for overall and 2-positional fatty acid composition (sections 3.8 and 3.9). The theoretical triacylglycerol compositions for completed random and 1,3-specific interesterification were calculated using the Lipolysis and Interesterification functions of the Tricalc program (Version 3, 1994). A sample of the extracted egg yolk lipid (2g) was interesterified in an 8mL glass reaction vial, according to the procedure described in 3.1 at a reaction temperature of 60°C using Lipozyme, 7.7 BAUN/g, (0.1361g – 6.5% dry wt/wt fat) with extra water (0.0063g) added. Samples were taken at 0, 2, 6 and 24 hours and analysed for triacylglycerol composition (section 3.10). Triacylglycerol reaction profile values were then calculated (section 5.3).

Results:

Table 5.4.2.1 The overall and 2-positional fatty acid composition (wt %) of extracted egg yolk lipids.

	C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2 ω6cis	C18:3 ω3cis	C18:2 (CLA)	C20:0
Overall	0.38	23.59	3.14	0.34	0.36	7.01	50.82	12.86	0.92	0.27	0.32
2-positional	0	5.33	2.06	0	0	3.36	60.97	28.27	0	0	0

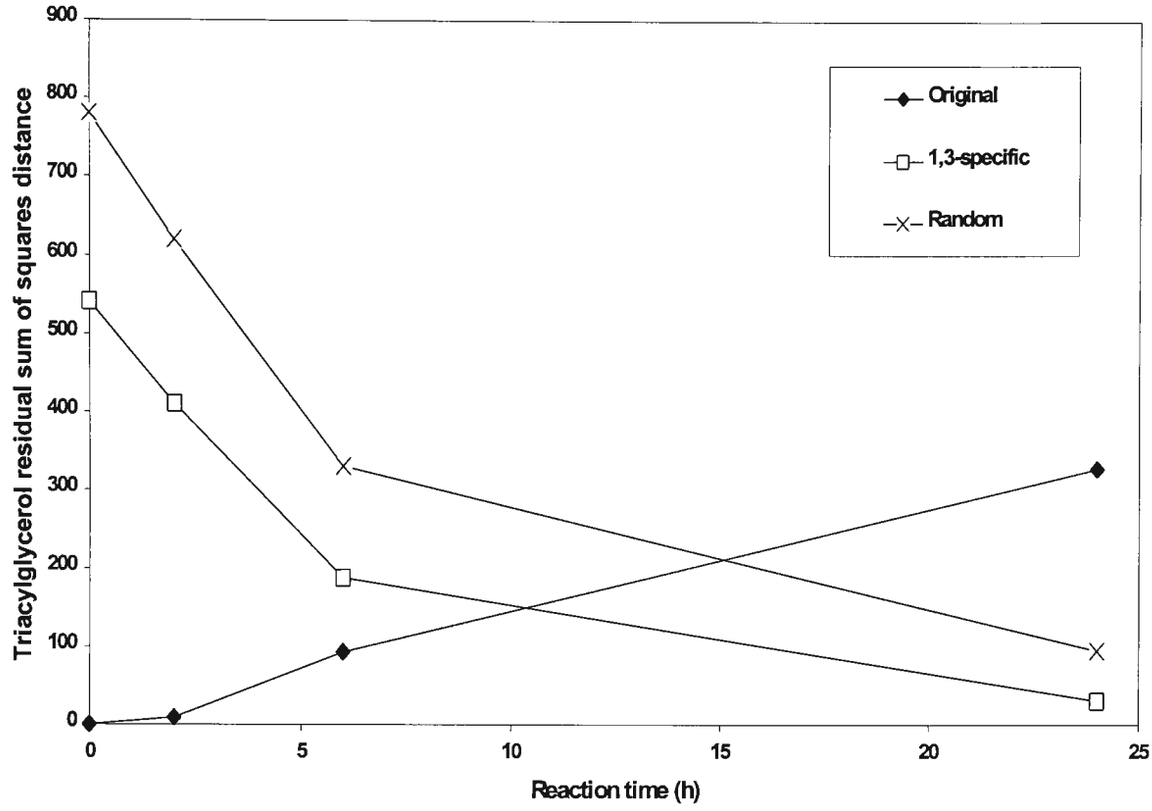
Table 5.4.2.2 Theoretically complete 1,3-specific and random interesterified egg yolk lipid triacylglycerol composition (relative wt %).

TG	Random	1,3-specific
T48	2.89	1.74
T50	18.14	18.61
T52	43.05	46.04
T54	35.48	33.13
T56	0.44	0.48

Table 5.4.2.3 Interesterified egg yolk lipid triacylglycerol results and triacylglycerol reaction profile data.

	Triacylglycerol composition (relative wt%)			
TG	**0 h**	**2 h**	**6 h**	**24 h**
T48	1.42	1.37	1.55	2.08
T50	11.11	11.71	13.01	14.95
T52	64.7	62.36	57.08	49.81
T54	21.18	22.94	26.74	30.7
T56	1.59	1.62	1.62	2.46
	Triacylglycerol reaction profile data			
	0 h	**2 h**	**6 h**	**24 h**
Original	0	8.9	92.6	327.5
Random	780.1	620.8	330.6	95.1
1,3-specific	541.0	411.3	187.5	30.8

Figure 5.4.2.1 The triacylglycerol reaction profile for the interesterification of extracted egg yolk lipids using Lipozyme (6.5%).



Discussion:

There were only relatively slight differences between the theoretical random and 1,3-specific triacylglycerol compositions and therefore the shape of the two triacylglycerol reaction profiles were similar. The triacylglycerol reaction profile distance values generated were extremely large compared to Malaysian cocoa butter, however, they followed the same general trends. In order to obtain sufficient quantities of egg yolk lipids, a large scale extraction would have to be carried out. The large scale extraction process, combined with the similarity of the random and 1,3-specific interesterified triacylglycerol compositions, limited the use of egg yolk lipids for further interesterification studies.

5.4.3 Cottonseed oil

Cottonseed oil is another fat source that has a reported asymmetry to the distribution of the fatty acids (Litchfield, 1972). The overall and 2-positional fatty acid composition of cottonseed oil was analysed and the expected triacylglycerol compositions for fully random and 1,3-specific interesterification were calculated. Cottonseed oil was enzymatically interesterified with samples taken over time, which were analysed for triacylglycerol composition, then a triacylglycerol reaction profile was generated.

Experimental Procedures:

Cottonseed oil was analysed for overall and 2-positional fatty acid composition (sections 3.8 and 3.9). The theoretical triacylglycerol compositions for completed random and 1,3-specific interesterification were calculated using the Lipolysis and Interesterification functions of the Tricalc program (version 3, 1994). The cottonseed oil (2g) was interesterified in an 8mL glass reaction vial according to the procedure described in 3.1 at a reaction temperature of 60°C, using Lipozyme, 7.7 BAUN/g, (0.1026g- 4.9% dry wt/wt fat) with extra water (0.0063g) added. Samples were taken at 0, 2, 6 and 24 hours, analysed for triacylglycerol composition (section 3.10) before triacylglycerol reaction profile distance values were calculated and plotted (section 5.3).

Results:

Table 5.4.3.1 Fatty acid composition (weight %) of cottonseed oil.

	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
Overall	0.54	21.20	0.46	2.40	17.53	57.87
2-posn	0.11	3.88	0.23	0.26	20.23	75.29

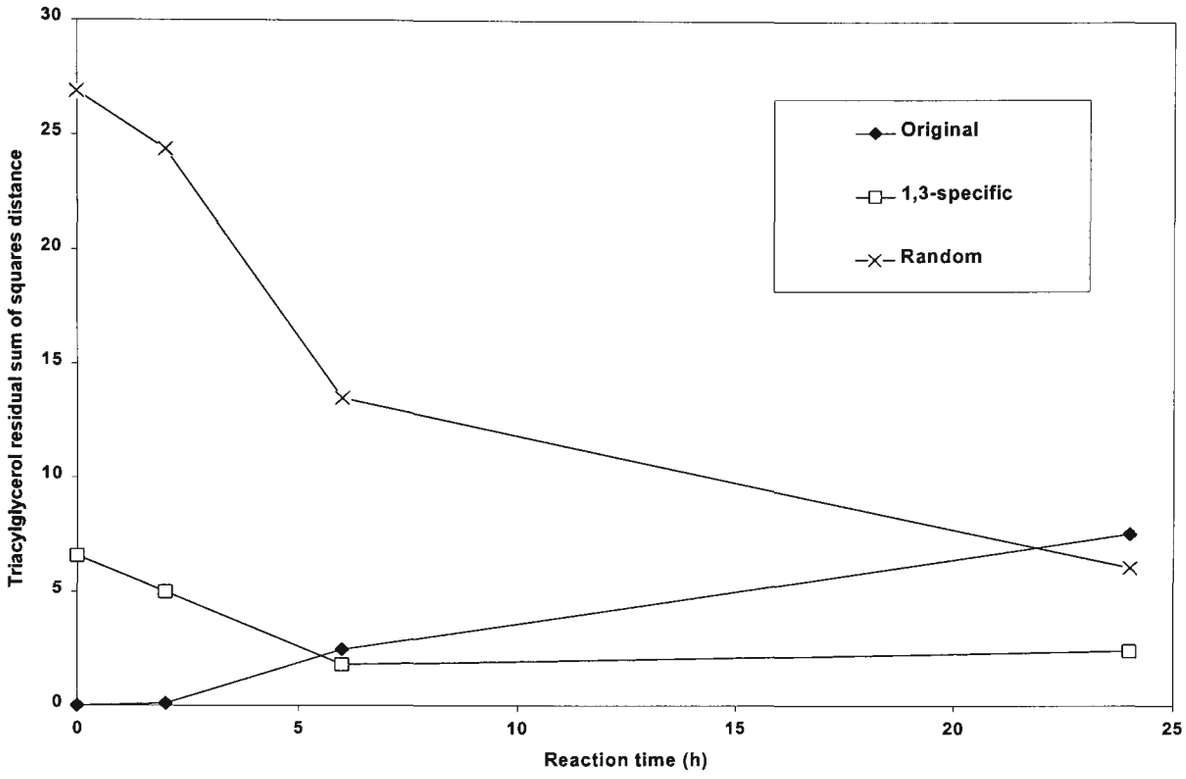
Table 5.4.3.2 Theoretically complete 1,3-specific and random interesterified cottonseed oil triacylglycerol compositions (relative weight %).

TG	Random	1,3-specific
T48	1.82	1.08
T50	12.98	12.82
T52	40.11	43.03
T54	45.08	43.07

Table 5.4.3.3 Interesterified cottonseed oil triacylglycerol composition and triacylglycerol reaction profile distance values.

	Triacylglycerol composition (relative wt%)			
TG	**0 h**	**2 h**	**6 h**	**24 h**
T48	1.15	1.11	1.23	1.50
T50	14.57	14.36	13.86	13.49
T52	43.08	43.07	42.45	41.71
T54	41.20	41.45	42.45	43.30
Triacylglycerol reaction profile distance values				
	0 h	**2 h**	**6 h**	**24 h**
Original	0.00	0.11	2.47	7.57
Random	26.87	24.37	13.51	6.09
1,3-specific	6.59	5.01	1.82	2.42

Figure 5.4.3.1 Triacylglycerol reaction profile for the interesterification of cottonseed oil using Lipozyme (4.9%).



Discussion:

From the triacylglycerol reaction profile (Figure 5.4.3.1), it can be seen that similar trends to that obtained from Malaysian cocoa butter were evident. The scale of the triacylglycerol reaction profile values were comparable to those obtained for Malaysian cocoa butter. This has shown that cottonseed oil could be used to characterise the interesterification process using the reaction profile technique. The expected changes in the physical properties of cottonseed oil are likely to be small because it is a liquid at room temperature. Therefore, cottonseed oil was not considered as suitable as cocoa butter for the substrate fat to be used for further interesterification studies.

From the fats studied in some detail, it was concluded that cocoa butter was the most suitable fat to continue to study interesterification reactions because of its ideal triacylglycerol composition and melting properties.

5.5 Chapter conclusions:

The work reported in this chapter has investigated the use of cocoa butter as a natural fat system for studying and characterising the enzymatic interesterification process under solvent-free conditions. Cocoa butter has a fatty acid composition and distribution that allows for changes in the triacylglycerols to be monitored using relatively simple techniques. The changes in the physical properties can also be assessed by measuring the solid fat contents of samples taken over time.

Malaysian cocoa butter was interesterified using several immobilised lipases, Lipozyme, Novozym and a prepared immobilised *C. rugosa* lipase. The changes in the triacylglycerol composition over time were monitored by analysing the triacylglycerol composition of samples taken during the reaction. The overall and 2-positional fatty acid compositions of Malaysian cocoa butter were determined then used to calculate the theoretical triacylglycerol compositions for completely 1,3-specific and random interesterified Malaysian cocoa butter using the Tricalc program.

A triacylglycerol reaction profile technique was developed as an effective new way to characterise and monitor the interesterification process using cocoa butter as a substrate, distinguishing between 1,3-specific and random interesterification. The triacylglycerol compositions of the interesterified samples were compared to the calculated triacylglycerol compositions for fully interesterified 1,3-specific and random interesterified cocoa butter. This was done by means of a 'sum of squares' calculation to generate a single number or 'distance value' that was representative of the likeness of the triacylglycerol compositions. The more alike the triacylglycerol compositions were, the smaller the distance value, with a value of zero resulting if the two triacylglycerol compositions were identical. A triacylglycerol reaction profile was then generated by plotting these distance values to provide information in a graphical form, relating to the specificity of the reaction products, as well as the rate and extent of the interesterification. Triacylglycerol reaction profiles were generated using the triacylglycerol compositions from the interesterification of Malaysian cocoa butter and the calculated triacylglycerol compositions for the fully 1,3-specific and random interesterified cocoa butter.

The potential of several other natural fats with different physical properties, milkfat, cottonseed oil and egg yolk lipids, for use as substrate fats to characterise the

interesterification process using the triacylglycerol reaction profile technique was also investigated. Cocoa butter was demonstrated to be the most suitable natural fat to use as a substrate in further investigations. In the next chapter, an investigation into the effects of enzymatic interesterification, as well as the presence of lipid by-products, on the physical properties of cocoa butter and other fats is reported.

Chapter 6

Investigation of changes in physical and chemical properties of fats due to enzymatic interesterification

It was demonstrated in chapter 4 that the physical properties of a milkfat and canola oil blend were altered during solvent-free enzymatic interesterification, by analysing and comparing the solid fat content of samples taken over the reaction time. The triacylglycerol reaction profile method developed in Chapter 5 characterised the changes in the triacylglycerol composition of interesterified cocoa butter and monitored the progress of the interesterification reaction. The work reported in this chapter investigated the relationship between changes in the triacylglycerol reaction profile and changes in the physical properties of the fats, as well as quantifying the levels of lipid class by-products.

The first section reports on investigations into the enzymatic interesterification of Malaysian cocoa butter, milkfat, canola oil and blends. These fats and blends were chosen because of the potential to improve their physical properties, which is of commercial relevance and although it was demonstrated in the previous chapter that the triacylglycerol reaction profile method was not as suitable for other fats, the interesterification reaction progress can be compared to that of cocoa butter. This was achieved by monitoring the changes in the physical properties through solid fat content analysis, as well as the chemical changes using the triacylglycerol reaction profile technique for the cocoa butter. In the second section, a method for the quantification of the lipid class levels is described. The third section details the effect of increasing free fatty acid levels on the solid fat curve of Malaysian cocoa butter. Consideration is given to several techniques for the removal of by-products from the interesterified products in the final section.

6.1 Effect of enzymatic interesterification on the physical properties of fats

The aim of the work in this section was to study the effect of enzymatic interesterification on the physical properties of a range of different fats. Malaysian cocoa butter, milkfat, a milkfat/canola oil (70/30) blend and a cocoa butter/milkfat (70/30) blend were interesterified using Lipozyme under solvent-free conditions. Samples were taken over time and analysed for solid fat content. The cocoa butter samples were also analysed for triacylglycerol composition then a triacylglycerol reaction profile was generated.

Experimental Procedures:

Lipozyme, 7.7 BAUN/g, was pre-hydrated to 10% water (w/w). The Malaysian cocoa butter, milkfat and canola oil were from the same batches as used in previous chapters. The milkfat/canola oil (70/30) blend and cocoa butter/milkfat (70/30) blend were prepared by melting the solid fats before combining on a weight basis. The substrate fats, in a batch size of 100g, were interesterified according to the procedure described in section 3.1, using Lipozyme (8g – 7.2% dry wt/wt fat) with a reaction temperature of 60°C. Samples were taken at 0, 2, 4, 8, 24 and 48 hours then analysed for solid fat content after the appropriate tempering procedure (section 3.7). For clarity, not all solid fat curves are shown in the figures. The cocoa butter samples were also analysed for triacylglycerol composition (section 3.10) which enabled a triacylglycerol reaction profile to be generated (section 5.3) using the theoretical triacylglycerol compositions for completely random and 1,3-specific interesterification calculated in section 5.2.

Results:

Figure 6.1.1 Solid fat content of samples taken during the interesterification of AMF using Lipozyme (7.2%) as a catalyst under solvent-free conditions.

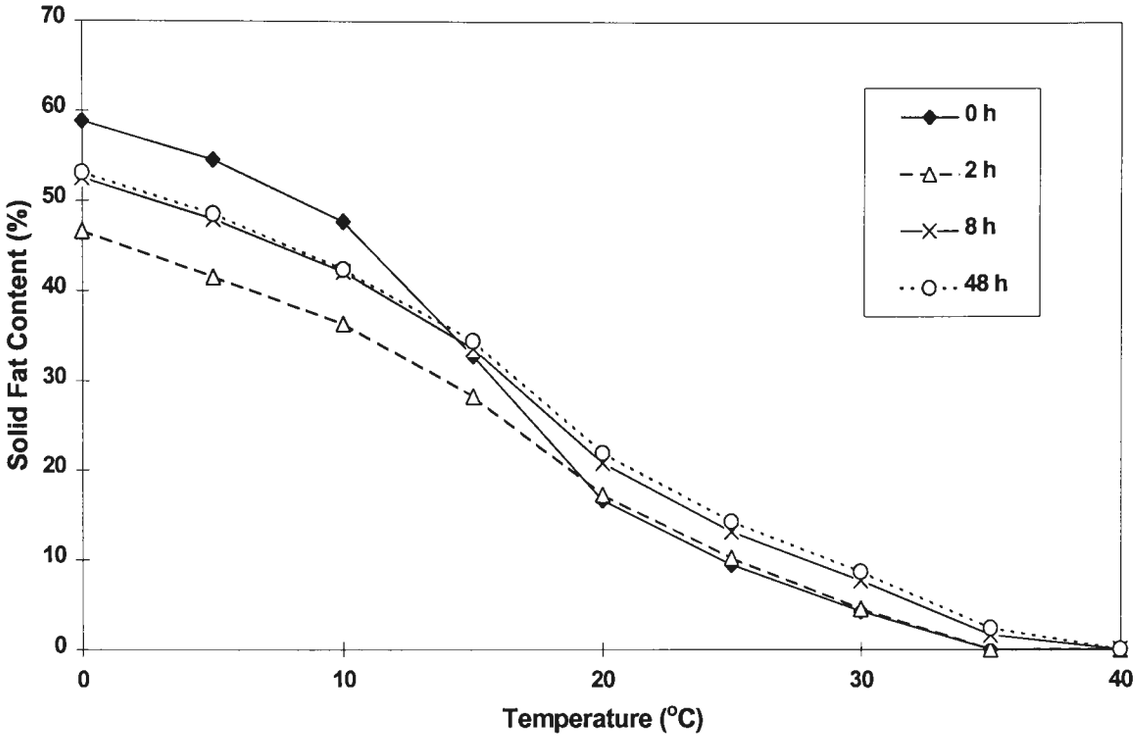


Figure 6.1.2 Solid fat content of samples taken during the interesterification of a 70:30 blend of AMF and canola oil using Lipozyme (7.2%) as a catalyst.

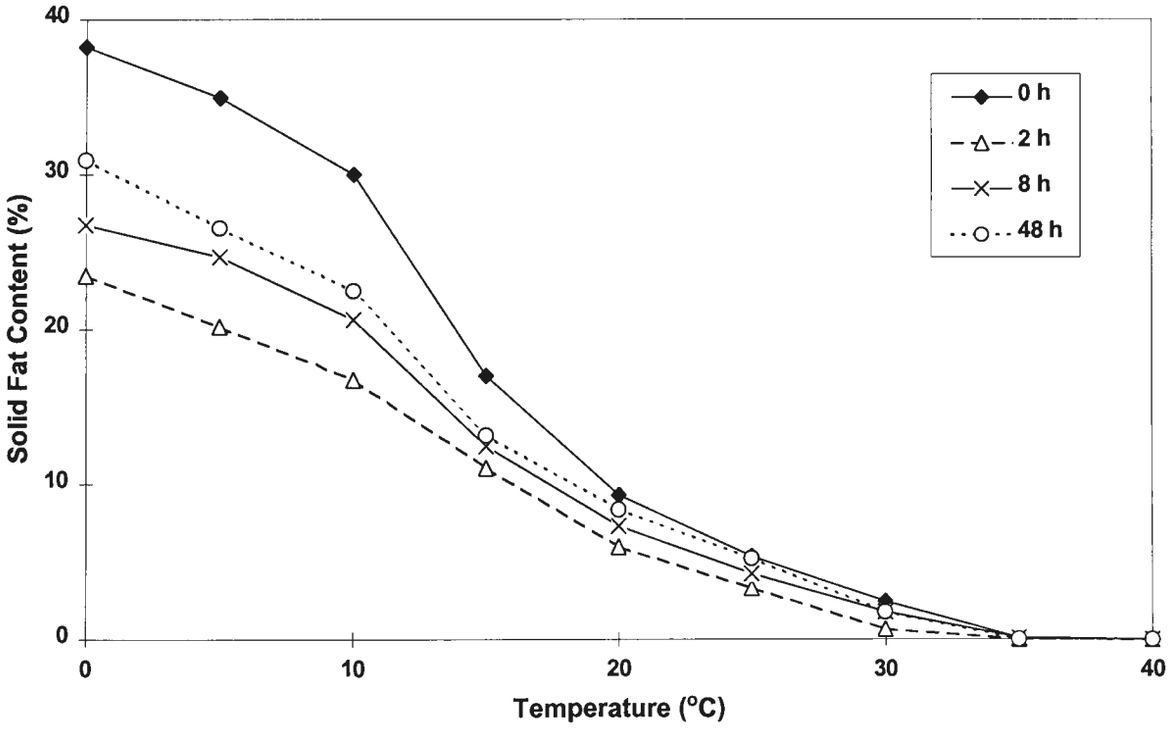


Figure 6.1.3 Solid fat content of samples taken during the interesterification of Malaysian cocoa butter using Lipozyme (7.2%) as a catalyst.

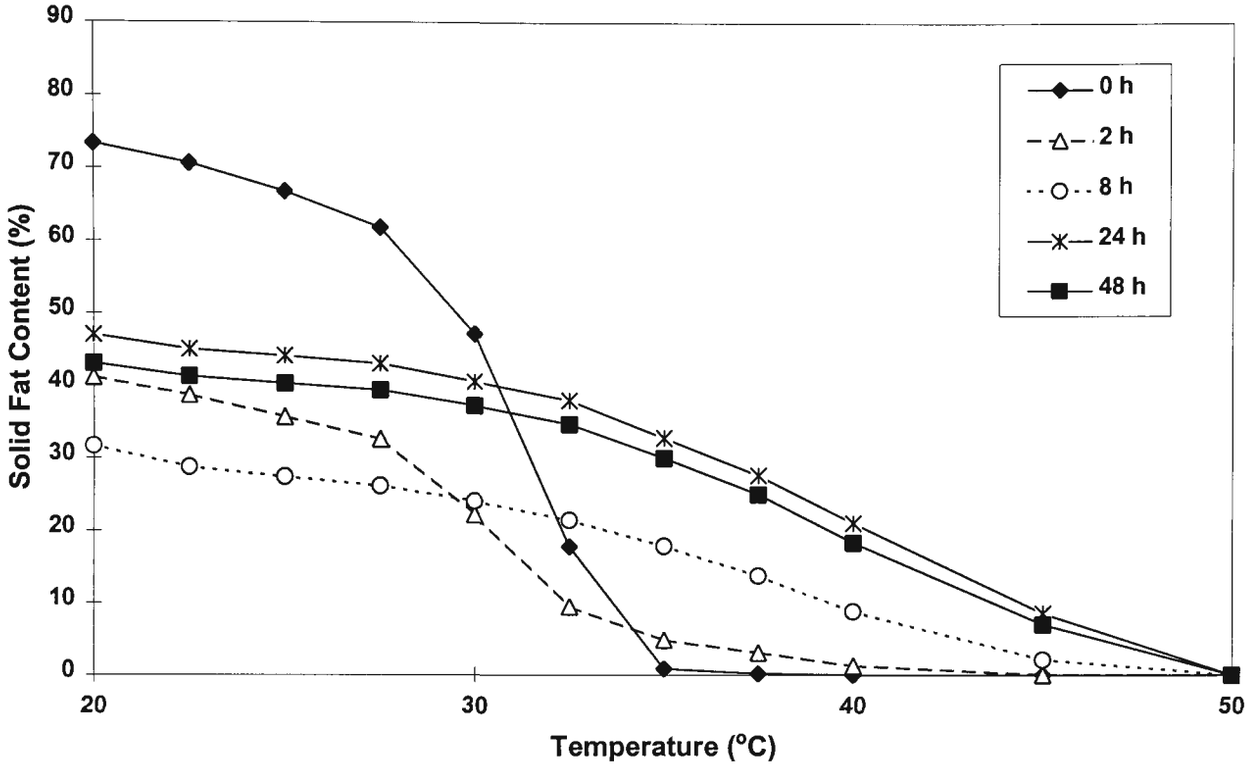


Figure 6.1.4 Solid fat content of samples taken during the interesterification of a 70:30 blend of Malaysian cocoa butter and AMF using Lipozyme (7.2%) as a catalyst.

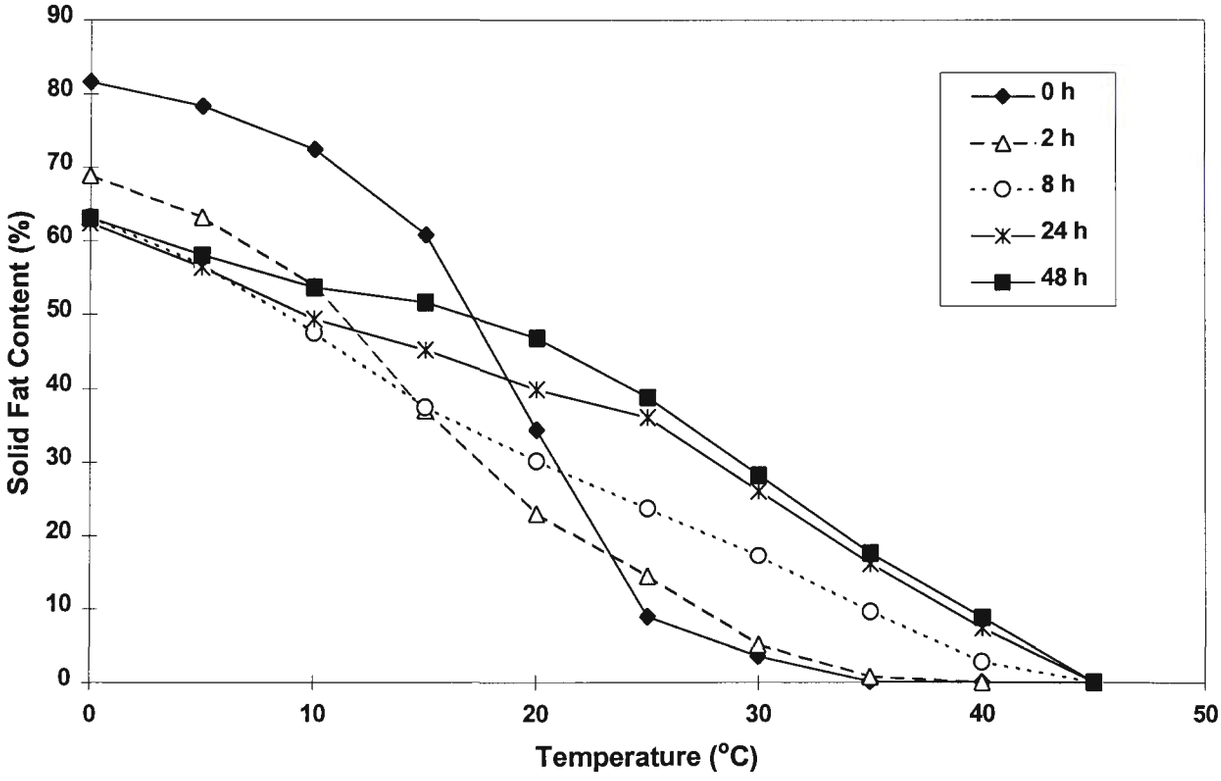
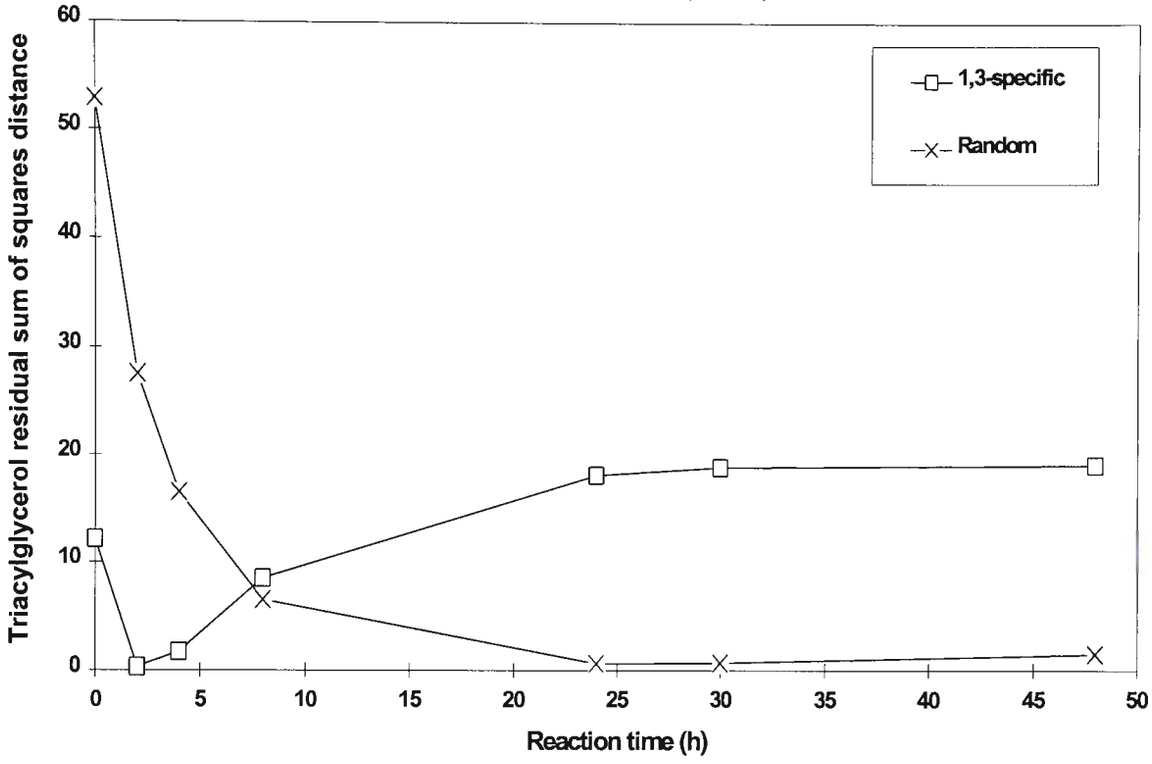


Figure 6.1.5 Triacylglycerol reaction profile of the interesterification of Malaysian cocoa butter using Lipozyme (7.2%) as a catalyst.
Standard deviations: Random (±0.25) and 1,3-specific (±0.09).



Discussion:

The solid fat content results (Figures 6.1.1-6.1.4) showed that the physical properties of all substrate fats changed substantially during the interesterification process. For AMF (Figure 6.1.1) it was evident that after 2 hours of reaction, the solid fat contents at the lower measuring temperatures, 0-15°C, were much less than those of the original AMF. The solid fat contents of the sample taken after 8 hours, however, were higher than those of the sample taken after 2 hours. The melting curves for the 8, 24 (not shown) and 48 hour samples were very similar suggesting an interesterification equilibrium was reached after 8 hours with no further changes in the physical properties occurring. The lowering of the solid fat content for milkfat at lower temperatures could result in a more spreadable product if used for a butter application.

Bornaz et al (1994) investigated the enzymatic interesterification of butter. They suggested that increases in the solid fat contents at temperatures greater than 20°C were

due to the formation of higher melting triacylglycerols, whereas the reduction in the solid fat contents for the lower temperatures may be due to the presence of minor lipid by-products. Minor lipids in milkfat:cocoa butter blends were found to affect the crystallisation and melting properties, which are important for chocolate applications (Tietz and Hartel, 2000). The effect of minor lipids on the physical properties of fats is discussed in further detail in the following sections of this chapter.

The solid fat contents of the AMF and canola oil (70/30) blend (Figure 6.1.2) were reduced at all measuring temperatures, with the largest reductions occurring at the lower measuring temperatures, less than 15°C. The greatest reduction was evident at 2 hours of reaction, with the solid fat increasing for samples taken after 8 hours. The melting curves of the 24 (not shown) and 48 hour samples were almost identical, suggesting that an interesterification equilibrium had been reached at or before 24 hours. The interesterification of a milkfat and canola oil blend could have potential application as a spreadable product that does not 'oil off'.

The solid fat contents of Malaysian cocoa butter (Figure 6.1.3) were measured from 20°C onwards, mainly in increments of 2.5°C, to observe the important changes occurring to the original sharp melting curve. Interesterification had a significant effect on the melting properties of Malaysian cocoa butter, lowering the solid fat content below 30°C and raising it at higher measuring temperatures. The melting curves of the interesterified products no longer resembled that of the original Malaysian cocoa butter. Instead of a sharp melting point, they had a long melting range. There were only small differences between the melting curves of samples taken at 24 and 48 hours, suggesting that an equilibrium composition was almost reached at 24 hours. The solid fat content method used specifically for cocoa butter, provided information at the most important temperatures for cocoa butter applications, however, it was expanded in further studies to include measuring temperatures from 0°C onwards.

The Malaysian cocoa butter and milkfat blend (70/30) solid fat content results in Figure 6.1.4 showed that the interesterified samples had a reduced solid fat content at measuring temperatures of 15°C or less, with an increased solid fat content at measuring temperatures of 25°C and above compared to the original blend. The pattern of changes in the melting curves over time was similar to those observed for Malaysian cocoa butter.

The Malaysian cocoa butter triacylglycerol composition results were used to generate a triacylglycerol reaction profile (Figure 6.1.5) that characterised the interesterification process. The 1,3-specific triacylglycerol reaction profile showed that after 2 hours, the sample was very much a 1,3-specific interesterified product, as the 'distance' was very close to zero. From 2 hours onwards, the triacylglycerol composition moved away from being 1,3-specific interesterified, with an equilibrium triacylglycerol composition reached by 24 hours. The random triacylglycerol reaction profile showed that the Malaysian cocoa butter became more randomised as the reaction continued, being almost completely random by 24 hours.

Some comparisons can be made between the changes occurring in the physical properties and the changes in the triacylglycerol composition, as illustrated by the triacylglycerol reaction profile for Malaysian cocoa butter. One interesting aspect to the changes in the physical properties of the fats investigated here was the extent of the initial reduction in solid fat content observed at 2 hours. After this time, increases in the solid fat contents were observed where the melting curve at interesterification equilibrium appeared to be somewhere in between that of the original melting curve and the melting curve for the sample at 2 hours. From the triacylglycerol reaction profile for Malaysian cocoa butter, it was seen that a 1,3-specific interesterified product was obtained after 2 hours, which was eventually randomised by 24 hours. This could mean that the physical properties of a 1,3-specific interesterified product are different to those of a random interesterified product, which may be especially true for cocoa butter. This may account for the lower solid fat contents at 2 hours due to 1,3-specific interesterification, followed by further increases, particularly at the higher measuring temperatures, as the product is randomised.

Monitoring changes in the physical properties of fats during interesterification can provide important information regarding the interesterification process. Solid fat content and crystallisation data were used to determine how efficient different enzymes were at randomising AMF:palm stearin blends (Lai et al, 2000a). They found that different lipases had different effects on melting and crystallisation, and concluded that *Pseudomonas sp.* more efficient in randomising the fatty acids than *R. miehei* lipase based on the physical property data.

This work described in this section has shown that the extent of the change in the melting curves and the rate at which the changes occurred during enzymatic interesterification was dependent on the nature of the initial fat. Care must be taken, however, when interpreting changes in the physical properties based on solid fat content analysis alone, particularly when comparing solid fat profiles of different fats (de Mann et al, 1989).

Milkfat and cocoa butter require different tempering procedures prior to solid fat content analysis because of their different polymorphic behaviour. Polymorphism is the term used to describe the different packing types of crystals within a fat structure. The three main polymorphs are alpha, beta-prime and beta, listed in order of stability and each will have a different melting point and physical properties (Chaiseri and Dimick, 1997). Fats will crystallise in the least stable form first before undergoing a phase transition, where the molecules pack more closely, to develop into a more stable configuration. For some products, only one particular crystal type, or polymorph, may be desired.

Cocoa butter is a highly polymorphic fat, due to the nature of its triacylglycerol composition. When used for chocolate, a tempering procedure is required to achieve an ideal crystal structure in the beta-prime polymorph. Over time during storage, the crystals will undergo a phase transition to the beta polymorph, which has been associated with bloom problems. Interesterification disrupts the triacylglycerol composition of fats, which may impact on the packing arrangements of the triacylglycerol crystals. The type

of crystal structure present in the interesterified cocoa butter samples may be affecting the solid fat content results due to the different melting points of the different polymorphs.

Changes in the melting and crystallisation behaviour may be better observed using differential scanning calorimetry (DSC). Differences in the heating and cooling rates, however, also effect the polymorphic forms for the same sample (Cebula and Smith, 1991). A study of polymorphism and the actual effect of the by-products on crystal types was considered beyond the scope of this thesis.

One other factor that may be influencing the solid fat content results is the presence of reaction by-products: free fatty acids, monoacylglycerols and diacylglycerols. The levels of lipid classes are important indicators of the degree of initial hydrolysis. A method for the quantification of the lipid classes was examined and reported in the next section.

6.2 Lipid class analysis

The amount of each lipid class (FFA, MG, DG, TG) at hydrolysis equilibrium of the interesterification reaction provides important information with respect to the degree of hydrolysis and yield of triacylglycerol. Separation of fats into lipid classes can be carried out using thin layer chromatography (TLC), as demonstrated in the 2-positional fatty acid composition analysis (section 3.9), thus providing a qualitative estimate of the amount of each lipid class. TLC techniques can be quantitative, via a Chromarod-Iatroscan method, where TLC is combined with flame ionisation detection (Tanaka et al, 1980). Quantitative analysis of lipid classes can also be achieved through high performance liquid chromatography (HPLC) or gas chromatography (GC) methods. A HPLC method was initially assessed based on published methods (Ergan and Andre, 1989; Ergan et al, 1991). Similar separations to the literature were achieved, however, problems were encountered due to the interesterified cocoa butter samples not being soluble in the solvent system used, so the method was not developed any further. This section describes a novel method using gas chromatography for the separation and quantification

of free fatty acids, monoacylglycerols, diacylglycerols and triacylglycerols in a single analysis.

For relatively simple fats such as cocoa butter, separation of the lipid classes can be achieved using gas chromatography where separations are based on molecular weight, as there is no overlap between lipid class groups. The triacylglycerol composition method (section 3.10) was modified by altering the column oven and injector port temperature programs to start at a lower temperature, 80°C, and increase at varying rates to 380°C. This allowed the lipid class groups of free fatty acids, monoacylglycerols and diacylglycerols to elute as discrete lipid class groups at earlier retention times than the triacylglycerols.

A derivatisation step was also included to derivatise the hydroxyl groups on the free fatty acids, monoacylglycerols and diacylglycerols to avoid peak tailing problems due to adsorption on the column (D'Alonzo et al, 1981) and the appearance of additional peaks (Khaled et al, 1993). The hydroxyl groups were derivatised using the silating agents (N,O)-bis(trimethyl silyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). BSTFA derivatisation produces derivatised glycerides and N-(trimethylsilyl) trifluoroacetamide, which is very volatile and co-elutes with the solvent causing no interference in the chromatogram.

There were several variations of the BSTFA derivatisation method in the literature (Kalo et al, 1990; Khaled et al, 1993; Kurashige et al 1993; Lee et al, 1988). These methods differed in the type of solvents and amounts of reagents used. The method reported by Siew and Ng (1994) for the analysis of lipid classes in palm oil was used as the basis for the derivatisation method described in section 3.11.

This method was ideal for the purpose of this thesis as it provided the quantification of the lipid classes during the same analysis for the triacylglycerol composition. The samples taken during the interesterification of cocoa butter in section 6.1 were analysed

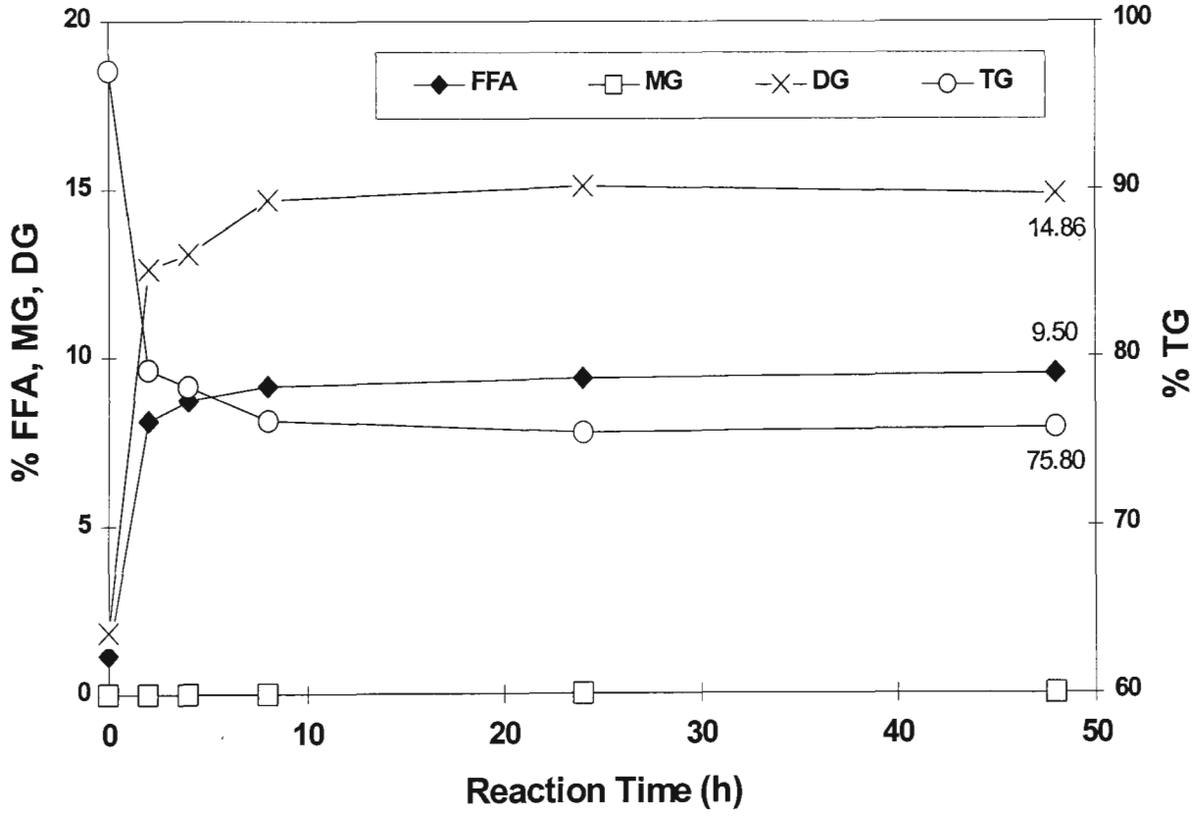
to gain an understanding of the changes in the lipid class levels throughout the interesterification process.

Results:

Table 6.2.1 Lipid class composition (relative wt %) of samples taken during the interesterification of cocoa butter using Lipozyme (7.2%) as a catalyst.
Standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

Lipid Class	0h	2h	4h	8h	24h	48h
FFA	1.13	8.11	8.71	9.11	9.37	9.50
MG	-	-	-	-	-	-
DG	1.85	12.61	13.07	14.67	15.09	14.86
TG	97.02	79.28	78.22	76.22	75.54	75.63

Figure 6.2.1 Lipid class profile of Malaysian cocoa butter interesterified using Lipozyme (7.2%) as a catalyst.



Discussion:

The changes in the lipid classes during the interesterification of cocoa butter under the conditions described in section 6.1 can be seen in Figure 6.2.1. The yield of the

triacylglycerols was around 75%, with a diacylglycerol level of nearly 15% and a free fatty acid level of almost 10%. Hydrolysis equilibrium was reached in a relatively short time, less than 8 hours, and the levels of by-products did not change significantly with prolonged reaction time. These results were similar to those of Macrae (1983), who found that most of the by-products were formed in the first hour of reaction during the transesterification of palm midfraction and stearic acid using immobilised *A. niger* lipase. It was demonstrated in studies on the interesterification of butterfat using Lipozyme in organic solvent mediums that the hydrolysis equilibrium was reached much faster than the interesterification equilibrium (Safari et al, 1993).

The monoacylglycerols were present in very small amounts, less than 0.1%, indicating that the secondary hydrolysis of diacylglycerols was limited. These results compare with the very low levels of monoacylglycerols, less than 1%, that have been reported in the literature for solvent-free interesterification (Kalo et al, 1990; Mohamed et al, 1993). The yield of triacylglycerols could be related to the water content, as the water content controls the hydrolysis equilibrium, and therefore the levels of each lipid class (Kalo et al, 1988b).

The Lipozyme used for this interesterification was pre-hydrated to 10% water, which was the recommended level for interesterification (Novo Industri, 1986b). The resulting level of free fatty acids at equilibrium of 9.5% for the interesterification of Malaysian cocoa butter, was much higher than the free fatty acid level of 3.7% found for the milkfat/canola oil blend, in section 4.1, where Lipozyme was used without additional water. This is consistent with the water content of the reaction system having an impact on the yield of interesterified triacylglycerols.

From the lipid class profile for the interesterification of cocoa butter, it can be seen that the initial change in lipid classes was significant. Comparatively, the initial changes in the physical properties and triacylglycerol composition shown in the previous section (6.1) were also significant. The increased levels of by-products may be contributing to

the initial changes in the physical properties. The effects of additional free fatty acids on the physical properties of cocoa butter were examined and are reported in the next section.

6.3 Effect of free fatty acids on the solid fat content of cocoa butter

The reaction by-products of free fatty acids and diacylglycerols may affect the physical properties of interesterified fat samples. Free fatty acids contribute to deterioration of product quality, for example through rancidity, and can be removed through a refining process (Zainal and Yusoff, 1999). In Malaysian cocoa butter, the main fatty acids present are palmitic (24.4%), stearic (37.3%) and oleic (33.8%) acid (data from section 5.2). These studies examined the effect of increased free fatty acid levels on the melting profile of Malaysian cocoa butter by adding palmitic, stearic and oleic acids to Malaysian cocoa butter individually, and as a mixture.

Experimental Procedures:

Standards of palmitic, stearic and oleic acid (Sigma) were added individually and as a one-third mixture of all three by weight to Malaysian cocoa butter at levels of 0, 2.5, 5, 7.5, 10, 20 and 30 % (wt/wt). All samples were analysed for solid fat content (section 3.7) using the tempering procedure for cocoa butter, although not all results are shown for clarity.

Results:

Figure 6.3.1 Solid fat content of Malaysian cocoa butter samples with palmitic acid.

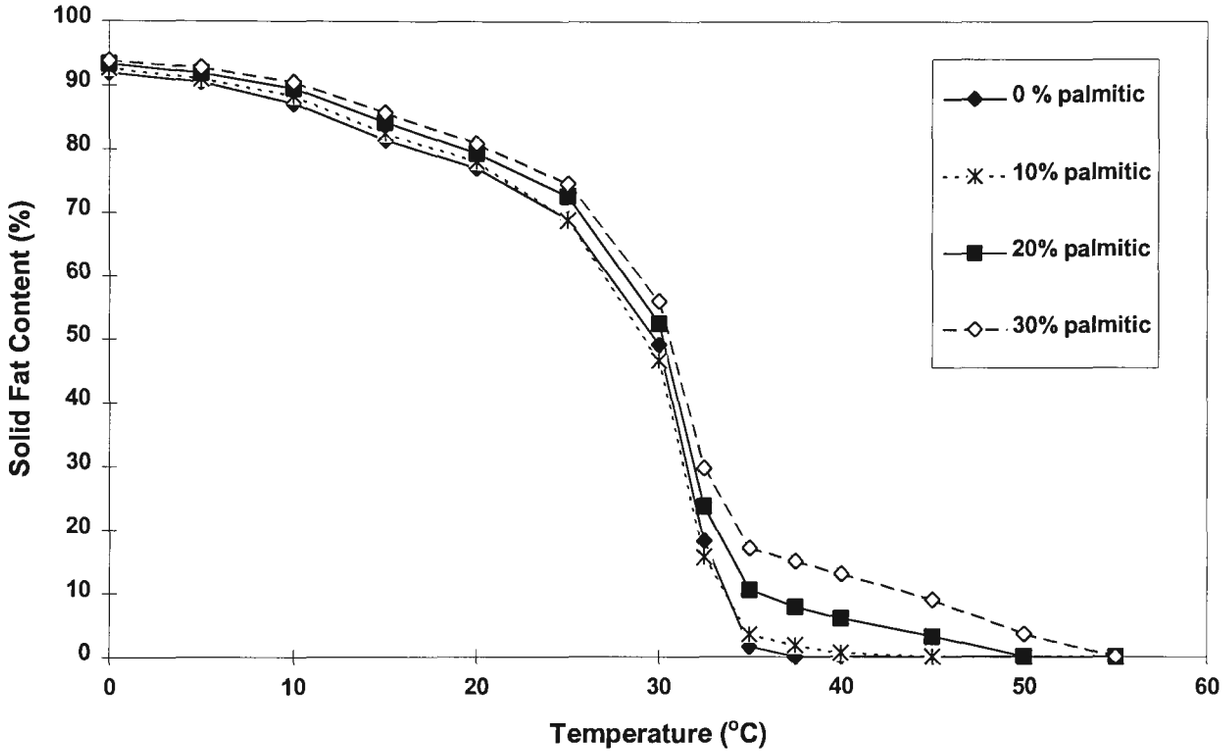


Figure 6.3.2 Solid fat content of Malaysian cocoa butter with added stearic acid.

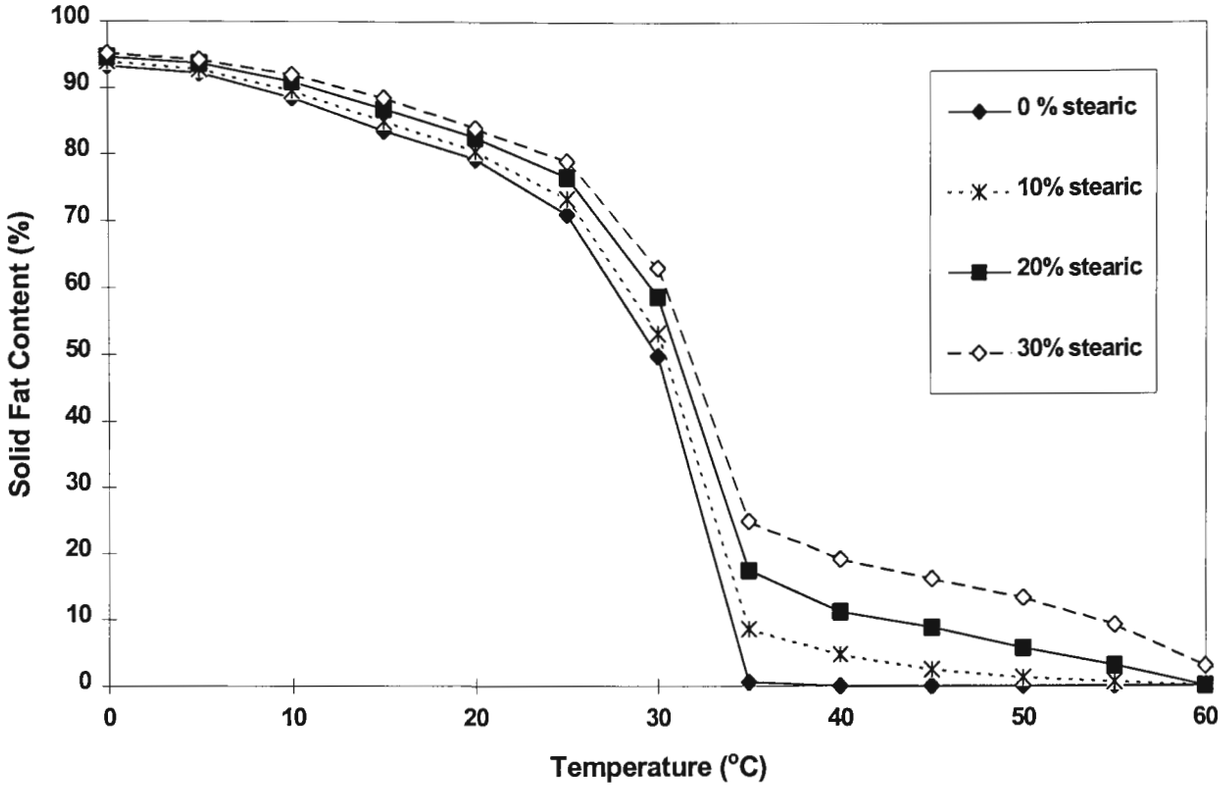


Figure 6.3.3 Solid fat content of Malaysian cocoa butter with added oleic acid.

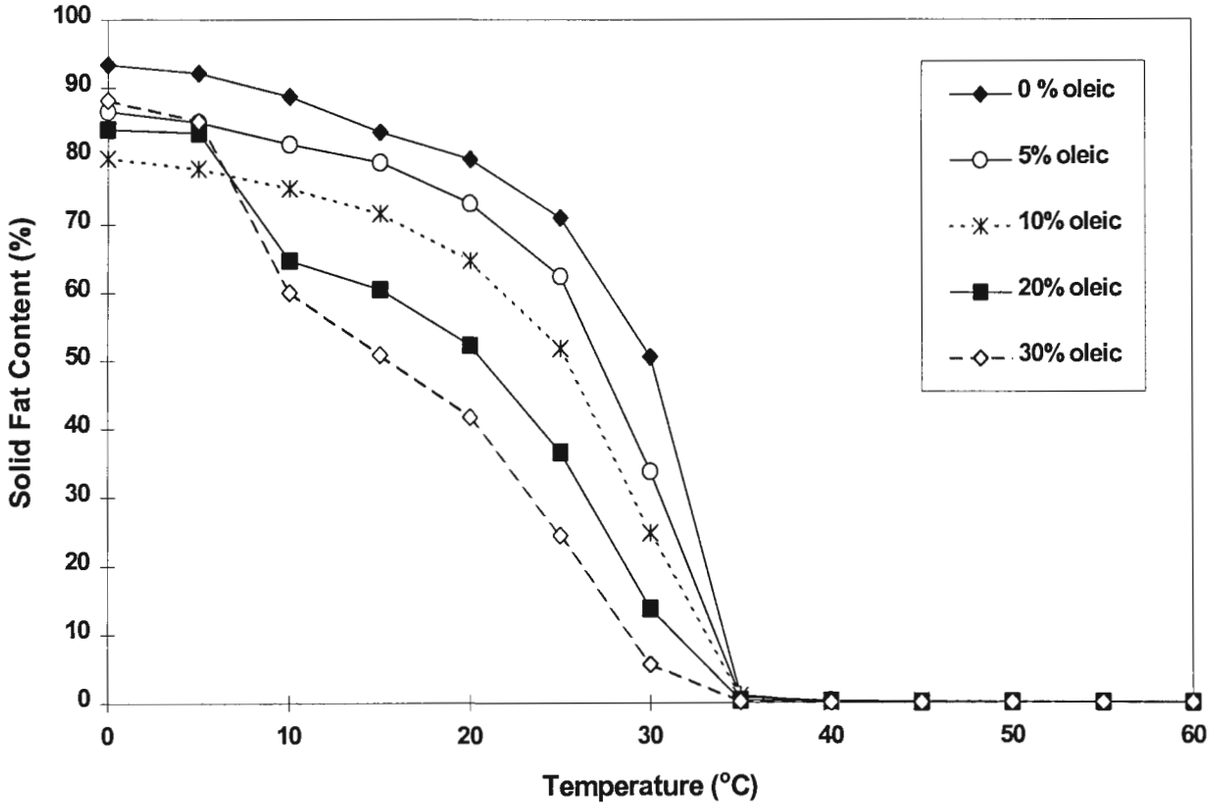
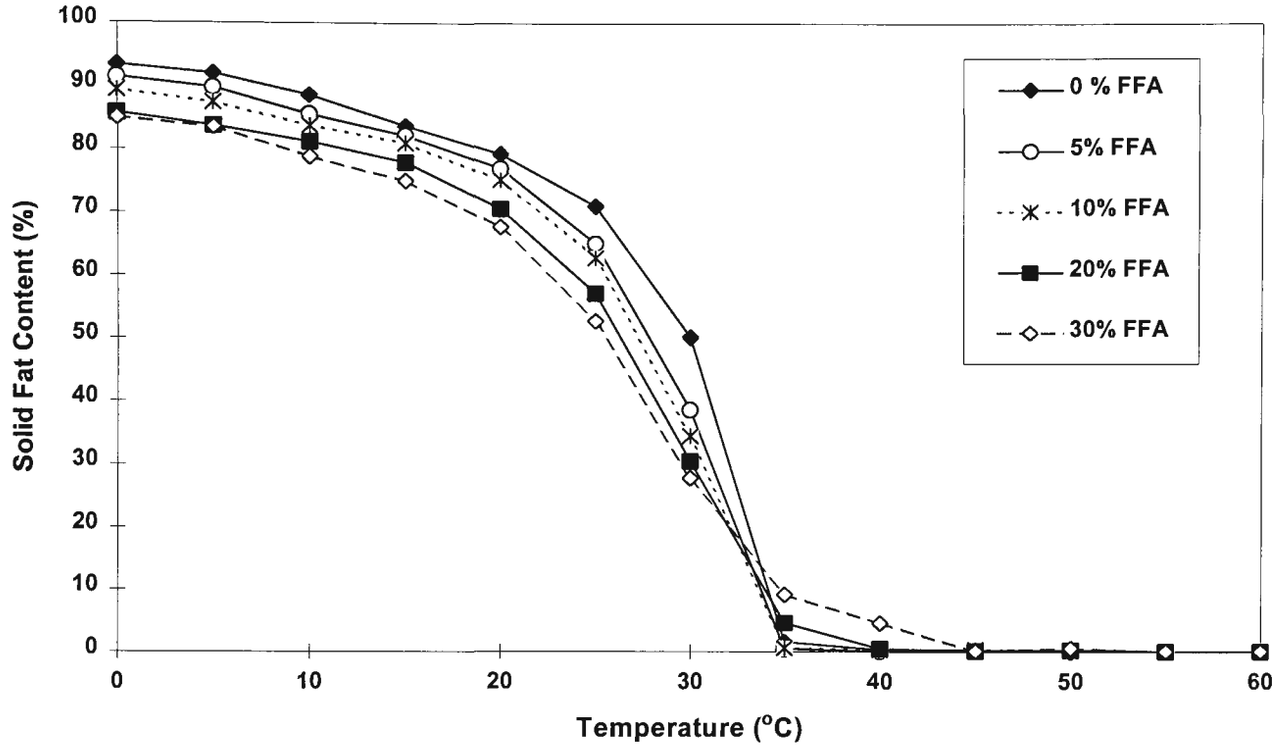


Figure 6.3.4 Solid fat content of Malaysian cocoa butter with added one-third mixture of palmitic, stearic and oleic acids.



Discussion:

The melting curves for Malaysian cocoa butter with added palmitic acid are shown in Figure 6.3.1. It can be seen that additional palmitic acid did not have a noticeable effect on the melting profile until levels of 20% palmitic acid or greater were reached. Palmitic acid, which is a saturated fatty acid with a melting point of 61-64°C, at levels of 20% or greater increased the solid fat content of Malaysian cocoa butter slightly at all measuring temperatures, with a more pronounced increase at higher temperatures (>35°C).

The melting curves for Malaysian cocoa butter with added stearic acid are shown in Figure 6.3.2. Stearic acid, a saturated fatty acid with a melting point of 67-69°C, had a similar effect to palmitic acid. The solid fat content was increased noticeably at levels of 10% stearic acid and higher, particularly at measuring temperatures over 30°C.

The melting curves for Malaysian cocoa butter with added oleic acid are shown in Figure 6.3.3. It can be seen that increasing oleic acid levels progressively decreased the solid fat

content of Malaysian cocoa butter at temperatures below the melting point of Malaysian cocoa butter (<35°C). The effect was noticeable at all levels of additional oleic acid. Oleic acid, an unsaturated fatty acid with a melting point of 13.4°C, is a liquid at room temperature. In comparison to palmitic and stearic acids, it appeared that oleic acid could affect the solid fat content of Malaysian cocoa butter to a greater extent, in terms of change in solid fat content values at measuring temperatures below 35°C.

In Figure 6.3.4, the melting curves for Malaysian cocoa butter with the added combination of free fatty acids are shown. It can be seen that increasing the combined free fatty acid levels progressively decreased the solid fat content of Malaysian cocoa butter at temperatures below 32.5°C for all samples, while the solid fat content values above 32.5°C were increased for the 20 and 30% FFA samples. This was an interesting result as the combined effect of the free fatty acids reflected a combination of the individual effects. Although oleic acid was only one-third of the free fatty acids, it had the dominant effect at temperatures less than 35°C.
These results have demonstrated that free fatty acids can affect the melting properties of Malaysian cocoa butter. The effect was minimal, however, at levels of 10% FFA or less. The effect will depend on the type and concentration of the fatty acids in the free fatty acid 'pool'. Hydrolysis equilibrium is reached fairly quickly in interesterification reactions, after which time the levels of FFA remain relatively stable. It is the composition of fatty acids in the pool of available free fatty acids that will change as the interesterification progresses towards equilibrium. Ledochowska (1999a and 1999b) found that when free fatty acids were removed from interesterified fat blends, the solid fat contents of the purified fats were slightly lower. Jacobsberg and Oh (1976) found that an increase in the free fatty acid and diacylglycerol content of palm oil decreased the solid fat content. They also found that elimination of free fatty acids by alkali refining methods produced a shift towards a higher melting range.

Free fatty acids are, however, only one of the by-products present, the diacylglycerols may exert an effect. Zainal and Yusoff (1999) found that high diacylglycerol contents

can lower the solid fat content of interesterified products. The issue of increased levels of diacylglycerols is addressed in the next section.

6.4 Consideration of the effects of by-products on physical properties

It was intended, in this work, to use cocoa butter as a model substrate only and that the characterisation of the interesterification reaction could be transferable to other natural fats, whose physical properties could be enhanced through interesterification. As discussed in section 6.1, cocoa butter is a polymorphic fat, therefore any change in the triacylglycerol composition, such as that occurring during interesterification, is likely to affect the melting and crystallisation properties. The initial changes in the physical properties of interesterified cocoa butter may also be influenced, to a small degree, by the presence of lipid by-products. It was shown in section 6.1 that changes in the physical properties continued after the levels of by-products had reached equilibrium. Studies reported by Zeitoun (1993) indicate that it was the triacylglycerol composition of the liquid oil portion of interesterified fat blends that significantly affected the physical properties, which can influence the choice of fats to use to produce a particular product such as margarine.

Ideally, for an optimum interesterification result, the amount of by-products would be minimal so that the interesterified product would require only a minor refining treatment. This section considers the effect of diacylglycerols on the physical properties of interesterified cocoa butter and describes several methods examined for their removal. The range of diacylglycerols that could be present in an interesterified cocoa butter sample would be quite large, including a mixture of 1,2-diacylglyerols and 1,3-diacylglycerols. It would therefore appear to be of benefit to isolate the diacylglycerols from an interesterified fat sample in order to examine the physical properties of the purified sample.

In certain cases, however, diacylglycerols may be desired due to the influence on the transition from beta-prime to beta crystal structure or their emulsification properties

(Hernqvist et al, 1981: Ledochowska, 1999a and 1999b). It has been found in studies of palm oil that diacylglycerols can effect the quality and physical properties of palm oil (Siew and Ng, 1995 and 1996). The diacylglycerols of palm oil delayed crystallisation time, although it was found different diacylglycerols had different effects. Diacylglycerols delayed the phase transition of lower melting crystal forms to higher forms of triacylglycerols (Yella Reddy and Prabhakar, 1986). Cebula and Smith (1992), however, found that diacylglycerols in confectionery fats caused crystallisation to occur sooner, perhaps due to formation of crystal nucleus, however crystal growth was slowed.

Several purification methods were investigated with limited success. Firstly, a silica gel adsorption technique was tested, where diacylglycerols and free fatty acids were adsorbed onto a silica gel, while the triacylglycerols remained in solution (Yella Reddy and Prabhakar, 1986). Molten fat was dissolved in hexane and silica gel was added to form a single slurry solution that was stirred for several hours before being filtered and the solvent evaporated off the filtrate. Several combinations of different amounts of silica gel and fat were also tested. Florisil was used as an alternative absorbant, as it reportedly adsorbs free fatty acids more strongly than silica gel (Litchfield, 1972). The amount of by-products before and after refining for the silica gel and florisil methods were either estimated by separation on a TLC plate or quantified using the GC method (section 3.11). It was found that if there was not enough silica gel present, not all the by-products were retained, while too much silica gel resulted in adsorption of all of the initial fat including triacylglycerols. It was also found that silica gel removed more diacylglycerols than florisil, which did adsorb more free fatty acids.

The adsorption method was time-consuming, involved the use of solvents and was not considered effective enough to apply to each sample prior to solid fat content analysis. In addition, it could not be determined whether the refining procedure itself would have an effect on the physical properties of the samples. These investigations demonstrated the difficulty and time requirements in removing the by-products from the every interesterified sample prior to analysis.

An alternative method was examined using an enzyme, Lipase G (Amano Pharmaceutical, Japan), which acts preferentially on diacylglycerols and monoacylglycerols to hydrolyse them into free fatty acids and glycerol. This reduces the purification problem to one of removing an increased amount of FFA and glycerol through alkali refining, rather than the more difficult task of removing the diacylglycerols. Using the lipase manufacturer's instructions for the refining of fats and oils, a preliminary investigation was carried out using an interesterified cocoa butter samples. The diacylglycerol content was reduced to about a third of the original content, while the free fatty acid content almost doubled. This result confirmed that Lipase G acted preferentially on diacylglycerols to reduce the amount present without affecting the triacylglycerol content. Although this method had potential for further development, it was also not considered an appropriate treatment for a large number of samples.

For comparison of samples from enzymatic interesterification of cocoa butter, the removal of the by-products would appear not to be essential because a similar range of by-products would be present in the samples. The samples are all subjected to the same tempering procedure when measuring the physical properties. The levels of by-products can be quantified and any large differences are allowed for when comparing the physical properties of different interesterified cocoa butter samples.

6.5 Chapter conclusions

This chapter has reported on further investigations into the process of enzymatic interesterification of fats under solvent-free conditions using a natural fat system. Changes in the physical properties of several fats, various combinations of cocoa butter, milkfat and canola oil, were examined by monitoring changes in the SFC melting profiles over time. Large differences in the physical properties were found between the untreated and interesterified fats, as a result of enzymatic interesterification using Lipozyme as the catalyst.

A method for determining the lipid class levels (FFA, MG, DG and TG), as well as the triacylglycerol composition, of interesterified cocoa butter samples in the same preparation and gas chromatographic analysis run was developed. This method enables the analysis of large numbers of samples and utilises relatively simple analytical techniques. The lipid class profile for the interesterification of Malaysian cocoa butter showed that hydrolysis equilibrium was reached within 8 hours of the start of the interesterification process.

It was found that additional free fatty acids could have an effect on the melting properties of cocoa butter, particularly at levels above 10%. A mixture of the three major fatty acids had much less effect and did not change the shape of the melting curve. Removing the diacylglycerols and free fatty acids from the interesterified samples was considered. The techniques were found to be labourious and not absolutely necessary for low levels of these by-products. In addition, the removal procedure itself may introduce further variations. It was acknowledged that diacylglycerols and free fatty acids do affect the melting and crystallisation properties of fats and oils, however, determining the exact effect on the physical properties of interesterified cocoa butter samples was not considered a priority for this thesis. Moreover, equilibrium in lipid class levels is reached within 2-8 hours, whilst interesterification generally continues much longer.

The solvent-free enzymatic interesterification process could be characterised with respect to changes in the solid fat content profiles, lipid class levels, triacylglycerol composition and the triacylglycerol reaction profile. The combination of these analyses was used in further studies to monitor the effects of different initial reaction conditions on the batch interesterification process. Some of these studies are reported in the next chapter.

Chapter 7

Investigations into the effects of the initial reaction conditions on the enzymatic interesterification process

A triacylglycerol reaction profile method for monitoring the solvent-free enzymatic interesterification of a natural fat was developed in Chapter 5 and the effects of interesterification and increased levels of by-products on the physical properties of fats were investigated in Chapter 6. The work in this current chapter aimed to investigate the effects that varying the initial enzyme and water contents had on the interesterification of cocoa butter, as observed through the changes over time in the physical properties, the lipid class levels and the triacylglycerol reaction profiles.

In these investigations, the effect of the reaction temperature was not studied. Several reports in the literature have suggested that the extent of the interesterification is independent of the reaction temperature in the range of 20 – 70°C (Foglia et al, 1993; Forssell et al, 1992; Lai et al, 1998: Thomas et al, 1988). The recommended optimum reaction temperature for Lipozyme, 60°C, was used in all enzymatic interesterifications carried out.

Experimental Procedures

The water contents of the cocoa butter and the Lipozyme, 7.7 BAUN/g, used were analysed according to the procedure described in 3.5 and were 0.04% for Malaysian cocoa butter and 4.22% for Lipozyme.

Table 7.1 outlines the experimental design of the different treatment combinations of initial enzyme and water contents used to interesterify Malaysian cocoa butter. This design also compared the location of additional water, which was added either to the enzyme or directly to the cocoa butter. Malaysian cocoa butter, batch size of 60g, was interesterified according to the procedure described in section 3.1, using the Lipozyme

and water contents in Table 7.1 at a reaction temperature of 60°C. Samples were taken at 0, 2, 4, 8, 24 and 48 hours and selected samples analysed for Solid Fat Content (section 3.7), lipid class and triacylglycerol composition (section 3.11) and triacylglycerol reaction profiles (section 5.3).

Table 7.1 Experimental design for the enzymatic interesterification of Malaysian cocoa butter using different treatment combinations of initial Lipozyme and overall water contents.

Trt	Lipozyme	Lipozyme (g)		Water (g)	Overall
	% w/w	4.22% Water	10% Water	Added	% water
1	1.6	-	1.0789	-	0.22
2	4.2	2.6273	-	-	0.22
3	4.2	2.6273	-	0.1525	0.46
4	3.9	-	2.6273	-	0.47
5	10.6	6.6667	-	-	0.46
6	10.6	6.6667	-	0.3878	1.03

Results:

Figure 7.1 Solid Fat Content curves for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 1.6% w/w and an overall initial water content of 0.22% w/w, including extra water added to Lipozyme (Treatment 1).

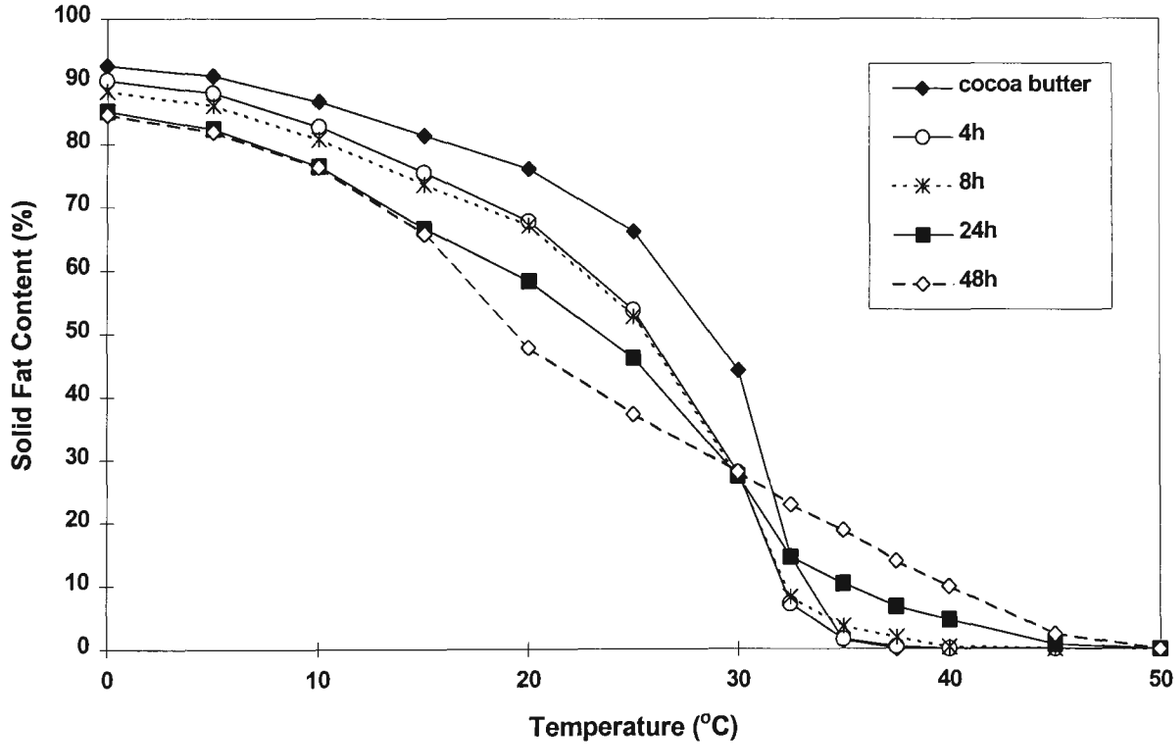


Figure 7.2 Solid Fat Content curves for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 4.2% w/w and an overall initial water content of 0.22% w/w, including extra water added to Lipozyme (Treatment 2).

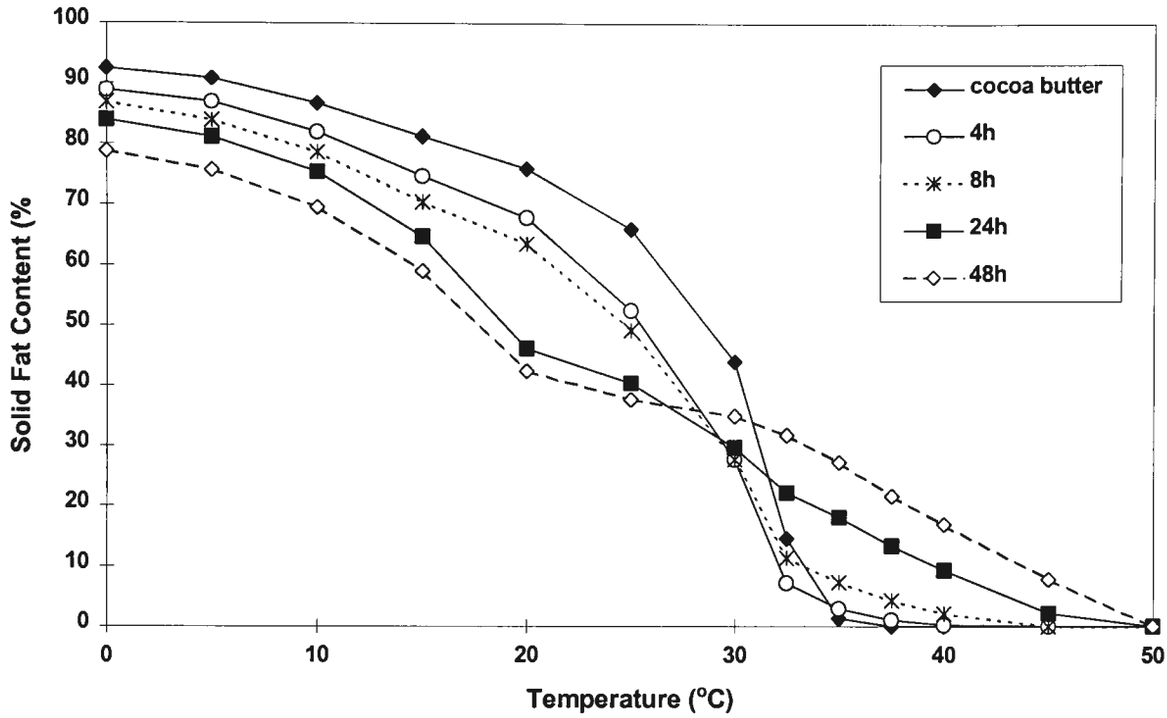


Figure 7.3 Solid Fat Content curves for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 4.2% w/w and an overall initial water content of 0.46% w/w including extra water added to the cocoa butter (Treatment 3).

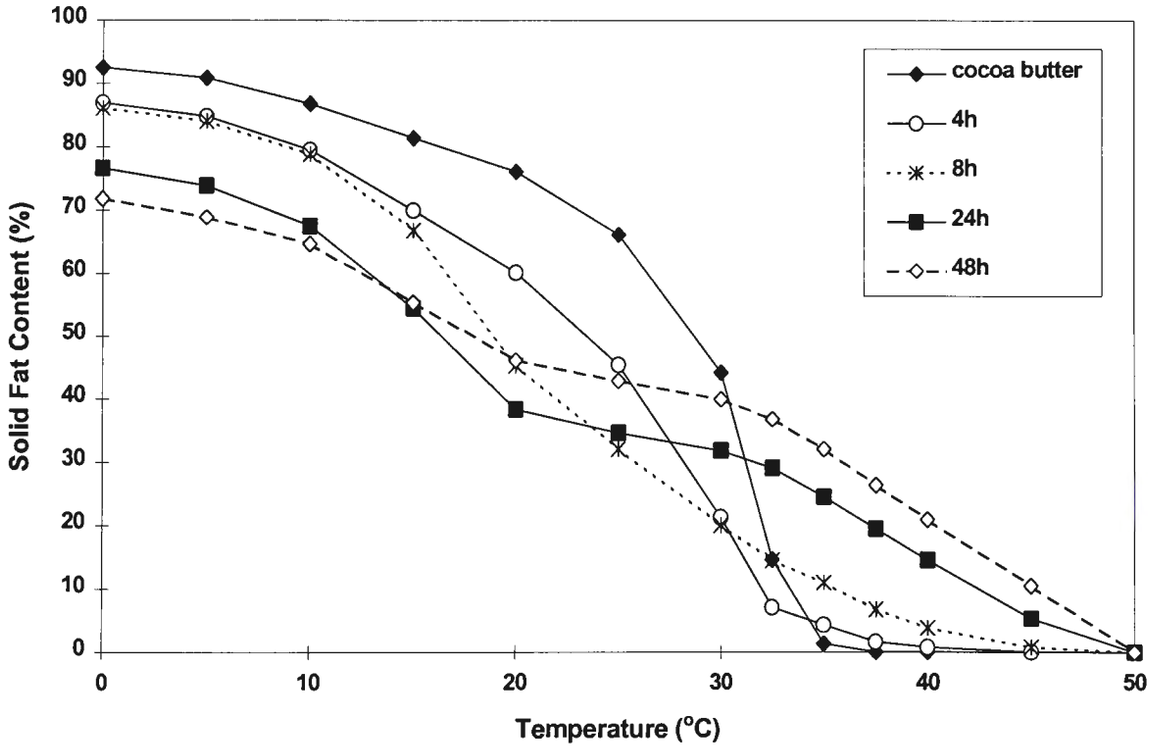


Figure 7.4 Solid Fat Content curves for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 3.9% w/w and an overall initial water content of 0.47% w/w, including extra water added to Lipozyme (Treatment 4).

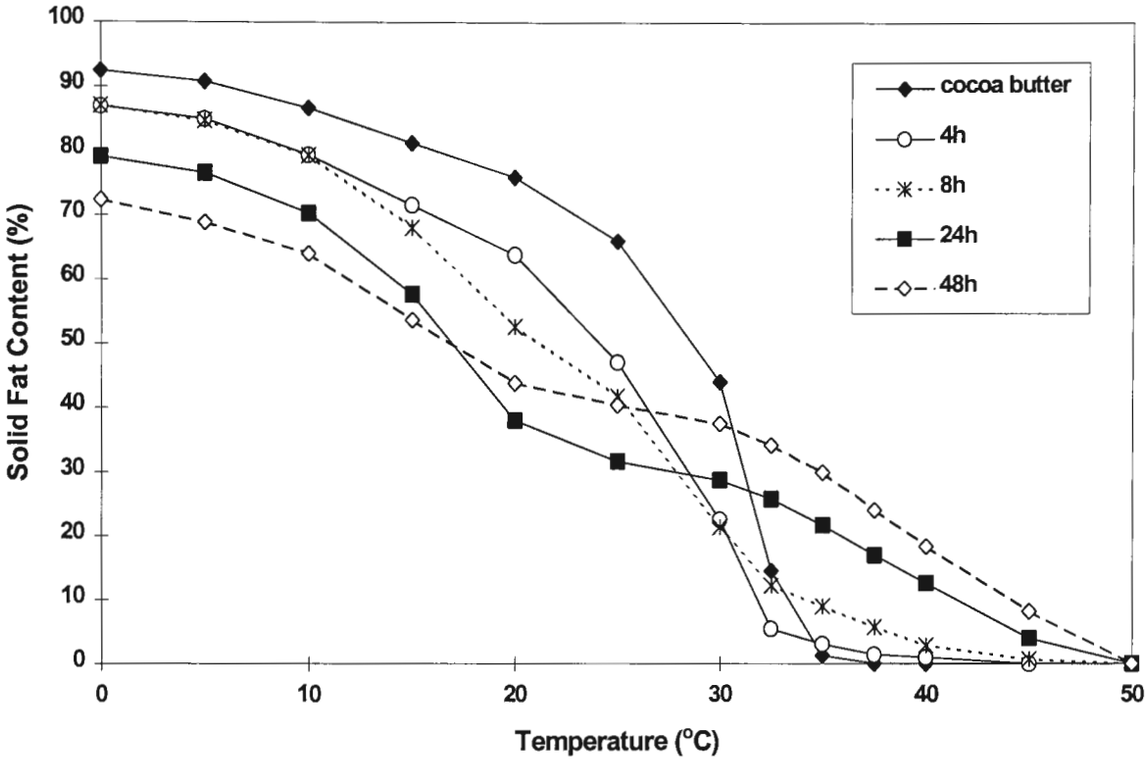


Figure 7.5 Solid Fat Content curves for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 10.6% w/w and an overall initial water content of 0.46% w/w (Treatment 5).

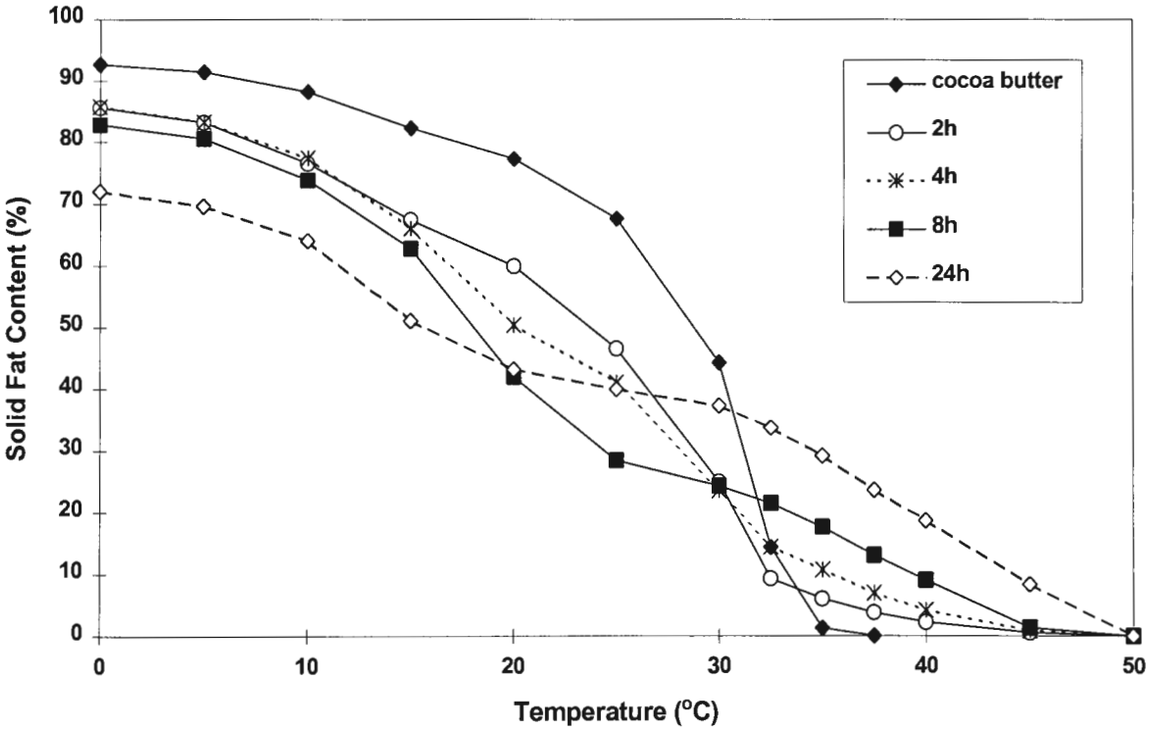


Figure 7.6 Solid Fat Content curves for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 10.6% w/w and an overall initial water content of 1.03% w/w, including extra water added to cocoa butter (Treatment 6).

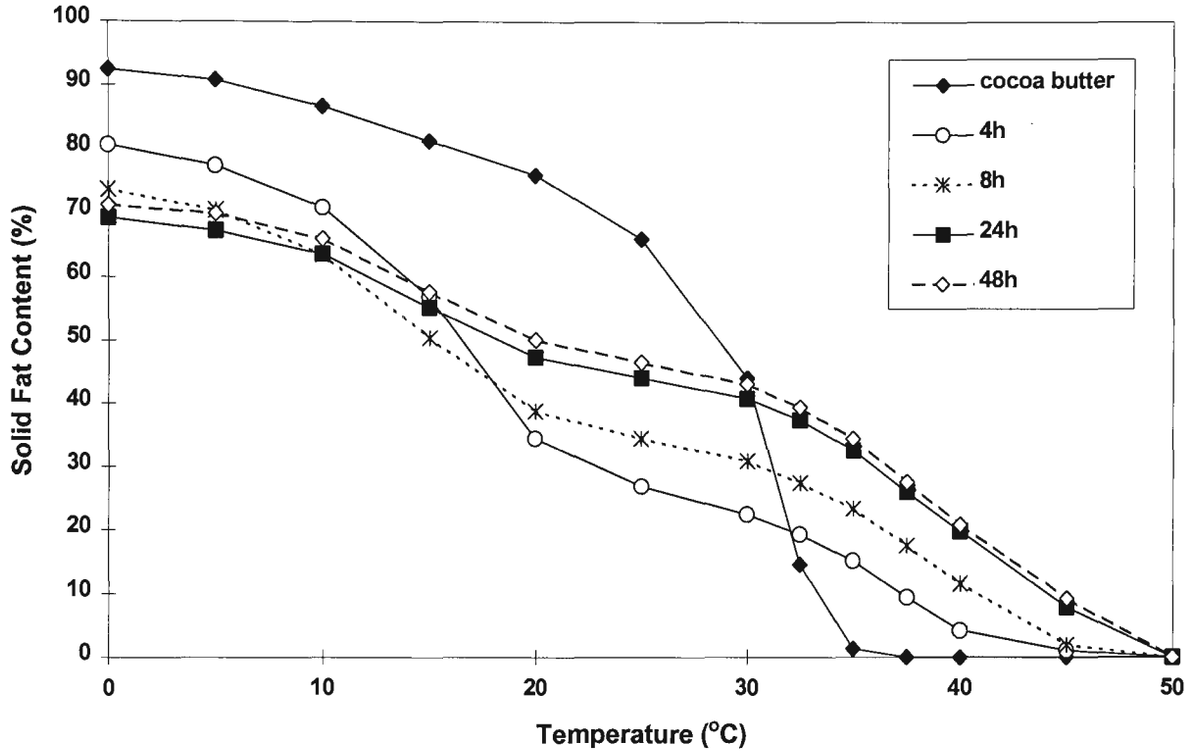


Figure 7.7 Lipid Class results for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 1.6% w/w and an overall initial water content of 0.22% w/w, including extra water added to Lipozyme (Treatment 1). Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

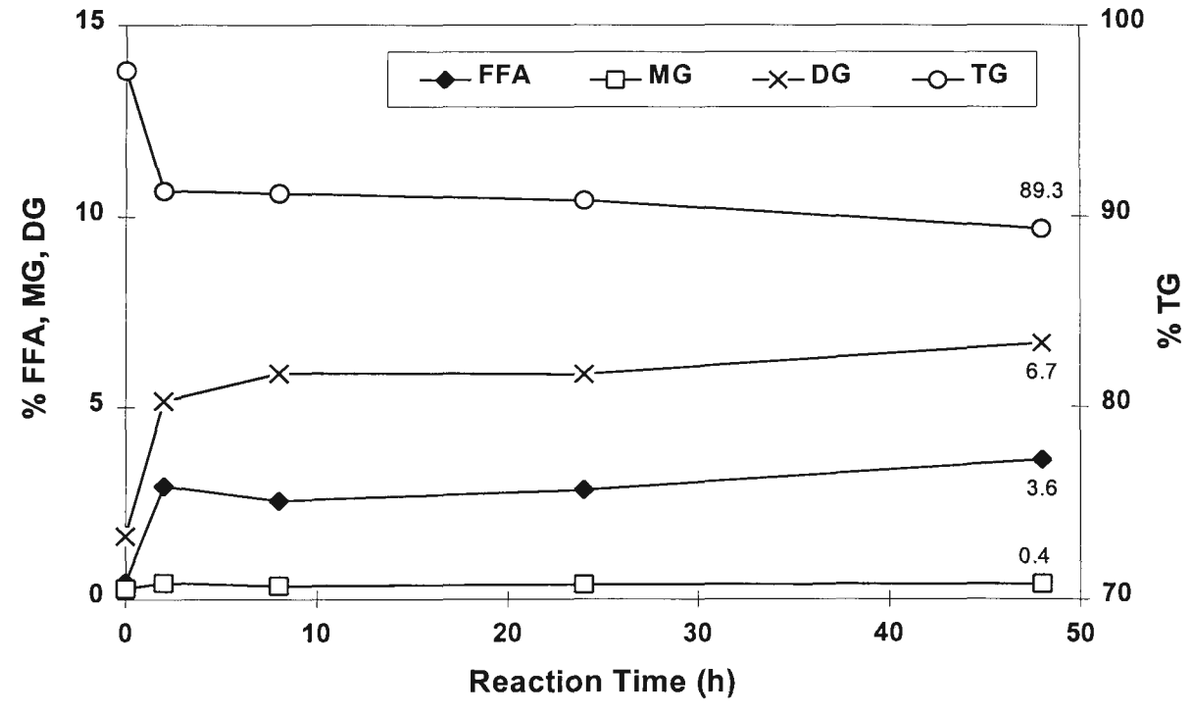


Figure 7.8 Lipid Class results for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 4.2% w/w and an overall initial water content of 0.22% w/w (Treatment 2). Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

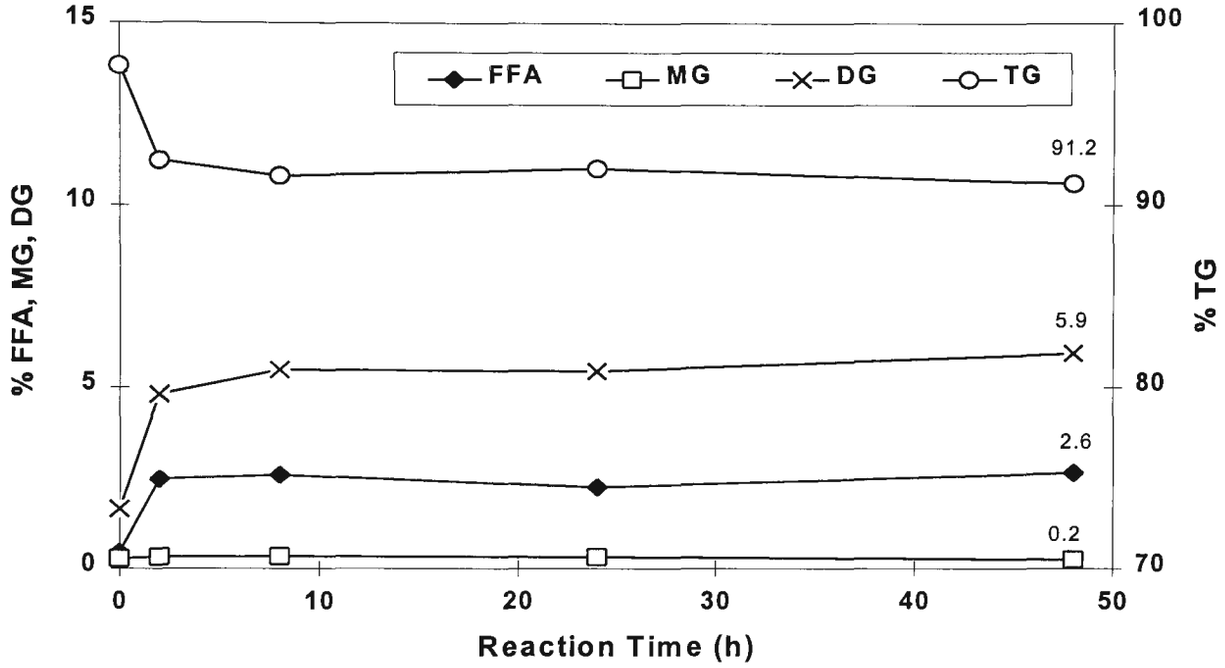


Figure 7.9 Lipid Class results for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 4.2% w/w and an overall initial water content of 0.46% w/w, including extra water added to the cocoa butter (Treatment 3). Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

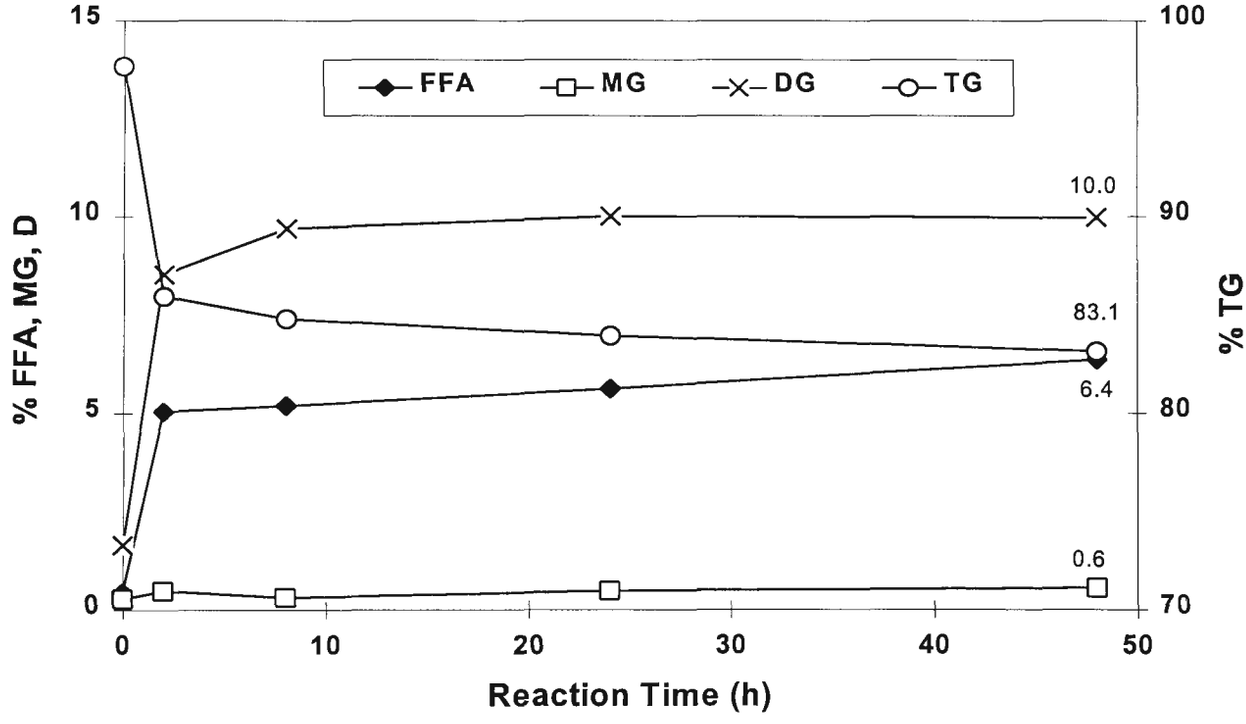


Figure 7.10 Lipid Class results for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 3.9% w/w and an overall initial water content of 0.47% w/w, including extra water added to Lipozyme (Treatment 4). Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

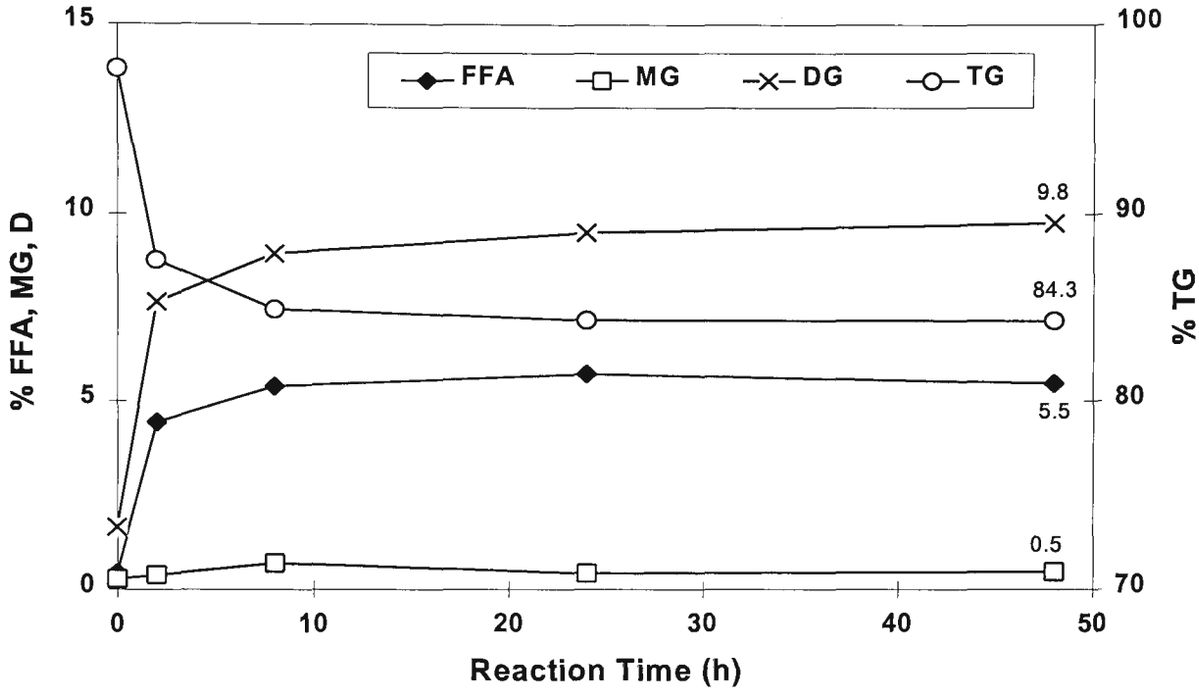


Figure 7.11 Lipid Class results for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 10.6% w/w and an overall initial water content of 0.46% w/w (Treatment 5). Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

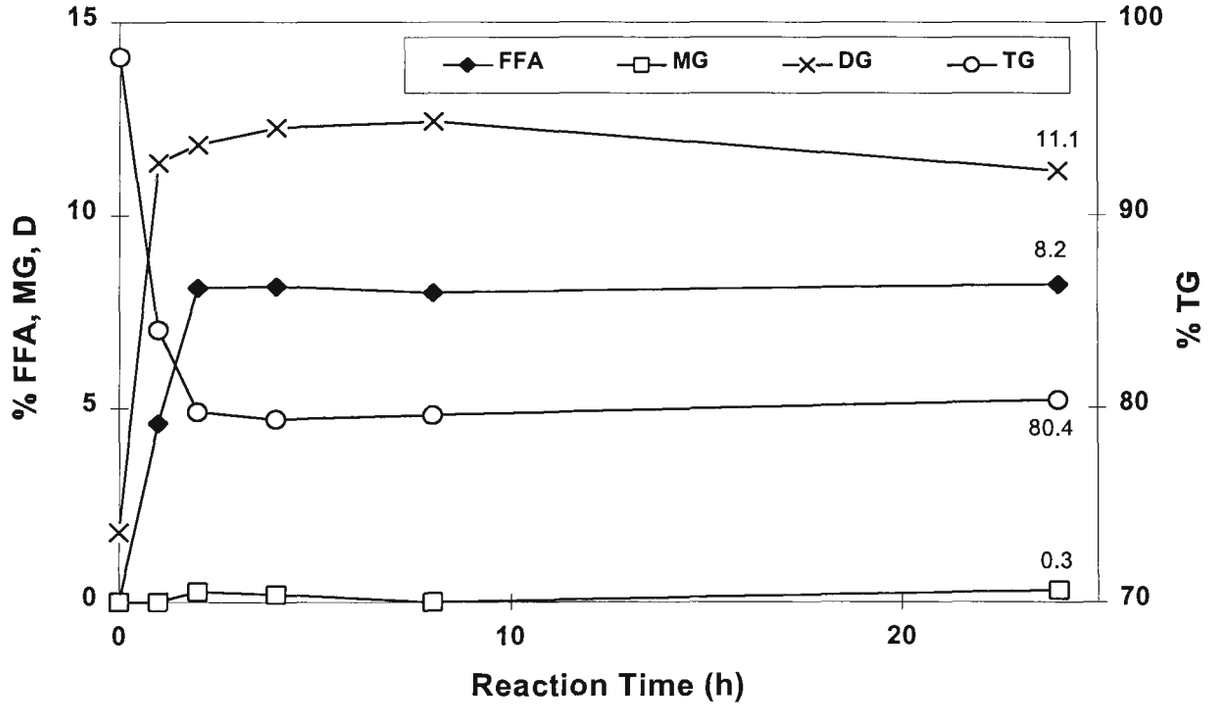


Figure 7.12 Lipid Class results for samples taken during the interesterification of cocoa butter using Lipozyme as a catalyst at 10.6% w/w and an overall initial water content of 1.03% w/w, including extra water added to cocoa butter (Treatment 6). Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

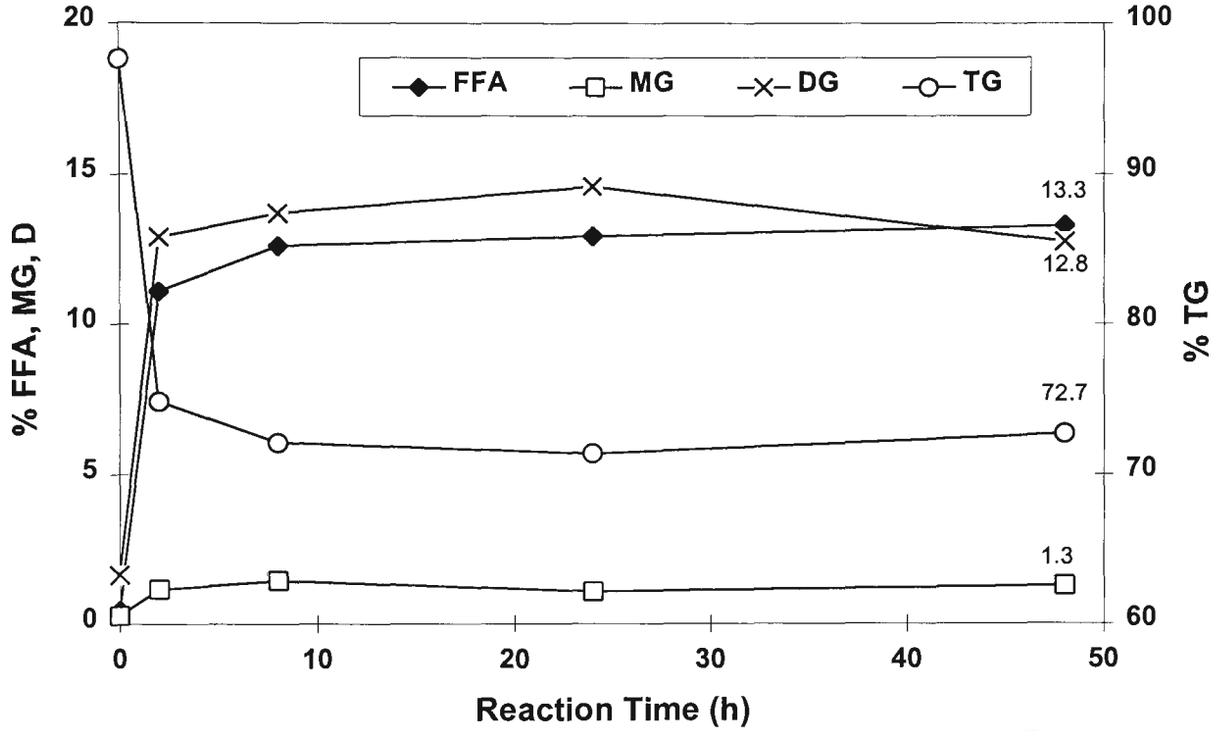


Figure 7.13 Triacylglycerol reaction profile for interesterification of cocoa butter using Lipozyme (1.6%) and an overall initial water content of 0.22% (Treatment 1). Standard deviations: 1,3-specific (±0.16) and Random (±0.26).

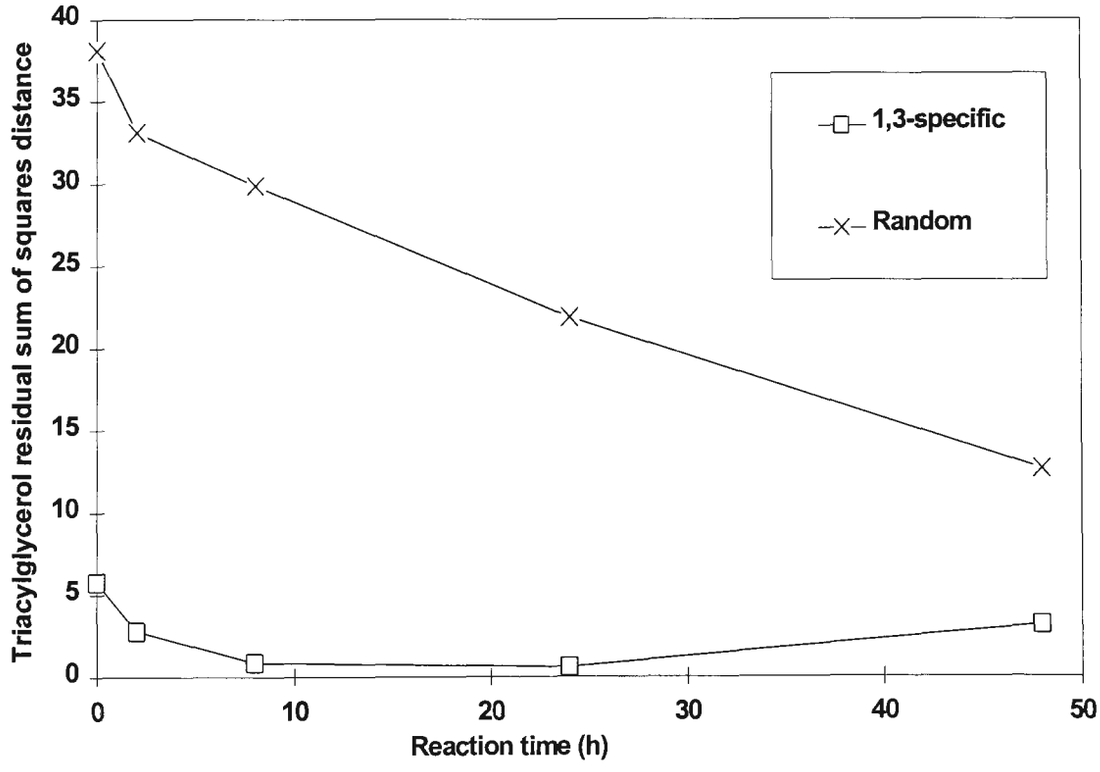


Figure 7.14 Triacylglycerol reaction profile for interesterification of cocoa butter using Lipozyme (4.2%) and an overall initial water content of 0.22% (Treatment 2). Standard deviations: 1,3-specific (±0.16) and Random (±0.26).

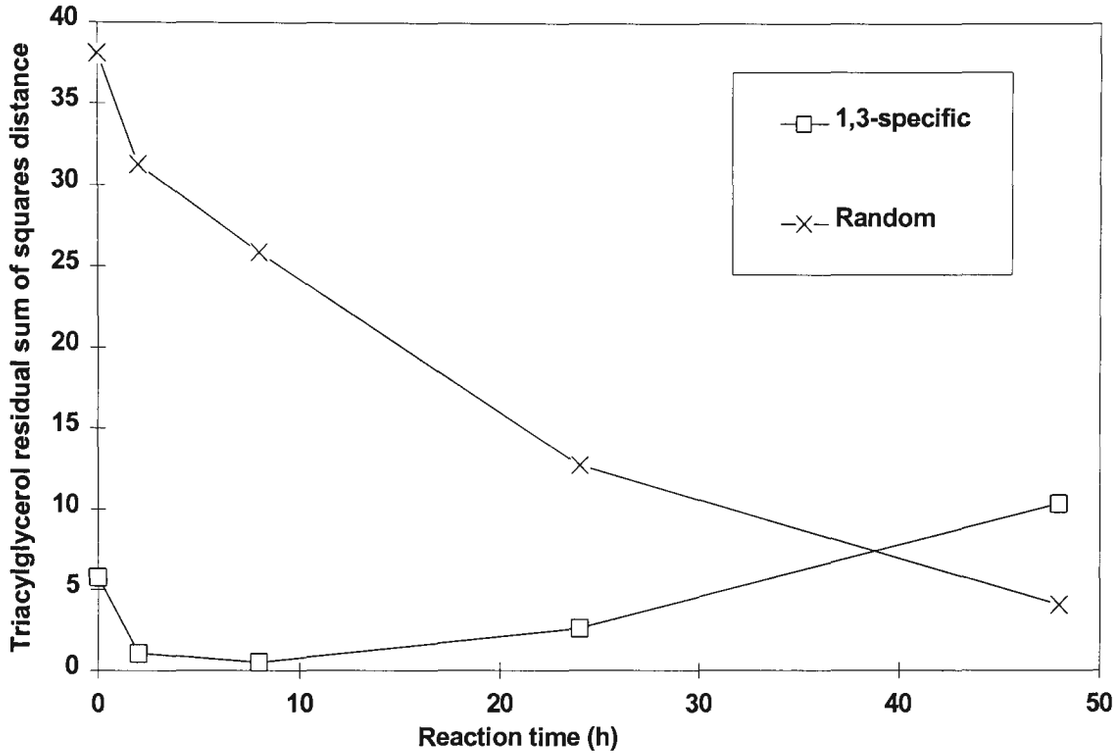


Figure 7.15 Triacylglycerol reaction profile for interesterification of cocoa butter using Lipozyme (4.2%) and an overall initial water content of 0.46% (Treatment 3). Standard deviations: 1,3-specific (±0.16) and Random (±0.26).

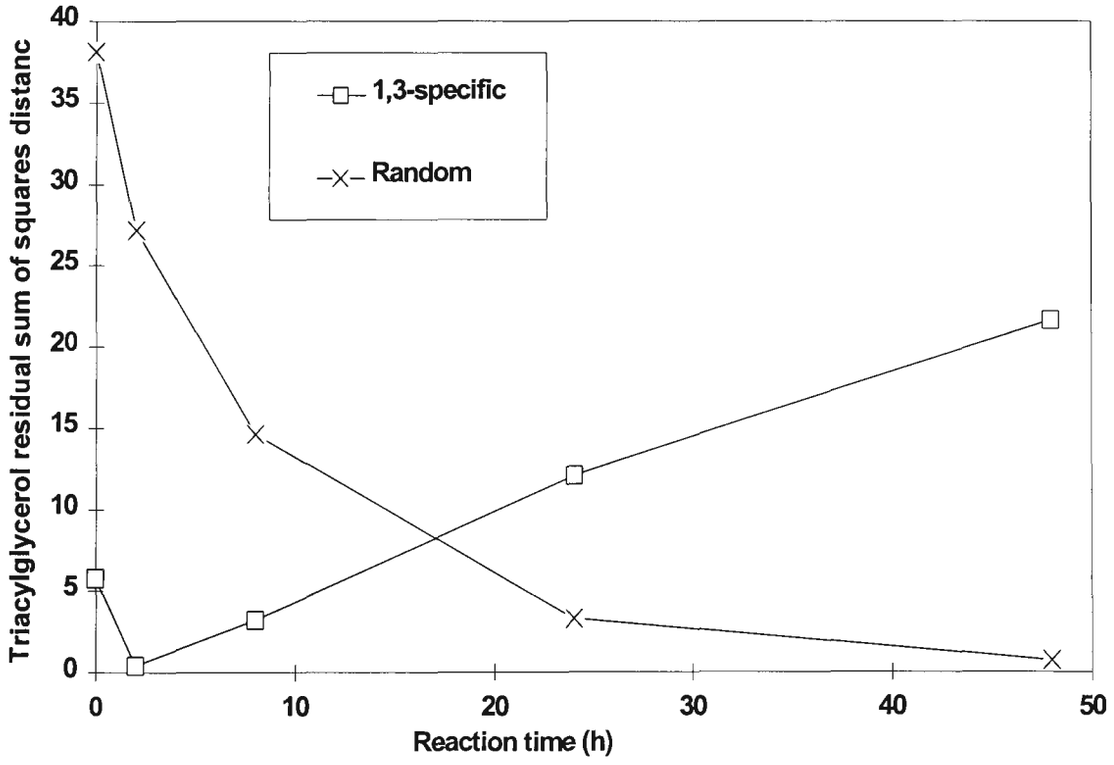


Figure 7.16 Triacylglycerol reaction profile for interesterification of cocoa butter using Lipozyme (3.9%) and an overall initial water content of 0.47% (Treatment 4). Standard deviations: 1,3-specific (±0.16) and Random (±0.26).

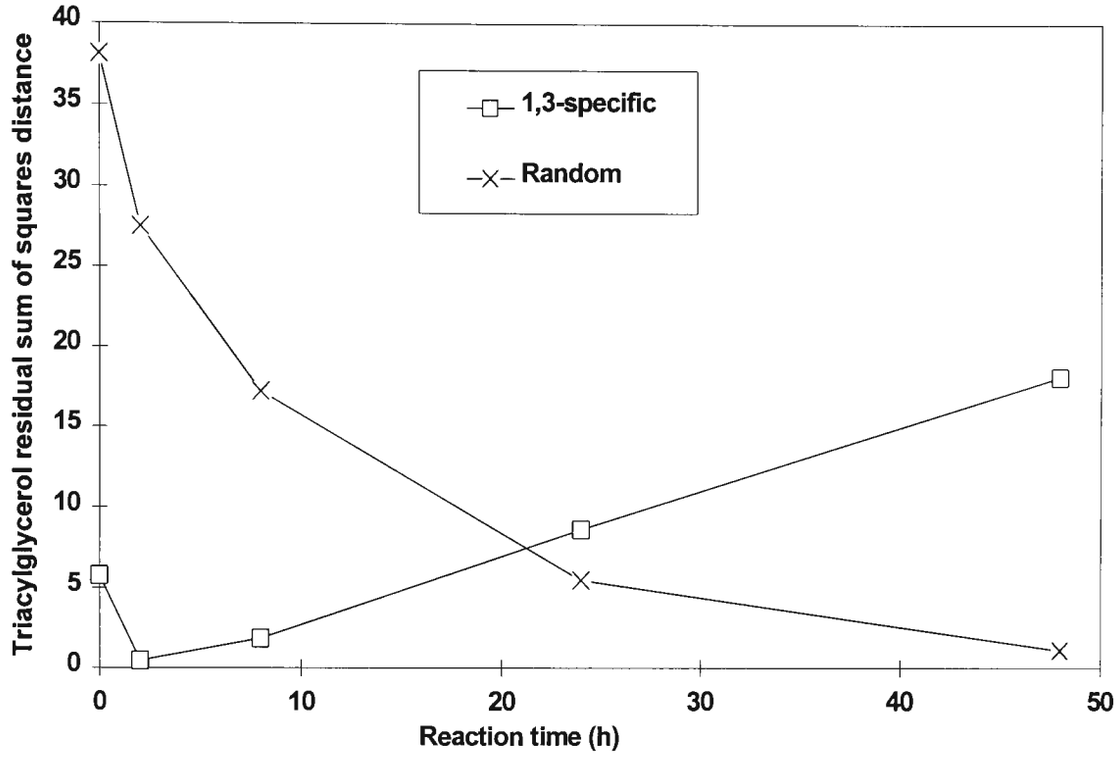


Figure 7.17 Triacylglycerol reaction profile for interesterification of cocoa butter using Lipozyme (10.6%) and an overall initial water content of 0.46% (Treatment 5). Standard deviations: 1,3-specific (±0.16) and Random (±0.26).

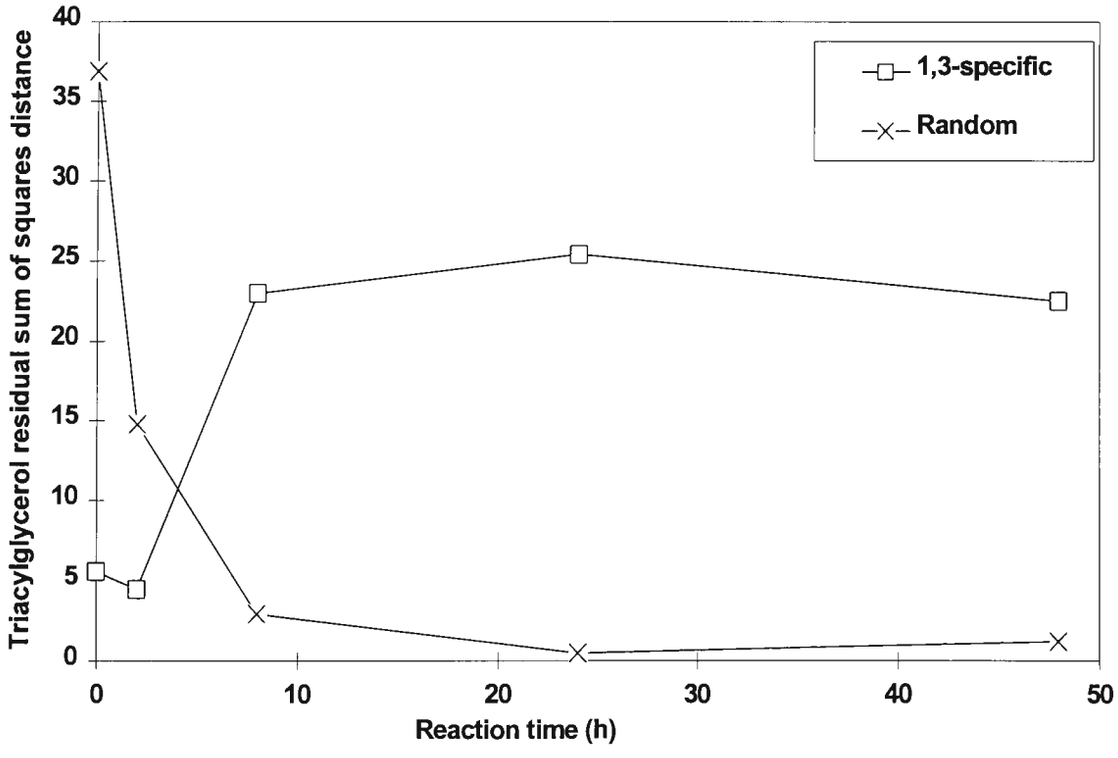
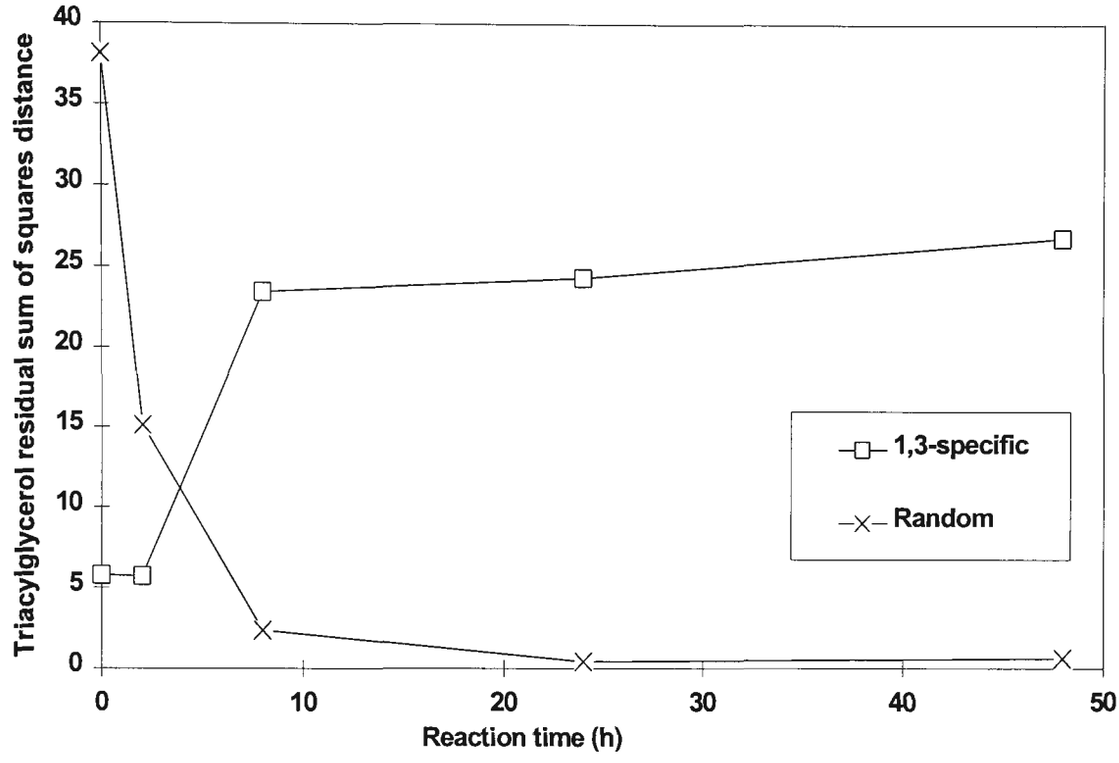


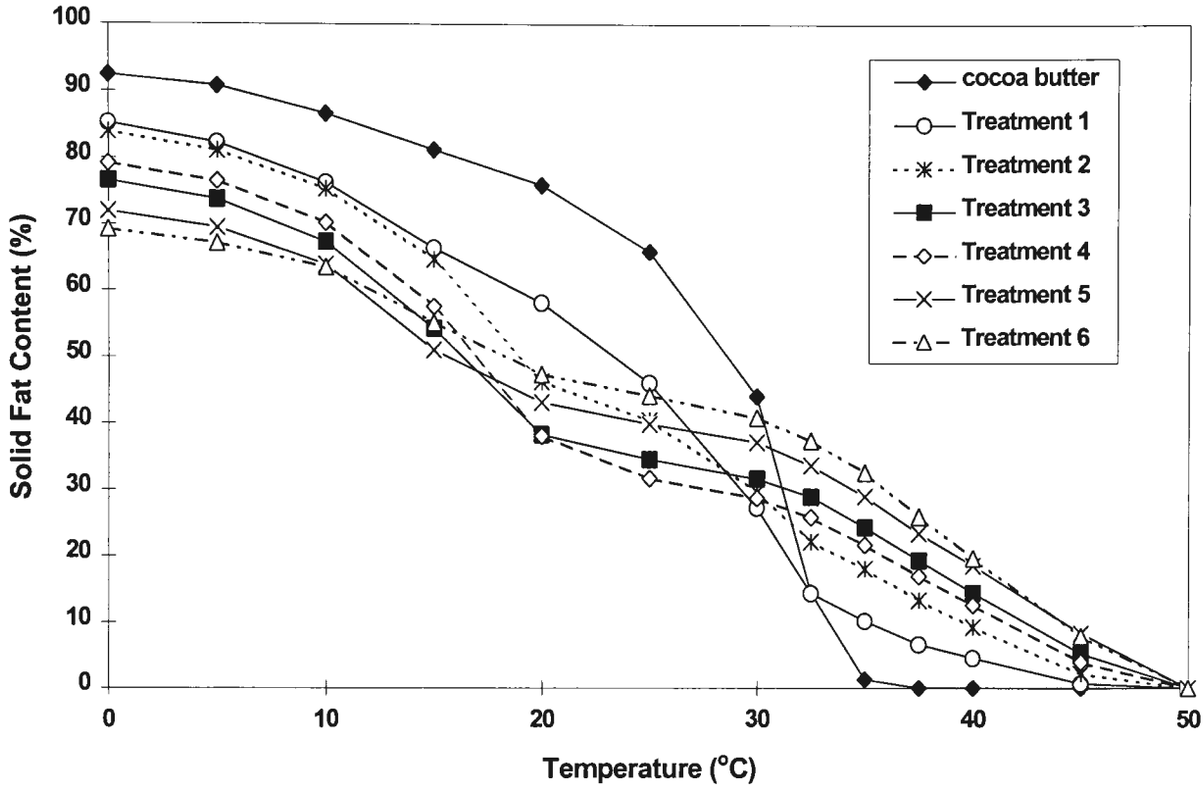
Figure 7.18 Triacylglycerol reaction profile for interesterification of cocoa butter using Lipozyme (10.6%) and an overall initial water content of 1.03% (Treatment 6). Standard deviations: 1,3-specific (±0.16) and Random (±0.26).



Discussion:

The changes in the physical properties of the interesterified Malaysian cocoa butter over time, according to the solid fat content melting profiles were shown for all six treatments in Figures 7.1 to 7.6. In general, the higher the enzyme and initial water content, the faster the changes occurred. This observation was evident in a comparison of the solid fat content melting curves of samples taken at 24 hours for all treatments, shown in Figure 7.19, where the different treatments had resulted in changes to the physical properties of Malaysian cocoa butter to different extents.

Figure 7.19 Comparison of solid fat curves of samples taken after 24 hours of enzymatic interesterification under different treatments of enzyme and water contents as described in Table 7.1.



The lipid class profiles for all treatments were shown in Figures 7.7 to 7.12. For all treatments, there was an initial increase in by-products at 2 hours. With further reaction time, the lipid class levels were at equilibrium or increased only slightly. In general, the higher the water content, the higher the level of by-products at hydrolysis equilibrium and consequently the lower the yield of triacylglycerols. The lipid class levels at 24 hours are presented for comparison in Table 7.2. It can be seen that the treatments with the same initial water content had similar lipid class levels at equilibrium, despite differences in the amount of enzyme.

Mu et al (1999) reported high levels of free fatty acids and diacylglycerols for the first use of Lipozyme in a batch interesterification process, with the levels decreasing with subsequent use. They also found that the reaction temperature affected the diacylglycerol levels and rate of acyl migration, with temperatures above 60°C causing significant

increases. The repeated use of Lipozyme was not studied in this thesis, however, this would be an important area of interest for future work.

Table 7.2. Comparison of lipid class levels (relative wt%) of samples taken after 24 hours of enzymatic interesterification under different treatments.

Trt	%Lipozyme	%Water	FFA	MG	DG	TG
1	1.6	0.22	2.8	0.4	5.8	90.8
2	4.2	0.22	2.2	0.3	5.4	92.0
3	4.2	0.46	5.6	0.5	10.0	83.9
4	3.9	0.47	5.7	0.4	9.5	84.3
5	10.6	0.46	8.2	0.3	11.2	80.4
6	10.6	1.03	12.9	1.1	14.6	71.4

The location of the added water did not influence the lipid class levels as evident by comparing the results of treatments 3 and 4, where water was added to the cocoa butter and the Lipozyme respectively. This observation is in agreement with Forssell et al (1992), who reported that the degree of hydrolysis did not depend on whether the water was added with the enzyme preparation or with the substrate when studying the interesterification of tallow:rapeseed oil mixtures using Lipozyme.

The triacylglycerol reaction profiles for the treatments were shown in Figures 7.13 to 7.18. In general, the interesterification reactions progressed at a faster rate with increased enzyme and initial water content. The random and 1,3-specific triacylglycerol reaction profiles for all treatments are given in Figures 7.20 and 7.21 for comparison.

From the random triacylglycerol reaction profiles in Figure 7.20, it can be seen that treatment 1, which had the lowest enzyme content of 1.6% and an initial water content of 0.22% was randomised at a slower rate than the other treatments and after 48 hours was still not completely random. Treatment 6 however, which had the highest enzyme content of 10.6% and an initial water content of 1.03% was randomised at the fastest rate and was almost completely random at or before 8 hours of reaction, after which time an interesterification equilibrium was reached.

These observations were also reflected in the 1,3-specific triacylglycerol reaction profiles, shown in Figure 7.21. Treatment 1 results showed that the rate of 1,3-specific interesterification was much slower than that of other treatments. A 1,3-specific product was obtained under treatment 1 conditions by 24 hours and maintained until 48 hours. For treatment 6, a 1,3-specific interesterified product was obtained after 2 hours, which was subsequently randomised by 8 hours of reaction.

Figure 7.20 Comparison of random triacylglycerol reaction profiles for enzymatic interesterifications under different treatments of enzyme and water contents as described in Table 7.1

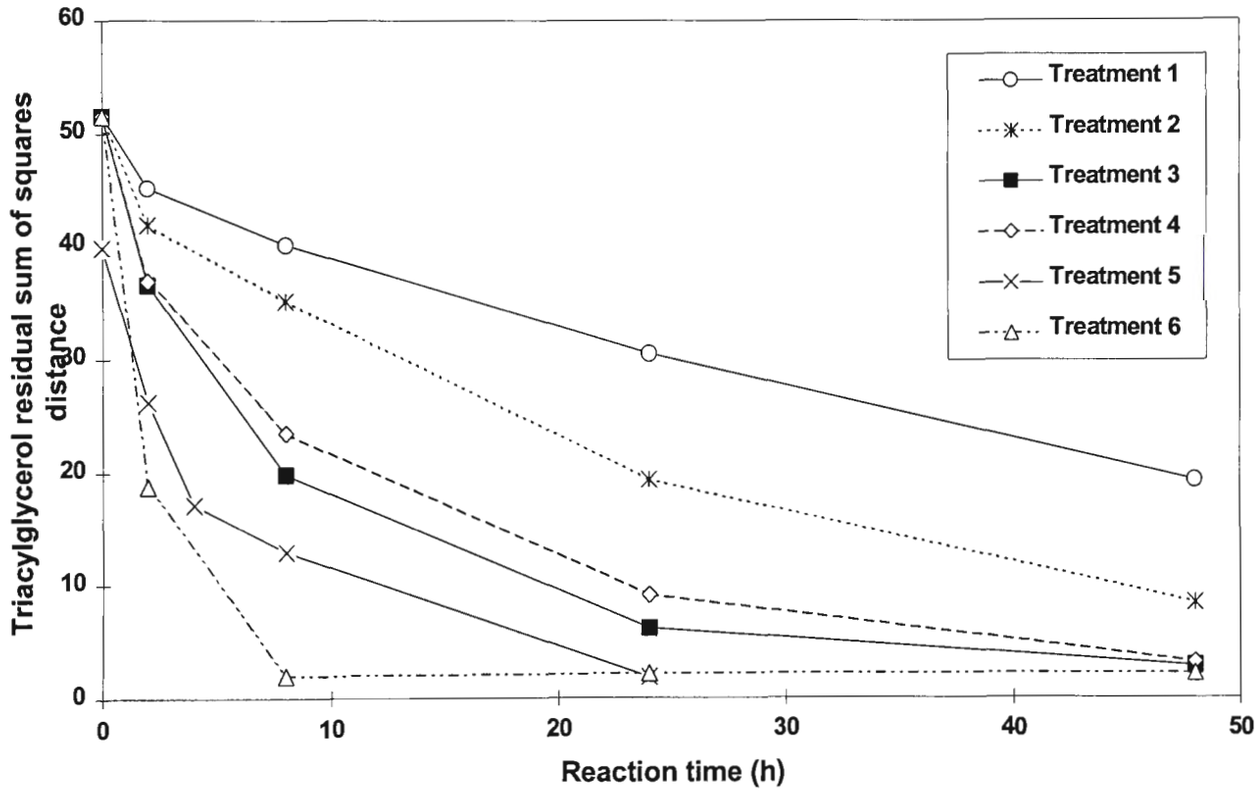
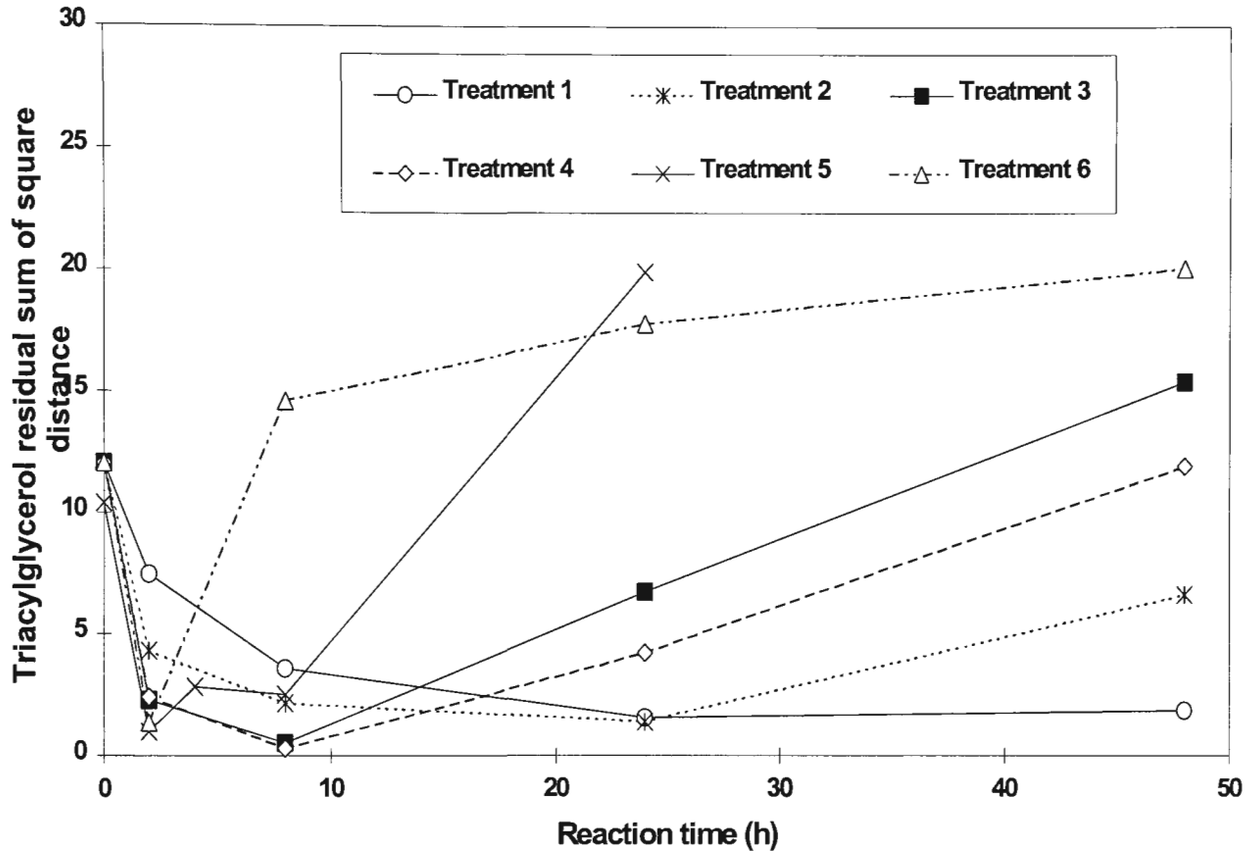


Figure 7.21 Comparison of 1,3-specific triacylglycerol reaction profiles for various interesterifications under different treatments of enzyme and water contents as described in Table 7.1.



For the treatments that had practically the same water and enzyme contents, treatments 3 and 4, it was found that the results were very similar. The results for treatment 2, which had a lower water content than that of treatments 3 and 4, showed much lower levels of by-products at hydrolysis equilibrium as well as a lower rate of interesterification, as seen by the triacylglycerol reaction profiles. The results for treatment 5, which had a higher enzyme content than that of treatments 3 and 4, showed slightly higher levels of by-products at hydrolysis equilibrium and a faster rate of interesterification.

The triacylglycerol reaction profile were used to identify the reaction times at which a 1,3-specific or randomised product was obtained. This was the point at which the triacylglycerol reaction profile distance values were closest to zero and the opposite profile was closest to the value calculated when comparing the two theoretical fully interesterified triacylglycerol compositions. In chapter 5, this value was calculated to be

24.0, which is still valid in these studies as the same source of Malaysian cocoa butter was used. The reaction times at which samples were determined to be close to 1,3-specific and random interesterified were identified for all treatments where practical, given the limited number of samples taken during the interesterifications.

The solid fat contents of the samples taken closest to the reaction times where the sample was identified as 1,3-specific and random were compared, in order to relate the changes in the solid fat profiles to changes in the triacylglycerol composition of the interesterified cocoa butter. The solid fat content results for the samples from the individual treatments at the reaction times identified as 1,3-specific and random are given in Table 7.3 and 7.4. It was found that similar melting profiles were obtained when the samples were compared that were at the same stage of the interesterification process, either 1,3-specific or random interesterified. The solid fat content values for each set of data were averaged and standard deviations calculated with results shown in Tables 7.3 and 7.4. These average values and their standard deviations for a 1,3-specific and random interesterified product were plotted along with untreated Malaysian cocoa butter and are presented in Figure 7.22.

Table 7.3. The solid fat content (%) results for the treatments specified at the reaction time closest to where a 1,3-specific interesterified product was identified.

		Temperature (°C)												
Trt	**Time (h)**	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**32.5**	**35**	**37.5**	**40**	**45**	**50**
1	**24**	85.22	82.29	76.37	66.46	58.16	46.04	27.28	14.42	10.21	6.64	4.54	0.66	0
2	**8**	86.84	83.91	78.64	70.47	63.59	49.28	27.76	11.31	7.26	4.22	2.11	0	0
3	**4**	86.89	84.78	79.45	69.81	59.95	45.26	21.19	7.02	4.31	1.6	0.75	0	0
4	**4**	86.99	85.02	79.43	71.63	63.85	47.15	22.56	5.43	3.09	1.38	0.96	0	0
5	**2**	85.55	83.17	76.49	67.31	59.89	46.5	24.84	9.25	5.97	3.77	2.21	0.49	0
Average		**86.30**	**83.83**	**78.08**	**69.14**	**61.09**	**46.85**	**24.73**	**9.49**	**6.17**	**3.52**	**2.11**	**0.23**	**0.00**
Std deviation		**0.84**	**1.13**	**1.54**	**2.18**	**2.51**	**1.52**	**2.87**	**3.54**	**2.76**	**2.15**	**1.51**	**0.32**	**0.00**

Table 7.4. The solid fat content (%) results for the treatments specified at the reaction time closest to where a random interesterified product was identified.

		Temperature (°C)												
Trt	**Time (h)**	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**32.5**	**35**	**37.5**	**40**	**45**	**50**
3	48	71.78	68.83	64.57	55.2	46.05	42.79	39.84	36.63	31.92	26.28	20.83	10.42	0
4	48	72.4	68.89	64.03	53.69	43.87	40.43	37.56	34.19	29.97	24.09	18.42	8.19	0
5	24	71.96	69.55	63.96	50.99	43.16	39.88	37.17	33.64	29.05	23.47	18.58	8.3	0
6	24	69.23	67.25	63.6	55.22	47.38	44.15	40.82	37.43	32.59	25.98	19.8	7.86	0
Average		**71.34**	**68.63**	**64.04**	**53.78**	**45.12**	**41.81**	**38.85**	**35.47**	**30.88**	**24.96**	**19.41**	**8.69**	**0.00**
Std deviation		**1.43**	**0.98**	**0.40**	**1.99**	**1.95**	**2.01**	**1.77**	**1.84**	**1.65**	**1.39**	**1.13**	**1.17**	**0.00**

Figure 7.22 Solid Fat Content curves for untreated Malaysian cocoa butter and the average data for samples taken during the interesterification of Malaysian cocoa butter under various initial reaction conditions at reaction times where the samples were identified as 1,3-specific interesterified and random interesterified.

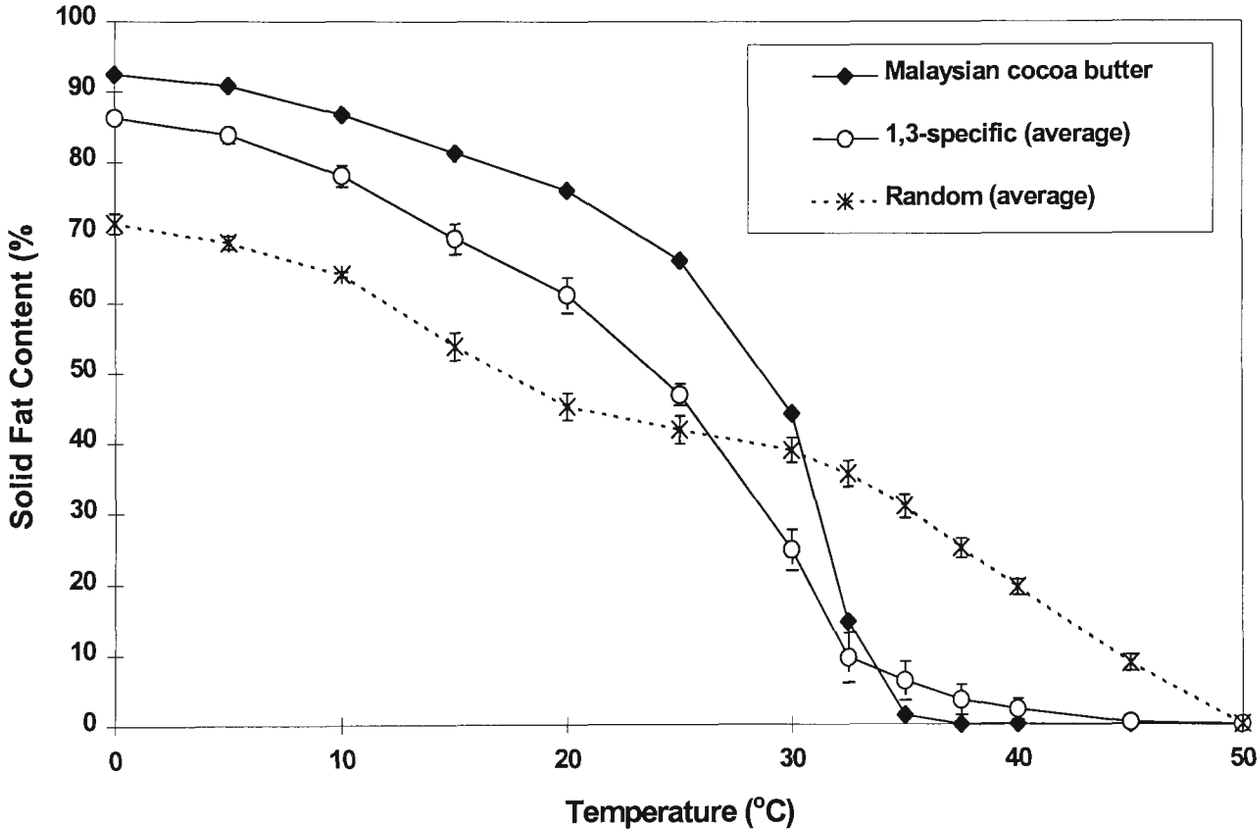


Figure 7.22 showed that there was a considerable difference between the melting profiles of a 1,3-specific and random interesterified cocoa butter product and the untreated cocoa butter. At a 1,3-specific interesterified triacylglycerol composition, solid fat contents were reduced at 30°C and below. At randomised interesterified triacylglycerol composition, the solid fat contents were reduced, further than for 1,3-specific

interesterification, at and below 30°C, while the solid fat contents increased significantly above these temperatures.

The averaged profiles appear to represent the solid fat profiles of a 1,3-specific and random interesterified product very well, with small standard deviations. The lipid class values for the samples that were identified as 1,3-specific or random were different, although this seems to have only a minor effect. This suggests that the degree of interesterification had a greater influence over the changes in the physical properties than the levels of by-products.

This type of information relating the solid fat content data to the stage of the interesterification process could be an additional tool to use when assessing the enzymatic interesterification of cocoa butter under different reaction conditions, as well as distinguishing between 1,3-specific and random interesterified products.

The triacylglycerol reaction profiles demonstrated that the Malaysian cocoa butter was eventually randomised, even though Lipozyme is a 1,3-specific lipase. This has also been found in other studies of enzymatic interesterification (Zhang et al, 2000). It is thought that the process behind this phenomenon is acyl migration, although the exact mechanism for the randomisation cannot be determined from these studies. The triacylglycerol reaction profile method, however, allows the randomisation process to be monitored and the factors affecting it examined. This may provide tools for controlling the enzymatic interesterification process where randomisation of the fat is undesired.

Mohamed et al (1993) found that 1,3-diacylglycerols were at a higher level than 1,2-diacylglycerols during interesterification under solvent-free conditions. They suggested that a 1,3-specific lipase was unable to deacylate 1,3-diacylglycerol. In other studies it was found that Lipozyme has a preference for 1,2-diacylglycerol over 1,3-diacylglycerol as a substrate, therefore isomerisation must first take place for reaction (Ergan et al, 1991). The formation of 1,3-diacylglycerols via acyl migration involves a number of

steps (Bloomer et al, 1992). Firstly, 1,2(2,3)-diacylglycerols are hydrolysed to form 2-monoacylglycerols. These monoacylglycerols are unstable and isomerise to form a 1(3)-monoacylglycerol. This 1(3)-monoacylglycerol is then re-esterified by the lipase to form a 1,3-diacylglycerol. This process can occur not only in interesterification, but also with transesterification and triacylglycerol synthesis (Soumanou et al, 1998).

Acyl migration can be influenced by a number of factors including; the water content; (Forssell et al, 1993; Lortie et al, 1993, Xu et al, 1998a); the hydrophobicity of any solvent used (Willis and Marangoni, 1997); reaction time, with longer times resulting in higher yields but increased acyl migration (Mu et al, 1998, Xu et al, 1998a); and reaction temperature, with increased temperatures associated with increased diacylglycerol levels (Mu et al, 1999).

It has been shown in the studies reported in this chapter that the initial enzyme and water contents can influence the outcomes of a batch enzymatic interesterification. The eventual randomisation of the cocoa butter, whether due to acyl migration or the specificity of the enzyme, can be monitored using the triacylglycerol reaction profile method. The distinction between 1,3-specific and random interesterification is very important when investigating the effects of reaction conditions on the interesterification process.

There were definite relationships between the enzyme and water contents and the measured interesterification outcomes. Lower enzyme and water contents produced a 1,3-specific interesterified product after a prolonged reaction time. To produce a 1,3-specific interesterified product in a shorter time, the water and enzyme should be increased, however, the levels of by-products will be greater, lowering the yield. These factors were also considered by Xu et al (1998a and 1999b) for the production of structured lipids using Lipozyme. They suggested that a continuous reactor may overcome the problem of acyl migration. In this thesis, only a batch reaction process was

used, however, studies utilising a continuous reactor system are recommended for future work.

7.1 Chapter conclusions

The effects of varying the initial enzyme and water contents on the enzymatic batch interesterification process were investigated by several interesterifications of Malaysian cocoa butter under various initial reactions conditions in the ranges of 1.6-10.6% for the enzyme content and 0.22-1.03% for the water content. For the enzymatic interesterifications where the initial water content was adjusted by introducing additional water to the reaction system, the location of this additional water was investigated. The work showed that whether the water was added to the enzyme, or directly to the cocoa butter prior to the addition of the enzyme, did not affect the outcomes of the interesterification.

In general, it was found that as the enzyme and water content were increased, the rate of the interesterification process also increased. This could be determined from comparing the triacylglycerol reaction profiles of the interesterifications carried out under different initial reaction conditions. The duration of the reaction was an important factor in determining whether a 1,3-specific or random interesterified product would be obtained. It was shown that 1,3-specific products were generated under certain conditions in a relatively short time, however if the reaction was allowed to proceed further, a randomised product resulted. This was also shown earlier in Chapter 5, p61.

The levels of lipid classes at hydrolysis equilibrium, which was reached after 2 hours of reaction in most cases, was related to the initial water content, with increased water contents resulting in increased levels of by-products and consequently lower yields of interesterified triacylglycerols. For example, at 24 hours of reaction for the treatments with an enzyme content of 4.2%, the treatment with a water content of 0.22% water had a triacylglycerol content of 92.0% compared to a triacylglycerol content of 83.9% for the treatment with a water content of 0.46%.

The solid fat content profiles of samples taken at 24 hours were compared and found to be different and dependent on the stage of the interesterification process when related to the triacylglycerol reaction profiles. The reaction times, at which the samples taken during the interesterification were 1,3-specific or random interesterified, were identified from the triacylglycerol reaction profiles, and the solid fat profiles of the samples taken closest to these times were averaged then compared. It was found that the solid fat profiles of the samples identified to represent 1,3-specific and random interesterified samples for each treatment were very similar, despite the considerable difference in the reaction times and lipid class levels. It was clearly demonstrated when these solid fat curves were compared, that the solid fat profiles of 1,3-specific and random interesterified Malaysian cocoa butter were quite distinct from each other and the original untreated cocoa butter. This information could be used as another tool for determining the stage of the interesterification reaction using a relatively simple analytical technique.

In summary, the studies in this chapter confirmed that there was a relationship between the initial reaction conditions of enzyme and water content and the outcomes of the interesterification as assessed by the solid fat content, the lipid class levels at hydrolysis equilibrium and the triacylglycerol reaction profiles. Further studies were undertaken to quantify this relationship by developing equations describing the outcomes of the interesterification process based on the initial reaction conditions. This work is reported in the following chapters.

Chapter 8

Preliminary investigations into the development of model equations to describe the outcomes of enzymatic interesterification based on the initial reaction conditions

In Chapter 7 it was found that the initial reaction conditions of enzyme and water content had an effect on the interesterification process when Malaysian cocoa butter was used as a substrate. The work in this current chapter was aimed at further investigating the effects of changing the initial reaction conditions and developing equations that describe the outcomes of enzymatic interesterification. The mathematical concepts were developed by the author, however Dr John Reynolds, a biometrician with the Victorian Department of Natural Resources and Environment, was consulted regarding the experimental design and the writing of the Genstat programs for analysing the data. Dr Reynolds provided several basic Genstat run programs in the Genstat code that were used and adapted appropriately, refer to Appendix 6. A number of enzymatic interesterifications were carried out under different initial reaction conditions and the interesterifications assessed through solid fat content, lipid class and triacylglycerol reaction profile analysis.

8.1 Malaysian cocoa butter interesterifications

A different batch of Malaysian cocoa butter (Mars Confectionery, Ballarat, Victoria) was used than that employed in Chapter 5. The Malaysian cocoa butter was characterised for overall and 2-positional fatty acid compositions, which are presented in Table 8.1.1, and the theoretical triacylglycerol compositions were calculated for fully 1,3-specific and random interesterified cocoa butter using the Tricalc program, which are presented in Table 8.1.2.

Table 8.1.1 Overall and 2-positional fatty acid composition (wt %) of Malaysian cocoa butter.

	Fatty acid (mol %)								
Cocoa butter	**C14:0**	**C16:0**	**C16:1**	**C17:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**
Overall	0.10	26.33	0.22	0.21	36.22	32.64	2.44	0.18	1.21
2-MG's	0.27	3.94	0.00	0.00	3.45	83.81	6.21	0.00	0.00

Table 8.1.2 Calculated theoretical triacylglycerol compositions for the 1,3-specific and random interesterification of Malaysian cocoa butter.

	Malaysian Cocoa butter (relative wt%)	
TG	**1,3-specific**	**Random**
T48	0.68	1.92
T50	15.39	15.13
T52	45.10	41.57
T54	36.62	39.37
T56	2.21	2.00

The fatty acid composition and distribution of this batch of Malaysian cocoa butter was found to be very similar to that of the Malaysian cocoa butter analysed previously in Chapter 5 (Table 5.2.1). The calculated theoretical triacylglycerol composition for completely random and 1,3-specific interesterified cocoa butter were also similar to the cocoa butter used in Chapter 5 (Table 5.2.2).

The water contents of the cocoa butter and the Lipozyme used were analysed according to the Karl Fischer procedure described in section 3.5 and were 0.06% for Malaysian cocoa butter and 3.76% for Lipozyme.

Experimental Procedures

Malaysian cocoa butter, batch size 80g, was interesterified according to the procedure described in section 3.1 and at the initial water and enzyme content treatments outlined in Table 8.1.3. A fresh batch of Lipozyme was used for these experiments, which had an activity of 7.0 BAUN/g according to the manufacturer. No checks were made on the relative activity between this batch and the old batch of Lipozyme. The experimental design included 3 levels of enzyme content and 7 different water contents. When extra water was required, it was added directly to the cocoa butter prior to the addition of the Lipozyme. The treatments were replicated entirely by repeating the experimental design

for treatments 1 to 12. One sample of untreated Malaysian cocoa butter was taken to represent the initial sample for all treatments and the replicates. Samples were taken after 2, 8, 24 and 48 hours and analysed for solid fat content (section 3.7), lipid class and triacylglycerol composition (section 3.11) and triacylglycerol reaction profiles (section 5.3). The results of these enzymatic interesterifications are presented in table form, with particular results and comparisons highlighted with further figures and tables in a discussion of the main findings.

Table 8.1.3 Experimental design outlining enzyme and initial water content treatments for interesterification of Malaysian cocoa butter.

Treatment number	Lipozyme % w/w	Overall Water %
1	0.65	0.08
2	0.65	0.11
3	0.65	0.13
4	0.65	0.20
5	2.00	0.13
6	2.00	0.20
7	2.00	0.27
8	2.00	0.48
9	5.48	0.27
10	5.48	0.48
11	5.48	0.66
12	0	0.06

Results:

Table 8.1.4 Solid fat content results (%) for samples taken during the interesterification of Malaysian cocoa butter under the initial reaction conditions specified for the 12 treatments, where %L = % Lipozyme and %W = % water.

		Temperature (°C)													
	Time (h)	0	5	10	15	20	25	27.5	30	32.5	35	37.5	40	45	50
Cocoa butter (0h)		92.45	91.18	87.95	82.56	78.15	68.39	60.62	43.43	15.98	1.38	0	0	0	0
Trt 1	2	91.17	89.27	84.99	80.39	74.83	63.47	55.82	40.03	13.49	1.19	0	0	0	0
0.65%L	8	89.99	88.02	83.59	78.75	73.03	61.74	53.73	38.01	13.02	2.12	0.33	0	0	0
0.08%W	24	88.25	86.18	81.32	75.37	70.16	58.46	50.41	35.58	14.17	5.69	3.37	1.37	0	0
	48	86.31	83.94	78.72	70.91	64.86	54.13	46.3	33.72	16.33	9.83	6.06	3.6	0	0
Trt 2	2	90.72	88.77	84.5	79.62	73.8	62.29	54.1	37.94	12.5	1.3	0	0	0	0
0.65%L	8	89.47	87.52	82.53	77.76	71.85	60.01	51.99	35.47	12.58	2.6	1.03	0.38	0	0
0.11%W	24	87.66	85.4	80.19	73.62	68.34	56.35	48.07	32.97	13.33	5.85	3.13	1.83	0	0
	48	85.52	82.96	76.92	68.81	61.79	51.45	44.46	32.18	16.99	10.82	7.31	4.33	0	0
Trt 3	2	90.63	88.78	84.26	78.68	72.86	61.17	52.36	35.83	11.42	0.61	0	0	0	0
0.65%L	8	89.47	87.31	82.44	76.85	71.08	58.91	49.89	33.6	10.75	2.33	0.87	0	0	0
0.13%W	24	87.44	84.94	79.5	72.78	67.04	55.23	46.8	32.53	13.48	6.96	4.25	2.05	0	0
	48	84.86	82.13	76.24	66.73	59.43	49.76	42.95	31.85	18.41	13.33	8.99	5.99	0.59	0
Trt 4	2	90.31	88.39	83.34	76.21	69.35	57.82	48.89	32.75	9.56	0.58	0.36	0	0	0
0.65%L	8	88.44	86.25	81.04	74.46	68.29	55.82	47.03	30.88	9.59	2.74	1.68	0.54	0	0
0.20%W	24	85.49	82.72	76.74	68.99	63.36	50.92	43.25	29.51	12.9	8.19	5.51	2.89	0	0
	48	83.1	80.11	73.66	63.04	51.82	44.72	38.78	30.68	20.16	15.32	11.08	7.44	1.1	0
Trt 5	2	89.4	87.24	81.99	77.34	71.03	59.23	50.55	34.18	11.04	1.83	0.4	0	0	0
2.0%L	8	87.68	85.33	79.95	74.13	68.62	56.7	48.07	32.59	11.57	4.29	2.78	1.29	0	0
0.13%W	24	84.58	81.57	75.53	66.76	59.36	49.83	42.9	31.67	17.29	12.04	8.11	5.31	0	0
	48	83.35	80.61	74.87	64.89	49.67	42.66	37.77	32.8	25.56	20.7	15.43	11.66	3.89	0
Trt 6	2	89.07	87.08	81.3	74.93	68.91	55.91	47.1	31.68	8.97	2.39	0.78	0	0	0
2.0%L	8	86.52	83.82	78.18	71.36	65.79	53.03	44.37	29.65	10.88	6.39	4.1	2.04	0	0
0.20%W	24	82.91	79.96	73.51	62.83	50.23	43.9	37.9	30.47	19.85	15.2	10.73	7.68	1.54	0
	48	80.8	77.99	71.79	60.98	43.89	34.89	34.06	31.85	29.15	24.25	19.15	14.71	6.26	0
Trt 7	2	88.66	86.46	80.79	73.09	66.66	54.3	45.27	29.98	8	1.86	1.14	0.3	0	0
2.0%L	8	85.44	82.71	76.81	69.03	63.18	50.71	42.05	27.82	10.99	7.39	5.03	3.12	0.43	0
0.27%W	24	83.81	81.11	75.05	64.1	46.67	35.72	32.74	27.17	21.91	17.64	12.93	9.28	2.25	0
	48	77.54	74.2	68.19	56.76	40.72	36.74	35.9	33.79	30.8	26.77	21.11	16.81	7.72	0
Trt 8	2	87.07	84.5	78.18	66.09	57.63	46.78	38.84	24.65	6.62	3.27	1.88	1.41	0	0
2.0%L	8	86.07	83.48	78	65.65	46.36	34.94	30.16	21.38	14.21	10.64	6.9	4.38	1.91	0
0.48%W	24	77.57	74.49	67.99	54.83	37.25	32.8	31.65	29.61	27.05	22.89	17.84	13.45	4.56	0
	48	71.38	66.99	61.89	52.25	43.72	40.9	39.79	37.89	35.02	30.32	24.74	19.34	9.6	0
Trt 9	2	86.43	83.63	77.36	71.46	65.6	53.27	44.44	28.95	8.34	3.61	1.97	0.95	0	0
5.48%L	8	82.52	79.31	72.6	62.33	54.65	45.24	38.62	28.75	16.43	11.99	8.6	6.41	1.28	0
0.27%W	24	78.28	75.16	68.68	57.47	40.81	35.26	34.4	32.45	29.4	24.88	19.98	15.47	7.05	0
	48	71.81	68.69	64.43	55.62	47.22	44.83	43.98	41.88	38.3	32.91	27.77	22.37	12.32	0.47
Trt 10	2	84.88	82.22	75.31	65.73	58.55	47.52	39.12	25.14	8.83	5.46	3.32	1.65	0	0
5.48%L	8	83.16	80.39	74.31	62.55	42.38	29.06	27.63	23.89	20.44	16.85	12.53	8.47	1.75	0
0.48%W	24	72.77	68.87	63.37	51.86	42.22	39.51	38.61	36.62	33.36	28.85	23.61	18.45	9.02	0
	48	72.14	70.36	65.69	57.5	48.95	46.32	45.4	43.46	39.34	34.24	28.71	23.11	12.14	0
Trt 11	2	86.34	83.63	77.54	63.75	44.57	35.36	29.6	20.9	11.46	7.81	4.68	2.77	0.96	0
5.48%L	8	79.31	76.22	69.32	56.52	36.22	29.5	28.11	26.09	23.17	19.44	14.3	9.98	2.38	0
0.66%W	24	69.91	65.67	61.58	52.52	44.43	41.77	40.83	38.72	35.39	30.77	24.74	19.55	9.46	0
	48	71.2	69.52	65.31	57.22	49.17	46.44	45.15	43.15	39.38	34.65	28.26	22.51	11.11	0
Trt 12	2	92.5	91.18	88.08	82.5	78.08	68.29	60.2	43.4	15.32	1.2	0	0	0	0
No L	8	92.63	91.23	87.95	82.55	77.94	68.06	60.49	43.63	16.01	2.01	0	0	0	0
0.06%W	24	92.71	91.26	87.97	82.3	77.95	68.03	60.25	43.63	16.07	2.2	0	0	0	0
	48	92.39	90.98	87.93	82.18	77.83	68.06	60.39	43.43	15.52	2.19	0	0	0	0

Table 8.1.5 Solid fat content results (%) for samples taken during the replicated interesterification of Malaysian cocoa butter under the initial reaction conditions specified for the 12 treatments, where %L = % Lipozyme and %W = % water.

		Temperature (°C)												
	Time (h)	0	5	10	15	20	25	30	32.5	35	37.5	40	45	50
Cocoa butter (0h)		92.34	91.13	87.9	82.41	77.76	67.91	43.26	15.04	1.27	0	0	0	0
Trt 1	2	91.18	89.42	85.61	80.59	75.1	64.18	41.1	13.71	1.2	0	0	0	0
0.65%L	8	89.91	87.9	83.58	78.89	73.34	61.75	38.8	13.73	1.91	0.41	0	0	0
0.08%W	24	88.43	86.31	81.56	76.05	70.61	59.02	36.89	14.17	4.94	2.91	0.29	0	0
	48	87.03	84.41	79.86	72.07	65.76	54.89	35.08	16.54	8.86	5.56	2.94	0	0
Trt 2	2	90.42	88.98	84.91	79.82	73.96	62.57	38.97	13.01	1.07	0	0	0	0
0.65%L	8	88.99	87.54	83.15	78.38	72.45	60.66	37.5	12.52	2.23	0.75	0	0	0
0.11%W	24	87.6	85.87	80.89	74.98	69.4	57.3	34.85	13.68	5.32	3.28	1.18	0	0
	48	85.77	83.31	78	70.44	63.34	52.75	33.66	16.78	10.1	6.69	3.69	0	0
Trt 3	2	90.68	88.86	84.31	79.17	73.07	61.4	37.89	12.2	0.96	0	0	0	0
0.65%L	8	92.34	90.94	88.08	82.68	77.94	68.08	44.18	15.65	1.61	0	0	0	0
0.13%W	24	87.15	85.33	80.04	73.16	67.57	55.39	33.56	13.26	5.99	3.71	1.39	0	0
	48	85.32	82.59	76.87	67.91	60.44	50.44	32.26	17.56	11.92	7.92	4.97	0	0
Trt 4	2	90.44	88.64	83.46	77.13	70.77	58.91	34.64	10.59	0.52	0	0	0	0
0.65%L	8	88.05	86.37	81.01	74.63	68.6	56.05	32.03	9.76	3.01	1.46	0.35	0	0
0.20%W	24	85.53	82.75	77.26	69.34	63.58	51.09	30.4	13.54	8.11	5.51	2.95	0.66	0
	48	83.08	80.24	74.04	63.76	52.42	45.12	30.92	19.88	15.16	10.84	7.26	1.44	0
Trt 5	2	88.99	86.39	81.41	77.13	70.9	58.82	34.73	10.34	1.32	0.21	0	0	0
2.0%L	8	86.67	84.6	78.84	73.55	67.9	55.67	33.03	11.57	4.38	2.63	1.05	0	0
0.13%W	24	84.17	81.14	75.35	66.64	58.94	49.43	31.73	17.08	11.67	7.82	5.17	0	0
	48	82.88	79.99	74.43	64.33	49.2	42.6	33.18	25.34	20.14	14.91	10.72	3.65	0
Trt 6	2	89.11	86.92	81.63	75.9	69.35	57.27	33.41	10.13	1.95	0.65	0	0	0
2.0%L	8	86.46	84.17	78.31	71.94	66.27	53.71	31.57	11.4	5.94	3.62	1.76	0	0
0.20%W	24	82.96	80.12	74	64.03	52.97	45.45	31.41	19.32	14.66	10.26	6.84	0.69	0
	48	81.39	78.76	72.8	62.74	45.43	35.05	31.99	28.8	24.34	19.04	14.28	6.44	0
Trt 7	2	88.7	86.72	80.84	73.78	67.39	54.99	31.66	8.77	1.8	0.47	0	0	0
2.0%L	8	84.91	82.59	75.99	68.9	62.82	49.98	28.62	11.27	7.46	4.73	2.68	0.55	0
0.27%W	24	83.54	80.96	74.96	64.03	47.09	37.23	27.93	21.73	17.51	13.1	8.86	2.13	0
	48	77.67	75.11	68.89	57.7	40.95	36.33	33.59	30.72	26.21	20.84	15.61	7.07	0
Trt 8	2	88.32	85.84	80.11	70.51	63.17	51.31	28.14	7.15	1.55	0.54	0	0	0
2.0%L	8	85.98	83.46	77.73	65.61	46.25	36.69	21.15	13.64	9.22	5.91	3.41	1.27	0
0.48%W	24	78.29	75.61	69.13	56.74	37.43	31.76	28.75	25.97	22.1	17.43	12.63	4.15	0
	48	71.33	67.01	62.26	52.45	43.6	40.58	37.72	34.54	30.13	24.89	19.18	9.36	0
Trt 9	2	87.12	84.7	78.8	73.39	67.51	54.57	31.16	9.17	3.37	1.74	0.66	0	0
5.48%L	8	82.76	79.81	73.44	63.98	55.51	45.91	29.86	16.78	12.17	8.45	5.28	0.43	0
0.27%W	24	79.22	76.62	70.34	60	42.65	35.64	32.7	29.65	25.25	20.21	14.77	6.83	0
	48	72.03	69.4	64.85	55.58	47.19	44.79	41.5	38.44	32.91	27.33	21.71	11.62	0
Trt 10	2	85.45	82.54	76.52	66.58	60.23	48.12	26.31	8.52	4.73	2.58	1.66	0	0
5.48%L	8	83.13	80.64	74.66	63.01	42.95	29.41	23.53	19.98	16.14	11.93	7.98	1.57	0
0.48%W	24	72.54	69.88	63.84	52.14	41.88	38.85	36.27	33.15	28.57	23.5	17.92	8.58	0
	48	71.64	70.68	65.96	57.6	48.71	46.14	43.18	39.35	34.1	28.64	22.83	11.7	0
Trt 11	2	85.12	82.1	75.01	61.72	48.87	41.39	24.03	8.25	4.75	2.31	1.62	0	0
5.48%L	8	79.41	76.3	69.32	56.69	35.73	27.86	24.76	21.96	18.31	13.59	9.21	2.1	0
0.66%W	24	69.51	65.3	60.86	51.96	44.08	41.2	38.39	35.2	30.75	25.45	19.33	8.55	0
	48	70.69	69.43	65.05	56.9	48.9	46.33	43.17	39.6	34.27	28.32	22.11	10.75	0
Trt 12	2	92.58	91.31	88.2	82.78	78.18	68.29	44.15	15.31	1.57	0	0	0	0
No L	8	89.21	87.19	82.4	76.84	70.8	58.69	34.87	11.48	2.64	1.18	0	0	0
0.06%W	24	92.37	91.1	87.84	82.56	77.8	67.8	44.02	14.99	1.21	0	0	0	0
	48	92.32	91.24	87.43	82.4	77.66	67.95	43.99	15.27	0.88	0	0	0	0

Table 8.1.6 Lipid class results (wt%) for the samples taken during the interesterification of Malaysian cocoa butter under the initial reaction conditions specified for the 12 treatments, where %L = % Lipozyme and %W = % water.
Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

	Time (h)	FFA	MG	DG	TG
cocoa butter (0h)		0.63	0.00	1.91	97.46
Trt 1	**2**	1.71	0.00	4.73	93.56
0.65%L	**8**	1.53	0.00	6.03	92.44
0.08%W	**24**	1.96	0.00	5.23	92.81
	48	2.15	0.14	6.24	91.46
Trt 2	**2**	1.93	0.00	4.68	93.39
0.65%L	**8**	1.74	0.00	4.64	93.62
0.11%W	**24**	1.58	0.00	4.91	93.51
	48	2.05	0.00	4.50	93.45
Trt 3	**2**	2.06	0.00	4.47	93.47
0.65%L	**8**	2.08	0.00	4.36	93.56
0.13%W	**24**	2.02	0.00	4.01	93.97
	48	2.41	0.00	4.68	92.91
Trt 4	**2**	2.53	0.00	6.35	91.11
0.65%L	**8**	2.60	0.00	7.52	89.88
0.20%W	**24**	2.53	0.00	7.70	89.77
	48	2.71	0.00	7.13	90.16
Trt 5	**2**	1.76	0.00	4.63	93.61
2.0%L	**8**	1.93	0.00	5.22	92.85
0.13%W	**24**	2.02	0.00	5.42	92.56
	48	2.01	0.00	5.09	92.90
Trt 6	**2**	4.45	0.00	8.74	86.82
2.0%L	**8**	4.69	0.00	9.05	86.26
0.20%W	**24**	4.49	0.00	9.01	86.50
	48	4.86	0.00	9.62	85.53
Trt 7	**2**	4.78	0.07	9.17	85.97
2.0%L	**8**	4.90	0.00	9.47	85.64
0.27%W	**24**	5.17	0.00	9.39	85.44
	48	5.31	0.06	10.10	84.53
Trt 8	**2**	6.10	0.18	12.13	81.60
2.0%L	**8**	6.34	0.27	10.98	82.41
0.48%W	**24**	7.25	0.17	12.50	80.08
	48	6.67	0.21	12.33	80.80
Trt 9	**2**	4.05	0.00	8.32	87.64
5.48%L	**8**	4.69	0.00	8.50	86.81
0.27%W	**24**	4.30	0.00	7.84	87.87
	48	5.04	0.03	9.09	85.85
Trt 10	**2**	7.16	0.14	13.17	79.54
5.48%L	**8**	8.25	0.17	13.79	77.79
0.48%W	**24**	8.33	0.17	14.00	77.50
	48	8.26	0.16	14.21	77.36
Trt 11	**2**	8.04	0.30	12.80	78.86
5.48%L	**8**	10.17	0.22	14.87	74.74
0.66%W	**24**	9.94	0.26	15.03	74.77
	48	9.30	0.24	14.67	75.78
Trt 12	**2**	0.90	0.00	2.78	96.32
No L	**8**	0.70	0.00	3.57	95.73
0.06%W	**24**	0.63	0.00	2.88	96.49
	48	0.90	0.00	2.81	96.29

Table 8.1.7 Lipid class results (wt%) for the samples taken during the replicated interesterification of Malaysian cocoa butter under the initial reaction conditions specified for the 12 treatments, where %L = % Lipozyme and %W = % water. Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

	Time (h)	FFA	MG	DG	TG
cocoa butter	**(0h)**	1.04	0.03	3.92	95.00
Trt 1	**2**	0.96	0.00	4.67	94.37
0.65%L	**8**	1.59	0.00	4.90	93.51
0.08%W	**24**	1.62	0.00	5.41	92.97
	48	1.61	0.00	4.83	93.56
Trt 2	**2**	1.96	0.00	6.97	91.07
0.65%L	**8**	2.08	0.00	7.01	90.91
0.11%W	**24**	1.99	0.04	7.07	90.89
	48	2.07	0.00	7.12	90.80
Trt 3	**2**	2.16	0.02	7.15	90.67
0.65%L	**8**	1.27	0.07	5.86	92.80
0.13%W	**24**	2.48	0.00	8.70	88.82
	48	2.79	0.00	8.68	88.53
Trt 4	**2**	2.86	0.08	7.88	89.18
0.65%L	**8**	3.22	0.03	8.17	88.57
0.20%W	**24**	3.20	0.17	8.41	88.22
	48	3.60	0.09	8.31	88.00
Trt 5	**2**	2.30	0.00	6.47	91.23
2.0%L	**8**	2.42	0.00	6.57	91.01
0.13%W	**24**	2.27	0.00	6.78	90.95
	48	2.60	0.00	7.49	89.92
Trt 6	**2**	3.26	0.05	9.67	87.02
2.0%L	**8**	3.67	0.02	10.57	85.74
0.20%W	**24**	3.48	0.02	10.38	86.11
	48	3.72	0.03	10.74	85.51
Trt 7	**2**	4.14	0.17	10.12	85.58
2.0%L	**8**	4.62	0.17	10.45	84.76
0.27%W	**24**	4.42	0.36	10.99	84.22
	48	5.22	0.19	11.20	83.39
Trt 8	**2**	5.43	0.14	11.25	83.18
2.0%L	**8**	7.23	0.41	14.57	77.78
0.48%W	**24**	7.45	0.36	15.09	77.10
	48	7.41	0.39	14.98	77.22
Trt 9	**2**	4.01	0.07	9.68	86.24
5.48%L	**8**	3.75	0.00	10.49	85.77
0.27%W	**24**	4.31	0.08	9.84	85.77
	48	4.43	0.03	10.67	84.87
Trt 10	**2**	6.96	0.65	14.49	77.89
5.48%L	**8**	7.11	0.29	15.24	77.36
0.48%W	**24**	7.78	0.50	15.55	76.17
	48	8.02	0.23	16.31	75.44
Trt 11	**2**	8.83	0.51	14.52	76.14
5.48%L	**8**	10.10	0.62	15.39	73.89
0.66%W	**24**	10.21	0.53	15.49	73.77
	48	9.83	1.18	15.58	73.41
Trt 12	**2**	0.68	0.00	2.99	96.33
No L	**8**	0.89	0.04	2.71	96.36
0.06%W	**24**	0.75	0.00	3.05	96.20
	48	0.69	0.00	3.25	96.05

Table 8.1.8 Triacylglycerol reaction profile distance values for samples taken during the interesterification of Malaysian cocoa butter under the initial reaction conditions specified for the 12 treatments and the replicated treatments, where %L = % Lipozyme and %W = % water. Standard deviations: Random (±0.32) and 1,3-specific (±0.17).

Treatment	Time (h)	Treatments		Replicated treatments	
		Random	1,3-specific	Random	1,3-specific
cocoa butter (0h)		43.09	8.75	51.88	13.85
Trt 1	2	38.73	7.50	48.81	10.38
0.65%L	8	36.20	6.58	46.95	8.44
0.08%W	24	35.50	4.24	42.15	6.34
	48	30.97	2.84	37.02	4.67
Trt 2	2	39.52	6.61	52.97	13.64
0.65%L	8	34.41	3.96	50.22	10.55
0.11%W	24	30.48	2.25	43.12	7.01
	48	25.14	1.38	39.76	4.68
Trt 3	2	40.14	5.80	71.35	22.47
0.65%L	8	37.90	4.11	74.58	25.98
0.13%W	24	33.05	1.83	56.63	13.31
	48	29.73	0.67	49.82	10.14
Trt 4	2	37.67	5.28	49.65	10.88
0.65%L	8	32.03	3.29	39.96	5.19
0.20%W	24	17.80	3.52	33.74	1.87
	48	17.36	2.55	25.30	0.77
Trt 5	2	34.29	3.43	49.35	9.57
2.0%L	8	32.53	2.52	42.89	6.16
0.13%W	24	22.83	1.44	34.67	3.34
	48	13.78	3.36	22.77	2.32
Trt 6	2	30.85	1.49	48.57	10.48
2.0%L	8	27.50	2.79	42.24	6.12
0.20%W	24	16.86	2.96	27.26	3.32
	48	9.06	5.50	16.11	3.10
Trt 7	2	34.11	4.36	62.15	17.00
2.0%L	8	26.24	1.79	48.97	7.55
0.27%W	24	12.90	2.66	30.48	3.67
	48	6.06	8.33	11.39	13.78
Trt 8	2	32.97	2.44	42.61	6.28
2.0%L	8	21.66	0.47	23.18	1.01
0.48%W	24	5.62	6.70	8.47	3.90
	48	1.57	12.21	3.12	11.15
Trt 9	2	32.51	2.21	43.63	6.39
5.48%L	8	22.34	1.63	31.38	1.84
0.27%W	24	7.22	5.96	11.82	3.65
	48	2.68	14.17	5.34	10.19
Trt 10	2	25.43	1.38	38.90	4.47
5.48%L	8	11.85	3.82	18.19	1.53
0.48%W	24	3.24	13.02	6.73	8.82
	48	2.95	17.15	5.73	11.32
Trt 11	2	25.57	0.55	45.89	6.59
5.48%L	8	8.00	6.09	16.49	0.82
0.66%W	24	1.27	14.14	8.43	10.54
	48	1.55	13.69	18.71	5.92
Trt 12	2	43.09	8.75	48.90	11.24
No L	8	39.24	7.01	47.38	10.37
0.06%W	24	41.43	8.01	49.90	11.73
	48	41.10	8.02	52.00	12.30

Discussion:

The solid fat content results for the treatments and their replicates were given in Tables 8.1.2 and 8.1.3. In general, similar patterns of changes were found to those in Chapter 7, with a flattening of the melting curve as the reaction progressed and larger changes occurring with increasing enzyme and water contents. The solid fat content at 5 and 35°C of samples taken after 24 hours of reaction for the treatments and replicates are given in Table 8.1.9. The solid fat contents of the samples taken at 24 hours for the treatment and replicate treatments were very similar. The results in Table 8.1.9 also highlight the fact that the different initial reaction conditions resulted in changes in the solid fat contents to different extents after 24 hours, which indicates that the interesterification process had progressed to different stages at this time.

Table 8.1.9 The solid fat content at 5°C and 35°C of samples taken after 24 hours of enzymatic interesterification for the treatments (A) and replicate treatments (B) under the initial Lipozyme and water contents specified.

			SFC @ 5°C		SFC @ 35°C	
Trt	**%Lipozyme**	**%Water**	**A**	***B***	**A**	***B***
Untreated cocoa butter	0	0.06	91.2	*91.1*	1.4	*1.3*
1	0.65	0.08	86.2	*86.3*	5.7	*4.9*
2	0.65	0.11	85.4	*85.7*	5.9	*5.3*
3	0.65	0.13	84.9	*85.3*	7.3	*6.0*
4	0.65	0.20	82.7	*82.8*	8.2	*8.1*
5	2.00	0.13	81.5	*81.1*	12.0	*11.7*
6	2.00	0.20	80.0	*80.1*	15.2	*14.7*
7	2.00	0.27	81.1	*81.0*	17.6	*17.5*
8	2.00	0.48	74.5	*75.6*	22.9	*22.1*
9	5.48	0.27	75.2	*76.6*	24.9	*25.3*
10	5.48	0.48	68.9	*69.9*	28.9	*28.6*
11	5.48	0.66	65.7	*65.3*	30.8	*30.8*
12	0	0.06	91.3	*91.1*	2.2	*1.2*

The lipid class results were presented in Tables 8.1.6 and 8.1.7. In general, the lipid class results also demonstrated similar trends to the findings in Chapter 7, where increasing enzyme and water content increased the levels of by-products and decreased the yield of triacylglycerols. For most treatments, hydrolysis equilibrium was reached after 2 hours of reaction. The levels of monoacylglycerols were very low (typically <0.5%) and therefore it would not be practical, nor appropriate to use the monoacylglycerol data for

equation development. The monoacylglycerols continued to be monitored to check for anomalous results.

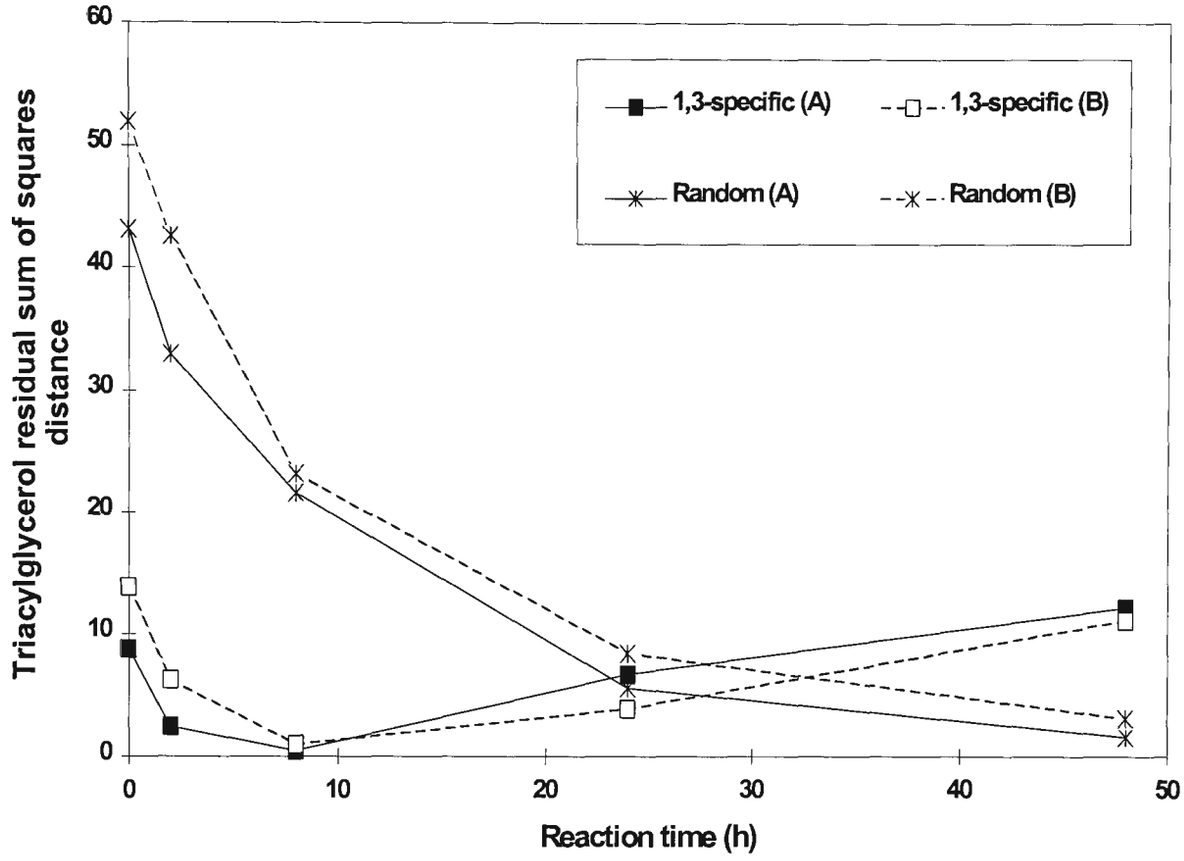
The lipid class results for FFA, DG and TG of samples taken after 24 hours of reaction for the treatments and replicates are given in Table 8.1.10 for comparison. It can be seen from this table that there was some variation in the levels of lipid classes between the treatments and the replicates.

Table 8.1.10 The lipid class results (wt%) of interesterified cocoa butter samples taken after 24 hours of reaction at the enzyme and water contents specified for each treatment (A) and replicate treatment (B).

Trt	Lipozyme (%)	Water (%)	FFA		DG		TG	
			A	*B*	A	*B*	A	*B*
Untreated cocoa butter	-	-	0.6	*1.0*	1.9	*3.9*	97.5	*95.0*
1	0.65	0.08	2.0	*1.6*	5.2	*5.4*	92.8	*93.0*
2	0.65	0.11	1.6	*2.0*	4.9	*7.1*	93.5	*90.9*
3	0.65	0.13	2.0	*2.5*	4.0	*8.7*	94.0	*88.8*
4	0.65	0.20	2.5	*3.2*	7.7	*8.4*	89.8	*88.2*
5	2.00	0.13	2.0	*2.3*	5.4	*6.8*	92.6	*91.0*
6	2.00	0.20	4.5	*3.5*	9.0	*10.4*	86.5	*86.1*
7	2.00	0.27	5.2	*4.4*	9.4	*11.0*	85.4	*84.2*
8	2.00	0.48	7.3	*7.5*	12.5	*15.1*	80.1	*77.1*
9	5.48	0.27	4.3	*4.3*	7.8	*9.8*	87.9	*85.8*
10	5.48	0.48	8.3	*7.8*	14.0	*15.6*	77.5	*76.2*
11	5.48	0.66	9.9	*10.2*	15.0	*15.5*	74.8	*73.8*
12	-	0.06	0.6	*0.8*	2.9	*3.10*	96.5	*96.2*

The triacylglycerol reaction profile distance values for the treatments and replicates were presented in Table 8.1.8. The triacylglycerol reaction profile results showed some variation between several of the treatments and their replicates. In general, the triacylglycerol reaction profile distance values for the replicate treatments were higher than for the first set of treatments. The shapes of the triacylglycerol reaction profiles were similar, when plotted and compared for example in Figure 8.1.1, which shows the triacylglycerol reactions profiles for treatment 8 and the replicate treatment 8. The reaction times at which 1,3-specific and random interesterified products were obtained and the rate of interesterification were similar for the treatment and the replicate.

Figure 8.1.1 The triacylglycerol reaction profiles for the interesterification of cocoa butter under the initial reaction conditions of treatment 8 (A) and the replicate treatment 8 (B), which was 2.0% Lipozyme and a water content of 0.48%.



In Chapter 7, the reaction times at which the interesterified samples were considered to be 1,3-specific and random were identified from the triacylglycerol reaction profiles and the melting profiles of the samples closest to these times were compared and found to be similar. This comparison between the physical properties and degree of interesterification was not made for these interesterifications due to the differences in the triacylglycerol reaction profiles between treatments and replicates, however this area was investigated further in the studies reported in Chapter 9.

The treatments at a level of 0.65% Lipozyme were anticipated to favour the 1,3-specific interesterification reaction. It was found that the interesterification process was quite slow at this level and that there was more variation in the triacylglycerol reaction profile results between treatments and replicate treatments. The very low water contents used may have limited the interesterification process by resulting in lower levels of diacylglycerols at hydrolysis equilibrium, the intermediates in the interesterification

process. The water content of the reaction system at such low levels may be more difficult to control, resulting in differences in the lipid class and triacylglycerol reaction profiles between treatments and replicates. No noticeable changes occurred in the control treatment that had no Lipozyme or water added, treatment 12.

It was also found that relatively small variations in the triacylglycerol composition between samples taken at the same reaction time for the treatments and replicate treatments were magnified into noticeable differences in the triacylglycerol reaction profile distances. It appeared that there might have been a systematic error influencing the results, which caused almost all of the triacylglycerol reaction profile distance values for the replicate treatments to be higher than those of the initial treatments.

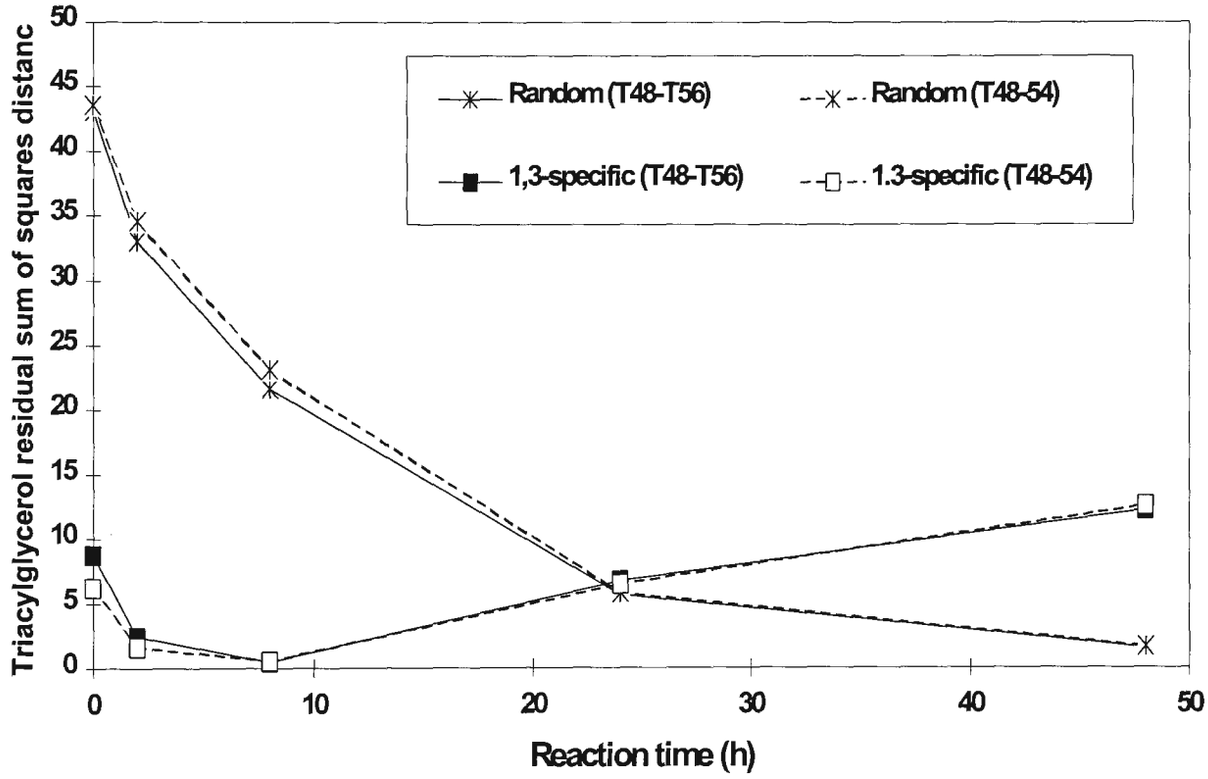
The triacylglycerol and lipid class method was modified to include an internal standard (section 3.12) in order to reduce any between run variation in results that may have been occurring. This internal standard method was used in future studies to analyse samples from enzymatic interesterification for triacylglycerol and lipid class composition.

The triacylglycerol reaction profile distance calculation was also simplified to involve less terms in the calculations by using the triacylglycerol results for the T48 to T54 peaks only, removing the T56 peak from the calculations. The T56 peak remains relatively the same throughout the interesterification process. The proportion of T56 expected in fully 1,3-specific (2.21) and random (2.00) interesterified Malaysian cocoa butter is very similar, and therefore not a major contributing factor to the triacylglycerol reaction profile distance values. This is unlike the T48 peak which, although also a small proportion, does increase with randomisation of the cocoa butter, with the proportions of T56 expected in fully 1,3-specific and random interesterified Malaysian cocoa butter being 0.68 and 1.92 respectively. Therefore, the results for the T48 peak contributes to a larger extent to the triacylglycerol reaction profile distance values than the T56 peak.

When the triacylglycerol reaction profiles are re-calculated without the T56 peak and compared to the triacylglycerol reaction profiles including the T56 peak, the distance values are only slightly different. For example, the triacylglycerol reaction profiles

calculated with and without the T56 peak are shown for treatment 8 in Figure 8.1.2. From this figure it can be seen that the triacylglycerol reaction profile distances decreased slightly for both random and 1,3-specific triacylglycerol reaction profiles. The calculations for the triacylglycerol reaction profile distance values using the results for the T48 to T54 peaks were used in all future interesterifications.

Figure 8.1.2 Comparison of triacylglycerol reaction profiles for treatment 8, the interesterification of cocoa butter under initial reaction conditions of 2.0% Lipozyme and overall initial water content of 0.48%, calculated using T48-T56 and T48-T54 triacylglycerol peaks.



These enzymatic interesterifications have highlighted the sensitivity of the triacylglycerol reaction profile method to accurate sample analysis. The following section reports on a further examination of the results regarding the development of equations to describe the outcomes of the reaction based on the initial reaction conditions.

8.2 Preliminary equation development

In the literature, there are several reports on optimisation and modelling studies relating to the use of immobilised lipases for the production of structured lipids via transesterification between a triacylglycerol source and a free fatty acid source. Approaches to modelling vary from response surface methodology (Shieh et al, 1995) to developing kinetic equations (Xu et al, 1998a). The input variables studied range from reaction time, temperature, the amount and type of solvent used, if any, together with substrate contents and ratios, as well as reactor configuration. The progress of the production of structured lipids via transesterification is usually monitored by analysing the amount of the free fatty acid that has been incorporated into triacylglycerol. There is a lack of modelling studies specifically relating to triacylglycerol:triacylglycerol interactions using a natural fat system.

The relationship between the water content and the lipid by-products appears to be linear in nature. This concurs with other studies where Forrsell et al (1992) also found that the degree of hydrolysis was linearly related to the initial water content for the batch interesterification of tallow and rapeseed oil using Lipozyme. More recently, Zhang et al (2000) in the batch interesterification of a palm stearin and coconut oil blend that addition of water to the enzyme increased the contents of the diacylglycerols and free fatty acids in the products linearly. No regression equations, however, were developed in their studies.

The approach in this thesis was to limit the studies to a minimum number of variables, then monitor the interesterification reaction, using methods that quantify the changes in the chemical composition. The results of these interesterifications were used to develop equations quantifying the relationships between the input variables and the outcomes of the interesterification process. The input variables for the development of equations included the initial reaction parameters of enzyme and water content, as well as the reaction time. The outcomes of the interesterification that the equations were based on included the lipid class composition and the triacylglycerol reaction profile distance values for random and 1,3-specific interesterification.

In this section, equations for the lipid class contents for FFA, DG and TG were developed. There were 4 data points (2, 8, 24 and 48 hours) for each treatment. The initial lipid class values (0 h) were not used at this stage due to the large increase in free fatty acid and diacylglycerol levels and subsequent decrease in triacylglycerol levels that occurred between 0 and 2 hours. There was also only one untreated cocoa butter sample taken to represent each group of treatments and the replicates, which was not statistically valid. The hydrolysis equilibrium was established relatively quickly (<2 hours) for most of the treatments, so that there was only slight changes to the lipid class levels between 2 and 48 hours. In other words, a linear (straight-line) relationship could be examined by using the data points from 2 hours onwards.

The lipid class results were averaged for the treatments and their replicates. The treatment variables were analysed using a Genstat software program using the data from treatments 1 – 11. The resulting linear equations included terms for the initial enzyme and water content, as well as the reaction time. The Genstat programs are given in Appendix 6.

Lipid class regression equations:

Where L = Lipozyme content (%), W = water content (%), H = Time (h)

Free fatty acids:

$$\%FFA = 0.29 + 0.123L + 12.9W + 0.012H \quad \text{.....8.2.1}$$

$R^2 = 96.6$ Standard error of observation (s.e) = 0.47

Diacylglycerols:

$$\%DG = 3.92 + 0.17L + 16.78W + 0.0181H \quad \text{.....8.2.2}$$

$R^2 = 90.7$ s.e = 1.05

Triacylglycerols:

$$\%TG = 95.91 - 0.275L - 30.64W - 0.0308H \quad \text{.....8.2.3}$$

$R^2 = 94.5$ s.e = 1.44

These preliminary equations highlighted the interactions between enzyme and water content as well as the effect of reaction time. The initial values for the lipid classes from these equations (when L, W and H = 0), were 0.29 for free fatty acids, 3.92 for diacylglycerols and 95.91 for triacylglycerols. Interestingly, these values were similar to the average values for the untreated cocoa butter (0.8 for free fatty acids, 2.9 for diacylglycerols and 96.3 for triacylglycerols) even though these points were not included in the results.

From the free fatty acid and diacylglycerol equations (Equations 8.2.1 and 8.2.2), it can be seen that any increase in enzyme, water or time results in an increase in the by-products. The water content has the largest coefficient term, by a factor of 10 over the coefficient for enzyme content, and therefore the largest influence on the levels of by-products. An increase in enzyme content, water content and time has the opposite effect for triacylglycerols, resulting in a lower level of triacylglycerols (Equation 8.2.3). Again, water has the largest influence on the level of triacylglycerols. In all of the equations, the effect of time was relatively small, as could be expected as the levels of lipid classes had almost reached equilibrium after 2 hours.

The percentage variance accounted for, reported as R^2, is actually an adjusted R^2 statistic and can be used as a guide to the fit of a model. The standard error of an observation is estimated by the square root of the residual mean square. These preliminary equations had relatively high R-squared values (> 90), and low standard errors. These equations suggested that the relationship between the initial reaction conditions and the lipid class levels could be described mathematically. The development of these equations was preliminary, in that a limited number of data points were used as well as averaged results for the treatments. In the next chapter, the development of model equations from further interesterifications is reported, with the suitability of these equations for predicting the levels of lipid classes assessed and reported in Chapter 10.

The triacylglycerol reaction profile results proved to be more complicated to develop equations that describe the outcomes based on the initial reaction conditions. As the profiles were curved in nature, rather than straight lines as in the lipid class results, the

equations needed to describe the curves would involve quadratic terms. The limiting factor with this data was that there were only four data points obtained, which prevented a more detailed analysis of the data due the higher degree of freedom required for more complex equations. These interesterification studies, together with the preliminary investigations into the development of model equations to describe the outcomes of interesterification based on the initial reaction conditions, formed the basis for further studies, where more samples were taken during the interesterification. These further interesterification studies are reported in the following chapter.

8.3 Chapter conclusions

This chapter reported on the preliminary studies into the development of equations for describing the outcomes of enzymatic batch interesterification based on the initial reaction conditions. Twelve interesterifications under different treatment conditions, at 3 different levels of enzyme (0.65, 2.00 and 5.48%) and water contents in the range of 0.08-0.66%, were carried out and replicated entirely, with samples taken after 2, 8, 24 and 48 hours.

It was found that the Malaysian cocoa butter used in these studies was similar to that used in Chapter 5, although they were from different batches. The initial levels of enzyme and water in the reaction system had a clear effect on the interesterification process and the levels of by-products at hydrolysis equilibrium. The enzyme content of 0.65% was too low to be of practical use, with a much slower reaction rate than the higher enzyme contents, however the amount of by-products was much lower. No significant changes were detected in the control treatment, with no added Lipozyme or water.

There were apparent similarities between the treatments and the replicates results for solid fat content and lipid classes, however, some variation was found in the triacylglycerol reaction profile distances. This variation appeared to be systematic, as the distance values for the replicate treatments were all higher than the corresponding treatment results. The curvature of the triacylglycerol reaction profiles was not affected and similar rates of interesterification occurred for the treatments and their replicates. This variation may have resulted from the triacylglycerol analysis, as relatively small

differences in the triacylglycerol composition translated into noticeable differences in the triacylglycerol reaction profile distance values. This also shows that the triacylglycerol reaction profile distances are sensitive analytical tools to characterise reactions of this kind. The analytical method for determining the triacylglycerol and lipid class composition was modified to include an internal standard to reduce any between run variation. The calculations for the triacylglycerol reaction profile distance values were simplified to include only the triacylglycerol peaks from T48 to T54, thus excluding the T56 peak.

The lipid class results showed that the level of monoacylglycerols were very low (<0.5%) and therefore it was not practical to develop equations or consider the results in further interesterifications. The monoacylglycerols continued to be monitored in case of any anomalous results. The relationships between the initial enzyme and water content and reaction times and the lipid class results for FFA, DG and TG linear equations were described by linear equations developed from the averaged data for the treatments conditions from 2 hours of reaction. These equations had R-squared values greater than 90, demonstrating by the size of the coefficient for each term that the water content had a much greater influence on the lipid class levels, than the reaction time or enzyme content. For example the equation for FFA was %FFA = 0.29 + 0.123L + 12.9W + 0.012H, where L = %Lipozyme, W = % water and H = reaction time (h).

The development of suitable equations for the triacylglycerol reaction profiles was more complicated, requiring more data points for meaningful analysis. These interesterification studies provided a basis for a revised experimental plan for further investigations into developing a predictive model for the solvent-free interesterification of fats and oils. The results of further investigations are reported in the following chapter.

Chapter 9

Development of model equations that describe the outcomes of enzymatic interesterification based on the initial reaction conditions

The work in this chapter builds on the preliminary studies in chapter 8, to develop several model equations that describe the outcomes of enzymatic interesterification based on the initial enzyme and water contents. Brazilian cocoa butter, which varied slightly in triacylglycerol composition from Malaysian cocoa butter, was used for these interesterification studies. In the first section of this chapter, the results of the interesterification of Brazilian cocoa butter under different initial reaction conditions are presented. In the following sections the model equations were developed that describe the lipid class levels as well as the triacylglycerol reaction profiles based on the initial reaction conditions. The author developed the mathematical concepts for this chapter, however Dr Reynolds provided assistance with the experimental design and the writing of the Genstat programs, which are given in Appendices 7 and 8.

9.1 Brazilian cocoa butter interesterifications

The overall and 2-positional fatty acid compositions of the Brazilian cocoa butter (Ballantyne Chocolates, South Melbourne, Victoria) were analysed according to the procedures described in sections 3.8 and 3.9, with the results presented in Table 9.1.1. The theoretical triacylglycerol compositions for completely random and 1,3-specific interesterified Brazilian cocoa butter were then calculated using Tricalc program (version 3, 1994) and normalised to 100% for the triacylglycerol carbon number groups T48, T50, T52 and T54, thus excluding T56, with the results presented in Table 9.1.2.

Table 9.1.1 Overall and 2-positional fatty acid composition (wt%) of Brazilian cocoa butter.

	C14:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0
Overall	0.05	25.39	0.26	0.26	36.22	33.52	2.91	0.18	1.20
2-Positional	-	4.91	-	-	4.69	83.11	7.30	-	-

Table 9.1.2 Calculated theoretical triacylglycerol compositions (relative weight %) for completely 1,3-specific and random interesterified Brazilian cocoa butter.

TG	1,3-specific	Random
T48	0.86	2.13
T50	16.77	16.32
T52	46.40	42.92
T54	35.98	38.63

The water contents of Brazilian cocoa butter and Lipozyme were analysed according to the Karl Fischer procedure described in section 3.5, and were 0.065% for cocoa butter and 4.42% for Lipozyme.

The overall and 2-positional fatty acid compositions of Brazilian cocoa butter (Table 9.1.1) were slightly different to that of Malaysian cocoa butter (Chapter 5, Table 5.2.1). This was to be expected as cocoa butters from different regions have slightly different lipid and hardness characteristics that can be related to the climatic variation in the growing regions (Chaiseri and Dimick, 1989). Malaysian cocoa butter is generally harder due to a uniform climate with consistent rainfall and temperature ranges, whereas in Brazil, the mean monthly temperatures fluctuate more, with lower temperatures from June through to September (Chaiseri and Dimick, 1989).

Experimental Procedures:

Brazilian cocoa butter, batch size 80g, was interesterified according to the procedure described in section 3.1, using the initial water and enzyme content treatments outlined in Table 9.1.3. Eight different enzyme and water combinations, with 4 combinations replicated were carried out for a total of 12 treatments. When extra water was required, it was added directly to the cocoa butter prior to the addition of the Lipozyme, 7.0 BAUN/g. The treatments were carried out in two batches, with treatments 1, 3, 9, 11 and 12 in the first batch and treatments 2, 4, 5, 6, 7, 8 and 10 in the second. A control, or blank treatment, where no enzyme or water was added to the cocoa butter, was included with each batch. This was to check for any reactions in the cocoa butter itself. There were, however, no significant changes found for the control, and the results are not reported here. Samples were taken after 0, 1, 2, 4, 6, 8, 24 and 48 hours. Selected samples (0, 2, 8, 24 and 48 hours) were analysed for solid fat content (section 3.7), while

all samples were analysed for lipid class and triacylglycerol composition (section 3.12) and triacylglycerol reaction profiles were developed for each treatment (section 5.3). The results for the solid fat content and lipid class contents are presented in Tables 9.1.4 and 9.1.5, and the triacylglycerol reaction profiles are presented in Figures 9.1.1 to 9.1.12.

Table 9.1.3 Experimental design outlining enzyme and initial water content treatments for interesterification of Brazilian cocoa butter.

Treatment	Lipozyme % w/w	Overall water %
1	1.25	0.12
2	1.25	0.20
3	1.25	0.20
4	1.25	0.40
5	2.5	0.17
6	2.5	0.30
7	2.5	0.30
8	2.5	0.48
9	4.0	0.24
10	4.0	0.24
11	4.0	0.48
12	4.0	0.48

Results:

Table 9.1.4A Solid fat content results (%) for samples taken during the interesterification of Brazilian cocoa butter under several initial reaction conditions (treatment 1: 1.25% Lipozyme and 0.12% water; treatments 2 and 3: 1.25% Lipozyme and 0.20% water; treatment 4: 1.25% Lipozyme and 0.40% water; treatment 5: 2.5% Lipozyme and 0.30% water; treatments 6 and 7: 2.5% Lipozyme and 0.30% water).

Trt	Time (h)	Temperature (°C)													
		0	5	10	15	20	25	27.5	30	32.5	35	37.5	40	45	50
1	0	91.02	89.74	85.81	80.16	74.53	64.20	57.10	41.90	11.25	1.15	0.36	-	-	-
1	2	89.28	86.74	81.88	75.53	68.43	55.83	46.59	31.24	7.04	0.55	-	-	-	-
1	8	87.08	85.36	80.13	73.19	66.72	53.32	43.92	29.16	7.98	2.44	1.61	0.78	-	-
1	24	85.95	83.17	77.34	68.47	61.24	49.15	40.46	27.84	10.36	6.18	4.17	2.12	-	-
1	48	83.50	80.34	74.53	64.23	52.75	44.36	37.43	28.44	16.31	10.87	7.95	5.80	-	-
2	0	91.48	89.63	85.61	79.80	74.51	64.88	56.66	40.49	12.62	1.06	-	-	-	-
2	2	89.30	86.84	81.48	74.12	66.86	54.47	43.94	27.99	6.46	0.46	-	-	-	-
2	8	86.91	84.00	78.39	70.98	64.29	52.49	42.58	27.22	7.78	3.17	1.25	0.66	-	-
2	24	83.84	81.02	74.82	64.15	53.77	45.46	37.48	26.87	13.83	10.19	6.61	4.07	-	-
2	48	83.57	80.94	74.82	64.65	45.75	33.08	30.67	26.01	21.68	17.66	12.93	8.97	1.67	-
3	0	91.02	89.74	85.81	80.16	74.53	64.2	57.10	41.9	11.25	1.15	0.36	-	-	-
3	2	89.35	86.79	81.76	73.42	65.26	52.98	42.75	27.94	5.8	0.70	-	-	-	-
3	8	86.36	84.39	78.63	70.95	63.78	50.42	41.23	26.37	7.36	3.72	1.90	0.76	0.24	-
3	24	84.11	81.53	75.09	64.81	56.07	44.43	35.97	25.63	11.92	7.96	5.81	3.08	-	-
3	48	84.10	81.21	75.75	64.37	46.59	35.16	31.56	25.90	19.77	14.4	11.35	8.25	0.56	-
4	0	91.48	89.63	85.61	79.8	74.51	64.88	56.66	40.49	12.62	1.06	-	-	-	-
4	2	88.52	86.29	80.49	67.65	52.23	43.18	33.56	21.64	3.87	1.59	0.60	-	-	-
4	8	86.40	83.65	77.98	65.02	40.09	24.97	22.30	16.52	11.15	8.06	4.50	2.70	1.17	-
4	24	77.17	74.68	67.65	54.18	34.9	30.12	28.70	26.88	23.97	20.20	15.33	10.49	2.55	-
4	48	70.57	66.36	60.71	50.35	41.15	38.42	37.12	35.29	32.41	28.15	22.71	17.53	7.24	-
5	0	91.48	89.63	85.61	79.8	74.51	64.88	56.66	40.49	12.62	1.06	-	-	-	-
5	2	88.70	86.52	81.12	75.2	68.5	55.65	45.33	29.77	7.79	1.83	0.71	0.69	-	-
5	8	85.74	83.58	77.98	70.53	63.99	52.16	42.78	28.50	10.05	5.91	3.57	1.61	-	-
5	24	83.08	80.23	74.09	63.42	49.88	43.72	37.34	29.04	18.84	15.30	10.75	7.40	0.55	-
5	48	80.59	78.19	72.36	61.88	44.11	33.81	32.72	30.7	27.75	23.40	17.98	14.1	5.23	-
6	0	91.48	89.63	85.61	79.8	74.51	64.88	56.66	40.49	12.62	1.06	-	-	-	-
6	2	87.94	85.61	79.69	71.55	64.40	51.83	41.62	25.62	4.70	1.56	0.48	-	-	-
6	8	83.95	81.42	74.87	65.22	56.31	46.19	37.51	25.85	11.47	7.97	5.22	2.3	0.59	-
6	24	82.05	79.31	72.96	61.54	41.47	28.45	27.09	24.74	22.02	18.21	13.38	9.23	1.81	-
6	48	74.67	71.68	65.35	53.78	40.33	37.23	35.98	34.15	30.97	26.92	21.62	16.78	7.37	-
7	0	91.48	89.63	85.61	79.80	74.51	64.88	56.66	40.49	12.62	1.06	-	-	-	-
7	2	87.69	85.64	79.97	71.80	64.28	51.93	41.93	26.06	5.15	1.63	0.44	-	-	-
7	8	84.37	81.52	75.09	65.31	56.51	46.19	37.07	25.63	10.55	7.35	4.71	2.8	-	-
7	24	82.02	79.56	73.36	61.93	41.94	28.71	27.26	24.8	21.62	18.02	13.55	9.34	1.51	-
7	48	74.33	71.79	65.26	53.70	40.34	37.05	37.11	34.16	31.25	27.00	21.90	16.87	7.32	-

Table 9.1.4B Solid fat content results (%) for samples taken during the interesterification of Brazilian cocoa butter under several initial reaction conditions (treatment 8: 2.5% Lipozyme and 0.48% water; treatments 9 and 10: 4.0% Lipozyme and 0.24% water; treatments 11 and 12: 4.0% Lipozyme and 0.48% water).

		Temperature (°C)													
Trt	**Time (h)**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**	**40**	**45**	**50**
8	0	91.48	89.63	85.61	79.80	74.51	64.88	56.66	40.49	12.62	1.06	-	-	-	-
8	2	86.87	84.67	78.55	66.61	56.66	46.30	36.60	22.00	4.06	1.88	0.41	-	-	-
8	8	85.14	82.47	76.78	64.58	41.65	26.7	24.37	18.99	13.89	10.38	7.22	4.16	1.05	-
8	24	75.54	73.30	66.56	53.50	36.87	32.96	31.85	29.65	26.86	22.81	17.72	13.12	4.24	-
8	48	70.34	66.43	61.92	52.67	43.78	40.98	39.36	38.07	34.69	30.31	24.90	19.05	8.61	-
9	0	91.02	89.74	85.81	80.16	74.53	64.20	57.10	41.90	11.25	1.15	0.36	-	-	-
9	2	87.63	85.36	79.27	72.66	65.27	51.75	42.39	27.22	6.1	2.16	1.15	0.86	-	-
9	8	83.90	81.63	75.24	66.10	58.13	45.73	37.13	26.13	11.41	7.15	6.90	3.59	0.63	-
9	24	82.70	79.81	74.05	63.35	44.03	30.72	29.14	26.11	22.62	17.03	13.93	9.97	1.91	-
9	48	75.09	72.25	66.11	54.85	41.04	37.94	36.8	35.28	31.39	24.90	21.62	17.69	7.53	-
10	0	91.48	89.63	85.61	79.80	74.51	64.88	56.66	40.49	12.62	1.06	-	-	-	-
10	2	87.72	85.47	79.90	72.85	66.05	53.02	42.09	26.63	6.60	1.96	0.89	-	-	-
10	8	84.00	81.29	74.97	65.63	56.75	46.68	38.34	26.73	12.93	9.39	6.10	3.71	-	-
10	24	81.55	78.50	72.79	62.16	43.32	31.57	30.39	27.75	24.93	20.82	16.16	11.57	4.06	-
10	48	73.96	71.13	65.17	54.27	42.91	40.05	38.78	37.19	33.7	28.89	23.64	18.54	9.08	-
11	0	91.02	89.74	85.81	80.16	74.53	64.2	57.1	41.9	11.25	1.15	0.36	-	-	-
11	2	85.60	83.65	77.26	65.72	56.17	44.28	35.37	21.82	5.09	2.53	1.55	0.93	-	-
11	8	84.30	81.36	75.74	62.95	40.65	26.1	23.84	19.51	15.25	10.11	7.93	5.41	1.1	-
11	24	73.97	71.53	65.03	51.33	37.77	34.5	33.51	31.30	28.00	22.47	18.75	14.99	4.96	-
11	48	70.08	67.51	63.31	53.74	45.11	42.26	41.25	39.42	35.49	28.75	24.62	20.15	9.38	-
12	0	91.02	89.74	85.81	80.16	74.53	64.20	57.10	41.90	11.25	1.15	0.36	-	-	-
12	2	86.28	84.01	77.83	65.49	55.74	44.39	35.12	22.09	5.93	3.33	2.28	1.77	0.38	-
12	8	84.31	81.41	75.83	63.03	40.28	25.41	23.37	19.24	15.6	11.25	8.20	5.38	1.38	-
12	24	74.14	71.24	64.73	50.99	37.53	34.37	33.58	31.59	28.21	22.04	18.8	14.64	5.12	-
12	48	70.15	67.15	63.29	53.93	45.00	42.16	41.33	39.42	35.21	28.52	24.7	19.92	9.33	-

Table 9.1.5A Lipid class results (wt%) for the samples taken during the interesterification of Brazilian cocoa butter under several initial reaction conditions as specified for the treatments used.

Treatment 1: 1.25% Lipozyme, 0.12% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.6	2.5	2.6	2.6	2.7	2.8	2.7	2.7
DG	2.9	4.8	5.1	4.8	5.6	5.4	5.4	5.5
TG	95.5	92.7	92.3	92.6	91.7	91.8	91.9	91.8
Treatment 2: 1.25% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.6	3.4	3.6	3.8	3.9	4.0	4.1	4.2
DG	2.7	5.8	6.8	7.4	6.8	7.7	7.3	8.2
TG	95.7	90.8	89.6	88.9	89.4	88.3	88.7	87.6
Treatment 3: 1.25% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.0	2.9	3.1	3.0	3.3	3.3	3.3	3.4
DG	2.3	5.7	6.5	6.5	7.1	6.8	7.3	7.3
TG	96.7	91.4	90.4	90.4	89.6	89.9	89.4	89.3
Treatment 4: 1.25% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.2	6.8	7.6	7.8	8.0	8.0	8.4	8.3
DG	2.7	10.7	13.1	13.9	14.2	14.4	15.2	15.2
TG	96.2	82.2	79.1	78.2	77.7	77.3	76.4	76.4
Treatment 5: 2.5% Lipozyme, 0.17% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.1	3.8	4.6	4.2	4.0	4.7	4.6	4.9
DG	4.6	7.9	8.7	9.7	8.8	8.8	9.4	11.3
TG	94.3	88.3	86.7	86.1	87.2	86.4	86.0	83.9
Treatment 6: 2.5% Lipozyme, 0.30% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.4	4.5	4.6	4.9	4.7	4.9	5.00	5.1
DG	2.7	8.1	8.8	9.2	8.9	9.6	9.5	9.8
TG	95.9	87.4	86.6	85.9	86.4	85.5	85.5	85.0

*Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

Table 9.1.5B Lipid class results (wt%) for the samples taken during the interesterification of Brazilian cocoa butter under several initial reaction conditions as specified for the treatments used.

Treatment 7: 2.5% Lipozyme, 0.30% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.2	4.6	4.7	4.9	4.8	5.1	4.9	5.3
DG	2.2	8.1	8.5	9.2	9.2	9.6	9.1	10.1
TG	96.6	87.2	86.8	85.8	85.8	85.2	85.9	84.5
Treatment 8: 2.5% Lipozyme, 0.48% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.6	6.5	7.1	7.3	7.3	7.8	7.6	7.8
DG	3.3	11.3	12.2	12.6	13.3	13.7	13.5	13.8
TG	95.1	82.1	80.6	80.0	79.2	78.4	78.7	78.2
Treatment 9: 4.0% Lipozyme, 0.24% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.1	3.5	3.6	3.7	3.3	3.9	4.0	4.0
DG	1.9	6.8	7.1	7.1	6.8	7.7	8.1	8.1
TG	97.0	89.7	89.3	89.2	89.8	88.5	87.9	87.8
Treatment 10: 4.0% Lipozyme, 0.24% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.2	3.6	3.7	3.9	3.8	4.1	4.1	4.3
DG	2.2	6.9	7.0	7.4	7.4	7.7	7.8	8.0
TG	96.6	89.5	89.3	88.7	88.8	88.3	88.0	87.7
Treatment 11: 4.0% Lipozyme, 0.48% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.6	7.5	6.8	7.4	7.3	7.5	8.2	7.9
DG	3.2	11.6	11.4	12.0	12.3	12.9	13.4	13.6
TG	95.2	80.9	81.8	80.5	80.3	79.5	78.4	78.5
Treatment 12: 4.0% Lipozyme, 0.48% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**48 h**
FFA	1.1	7.0	7.5	7.3	6.8	7.9	7.9	7.5
DG	2.3	11.9	12.0	12.4	12.6	13.1	13.5	13.5
TG	95.9	80.5	80.0	79.8	80.1	78.4	78.1	78.5

*Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2

Figure 9.1.1 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (1.25%) and water content of 0.12% (Treatment 1).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

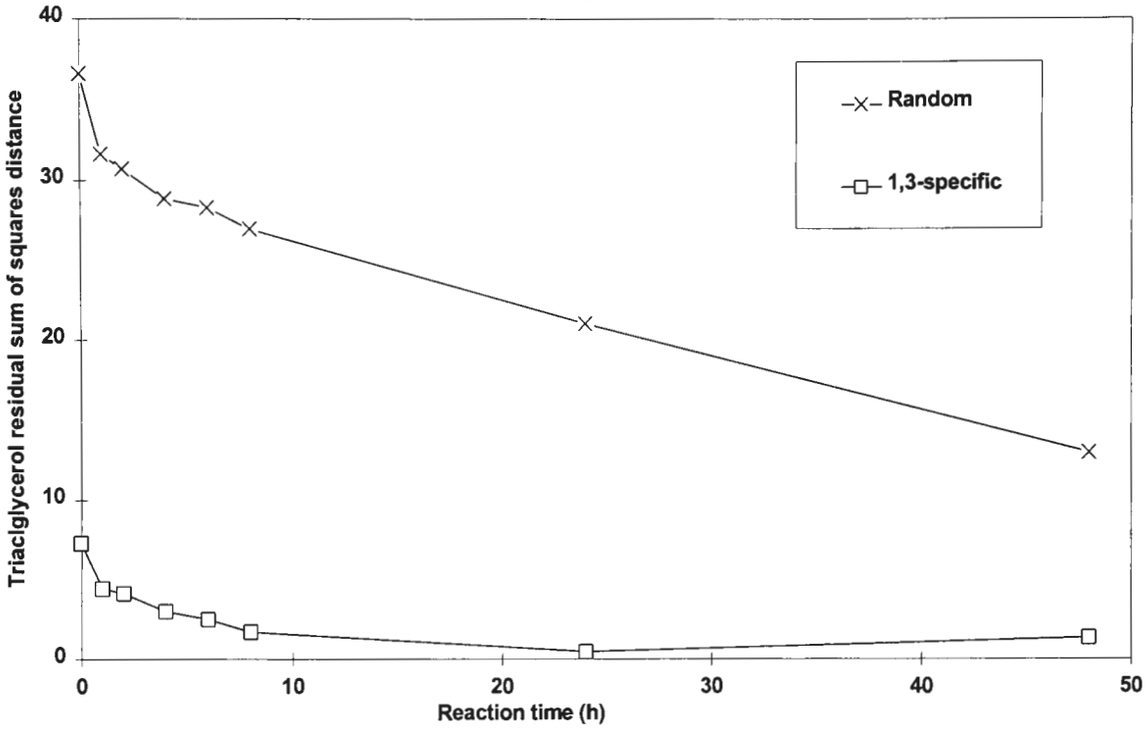


Figure 9.1.2 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (1.25%) and water content of 0.20% (Treatment 2).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

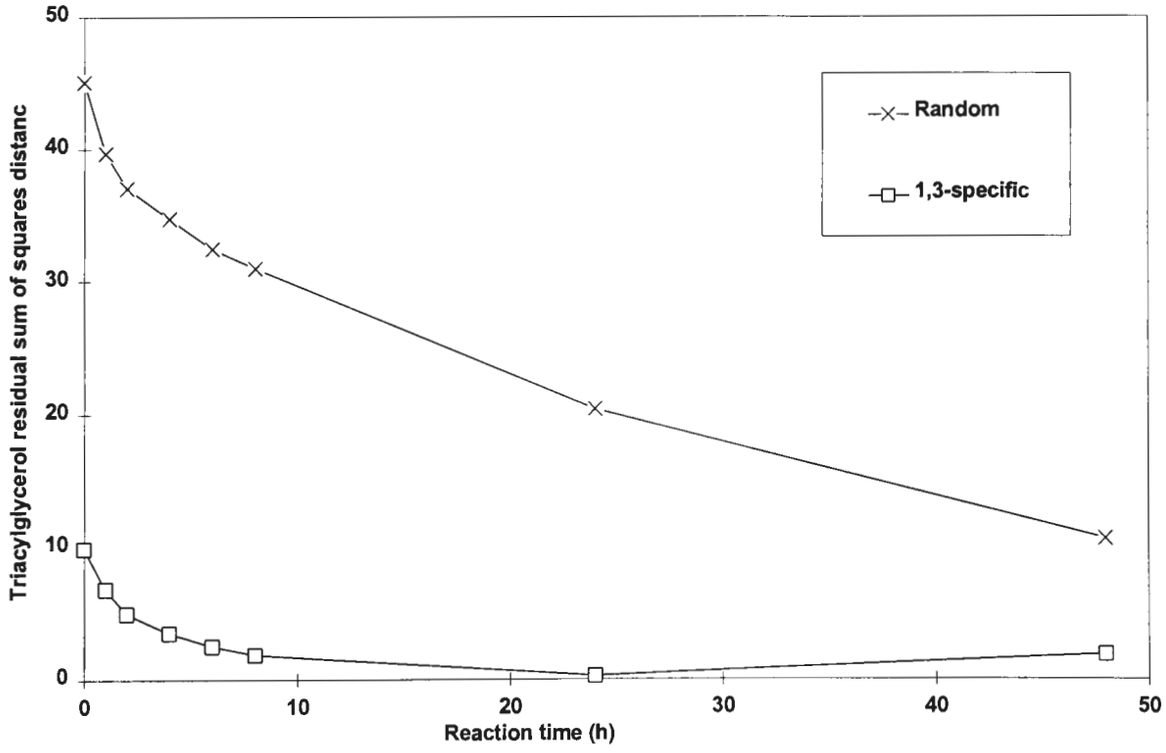


Figure 9.1.3 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (1.25%) and water content of 0.20% (Treatment 3).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

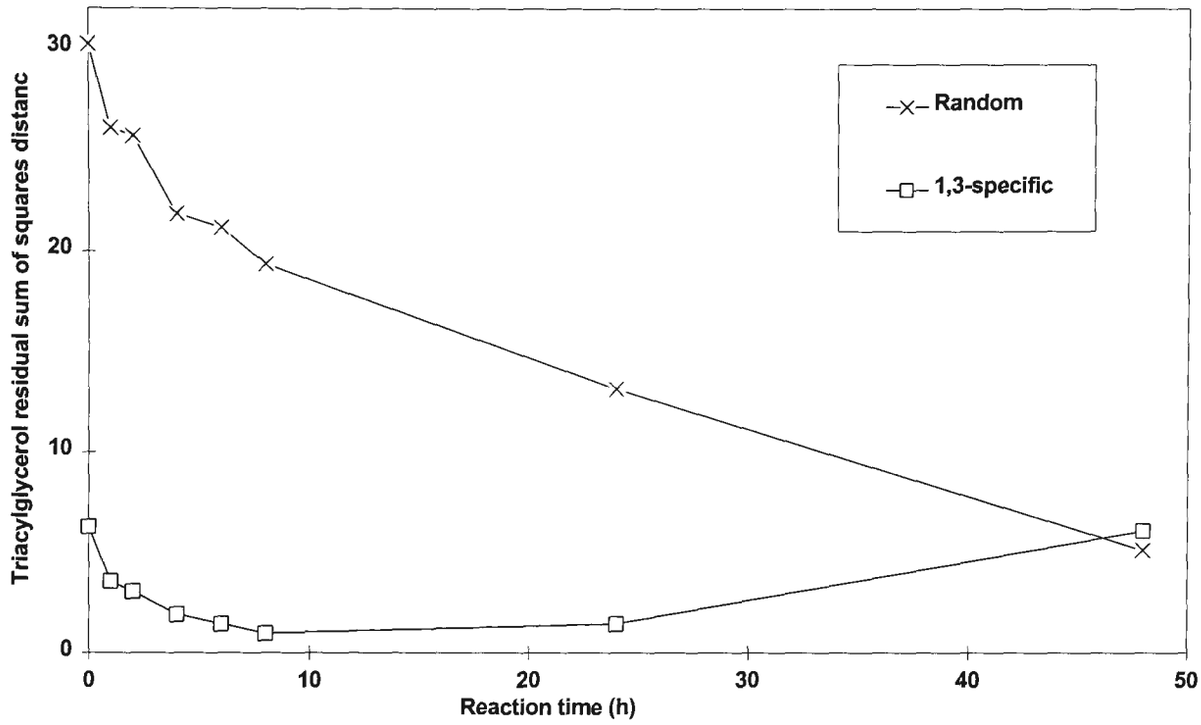


Figure 9.1.4 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (1.25%) and water content of 0.40% (Treatment 4).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

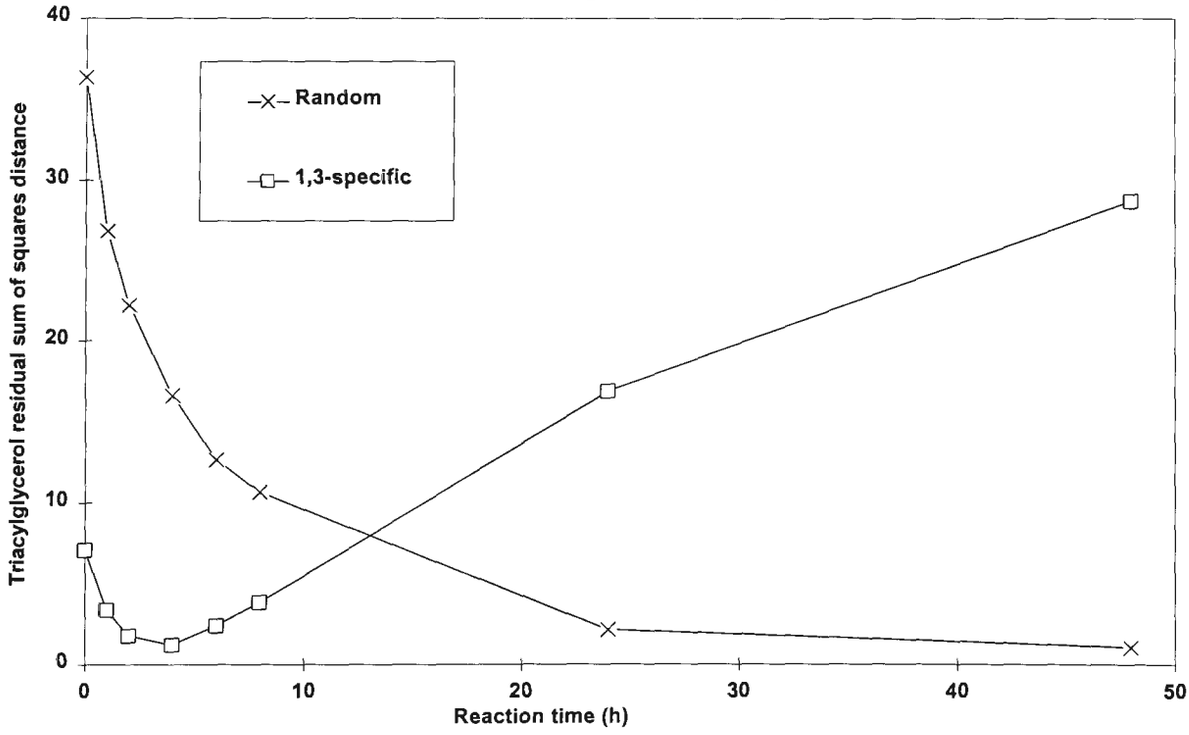


Figure 9.1.5 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (2.5%) and water content of 0.17% (Treatment 5).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

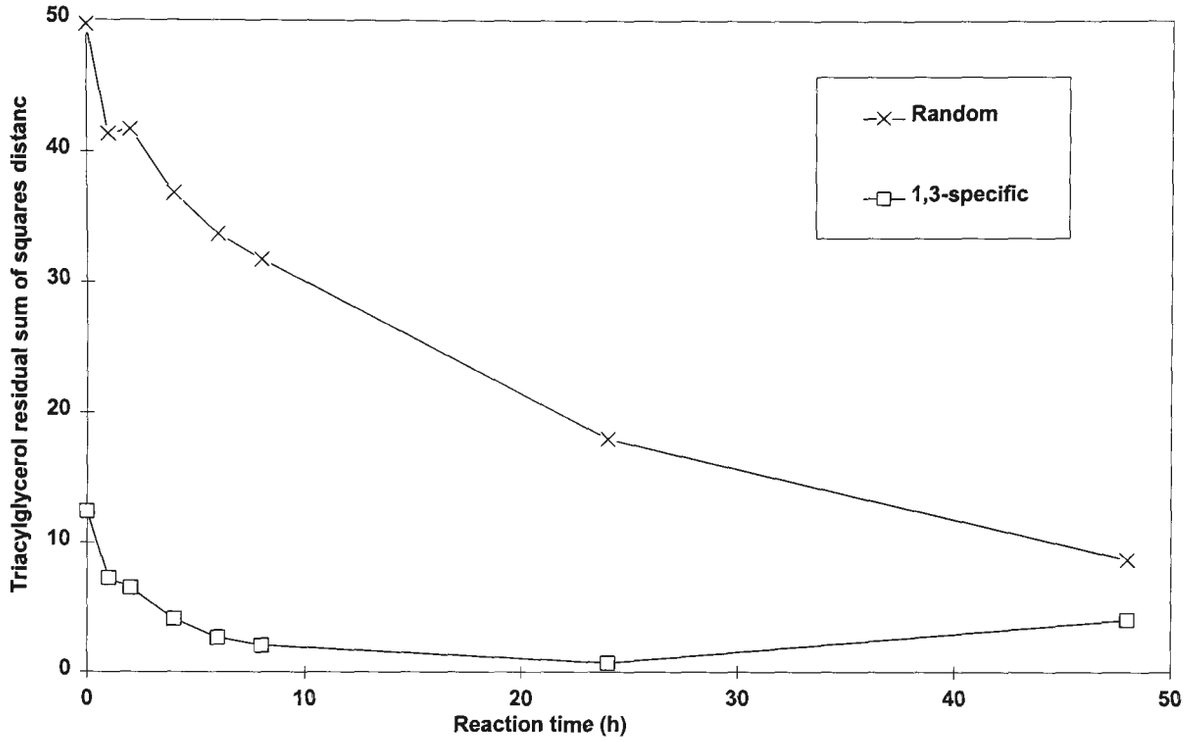


Figure 9.1.6 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (2.5%) and water content of 0.30% (Treatment 6).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

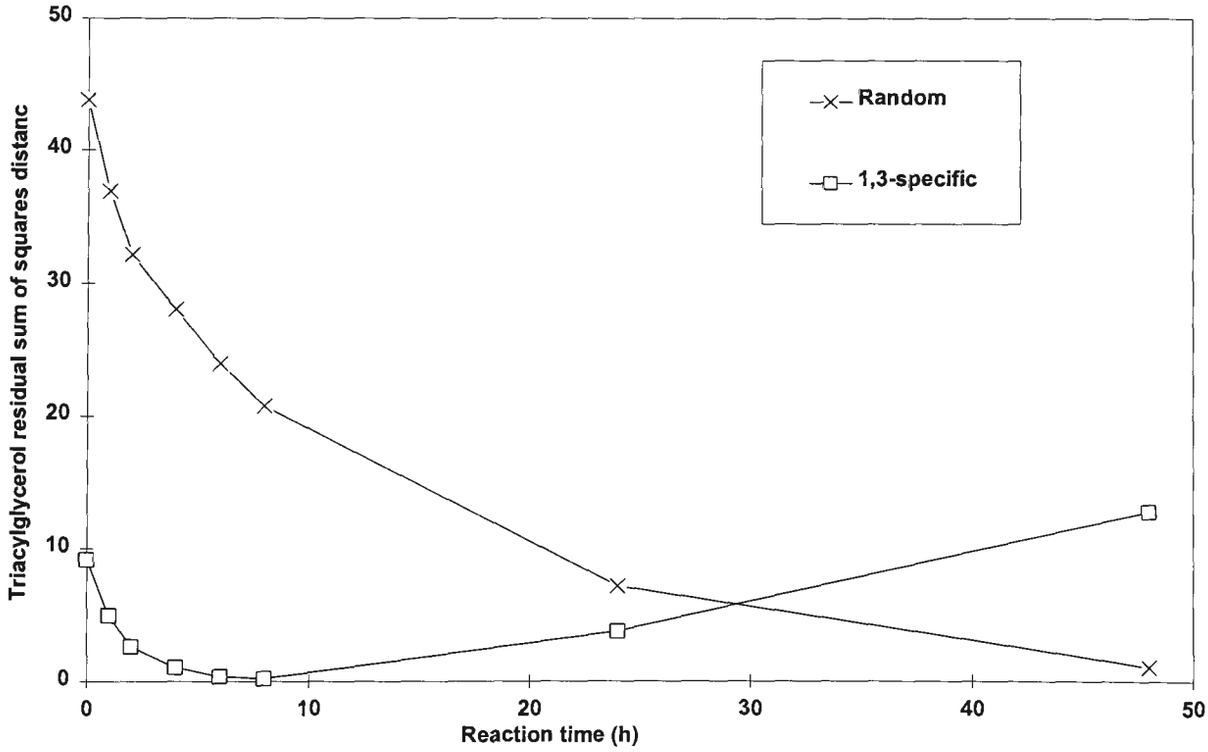


Figure 9.1.7 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (2.5%) and water content of 0.30% (Treatment 7).
Standard deviations: Random (±0.27), 1,3-specific (±0.11) and Original (±0.22).

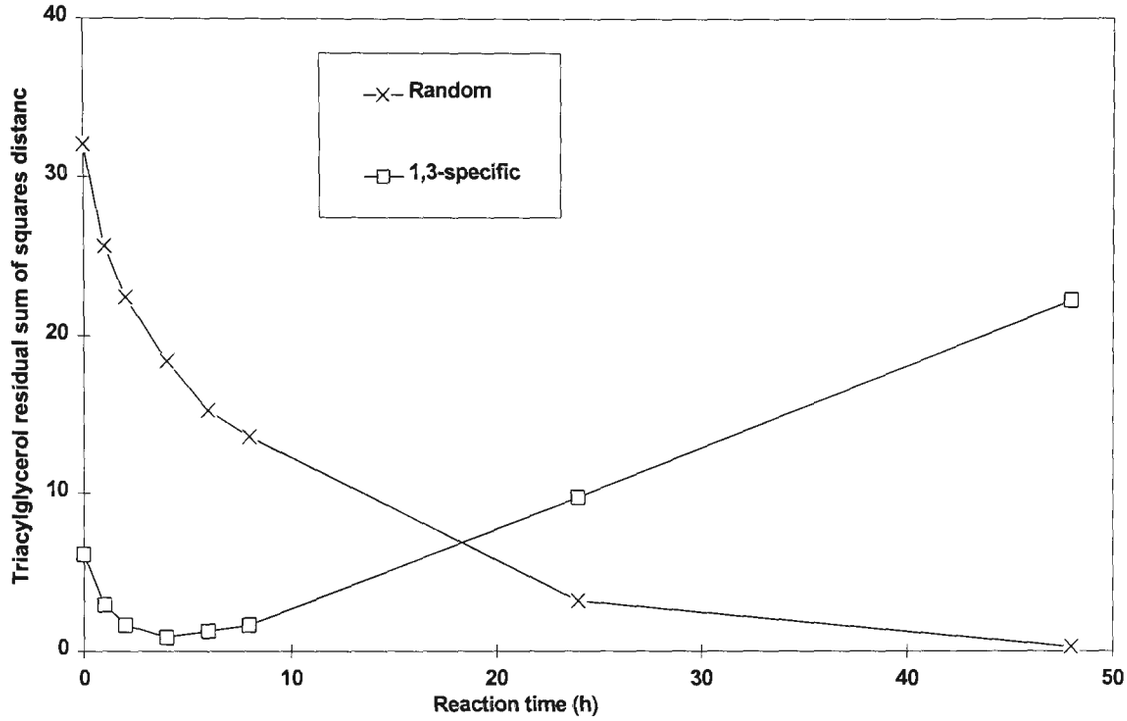


Figure 9.1.8 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (2.5%) and water content of 0.48% (Treatment 8).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

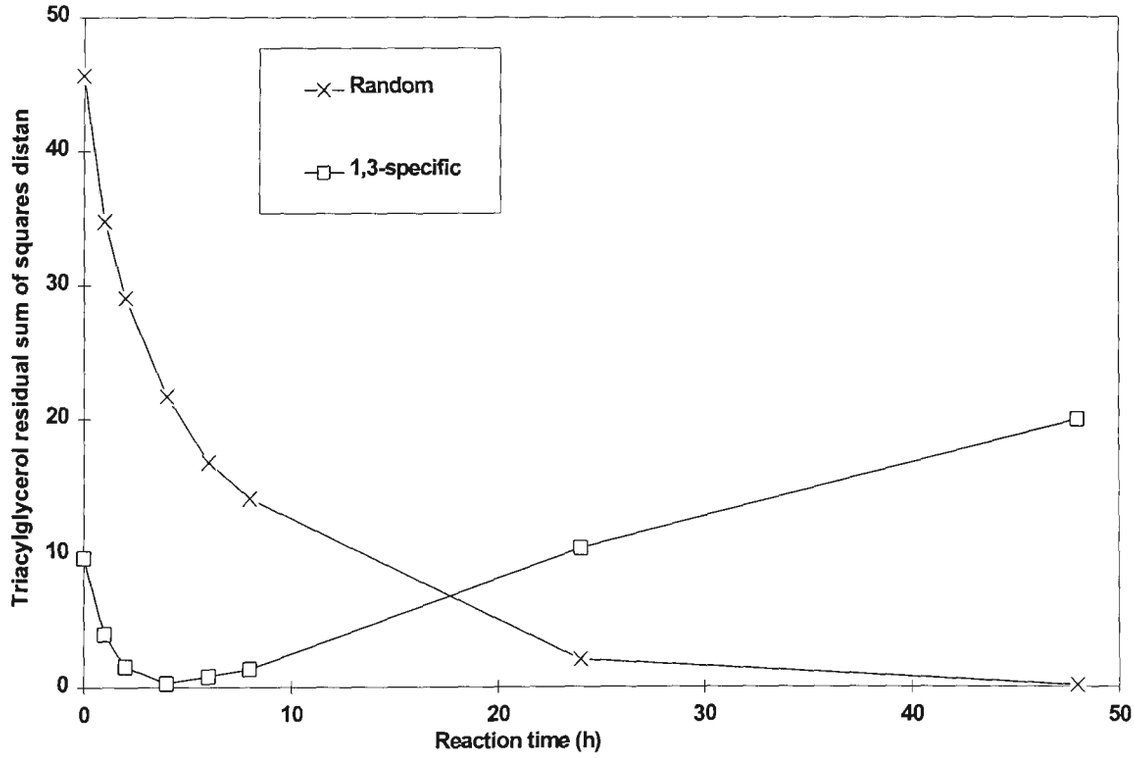


Figure 9.1.9 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (4.0%) and water content of 0.24% (Treatment 9).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

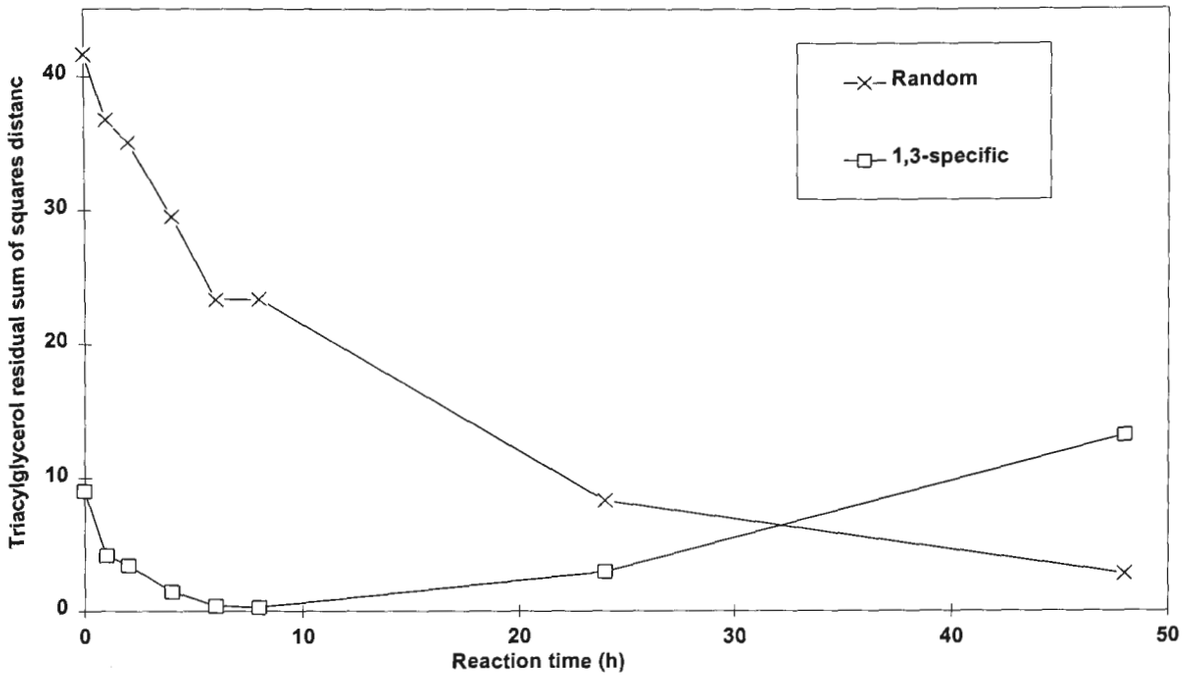


Figure 9.1.10 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (4.0%) and water content of 0.24% (Treatment 10).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

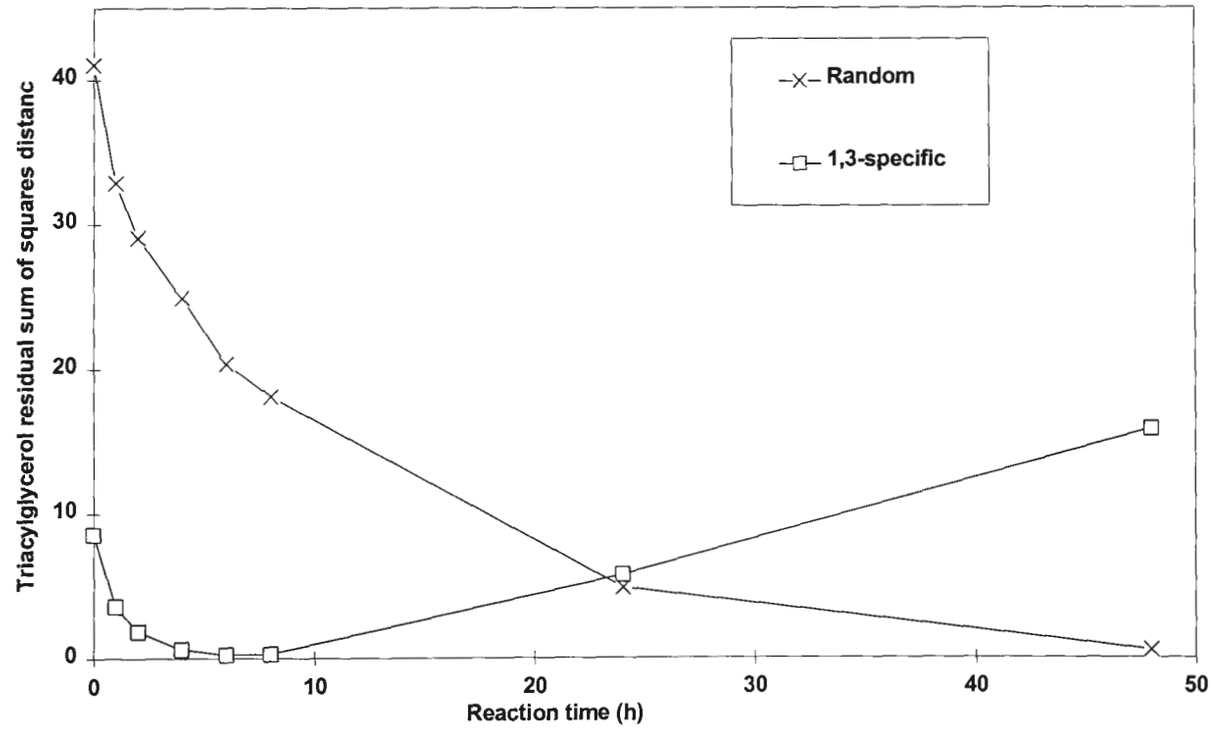


Figure 9.1.11 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (4.0%) and water content of 0.48% (Treatment 11).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).

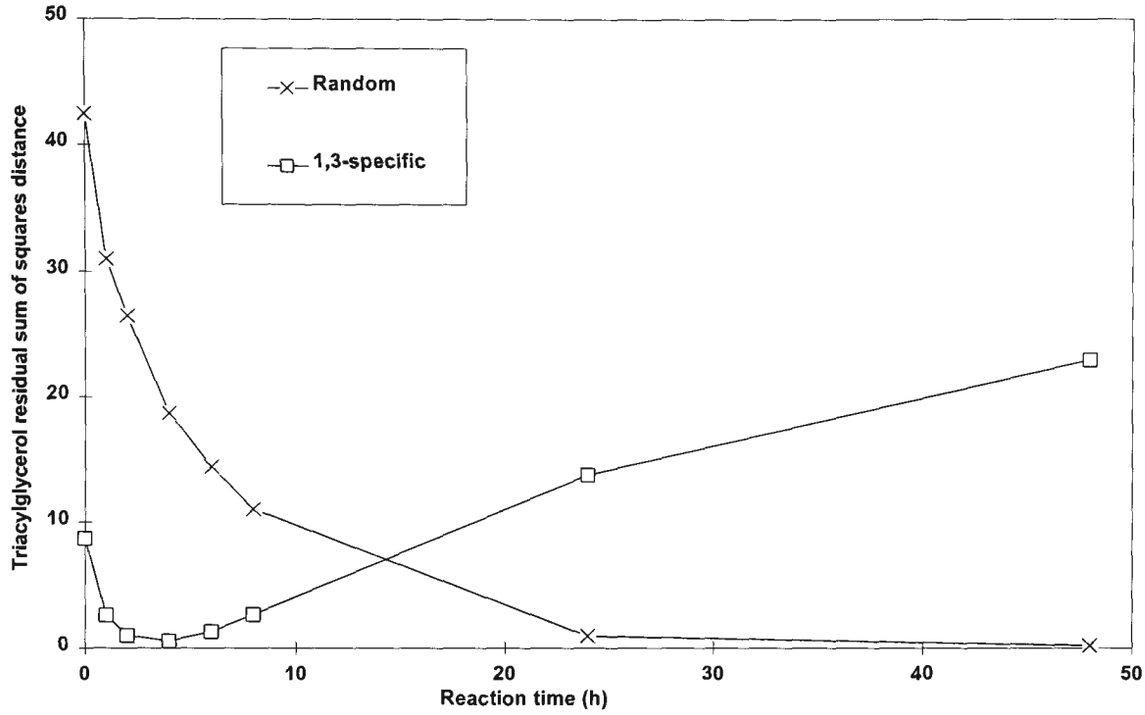
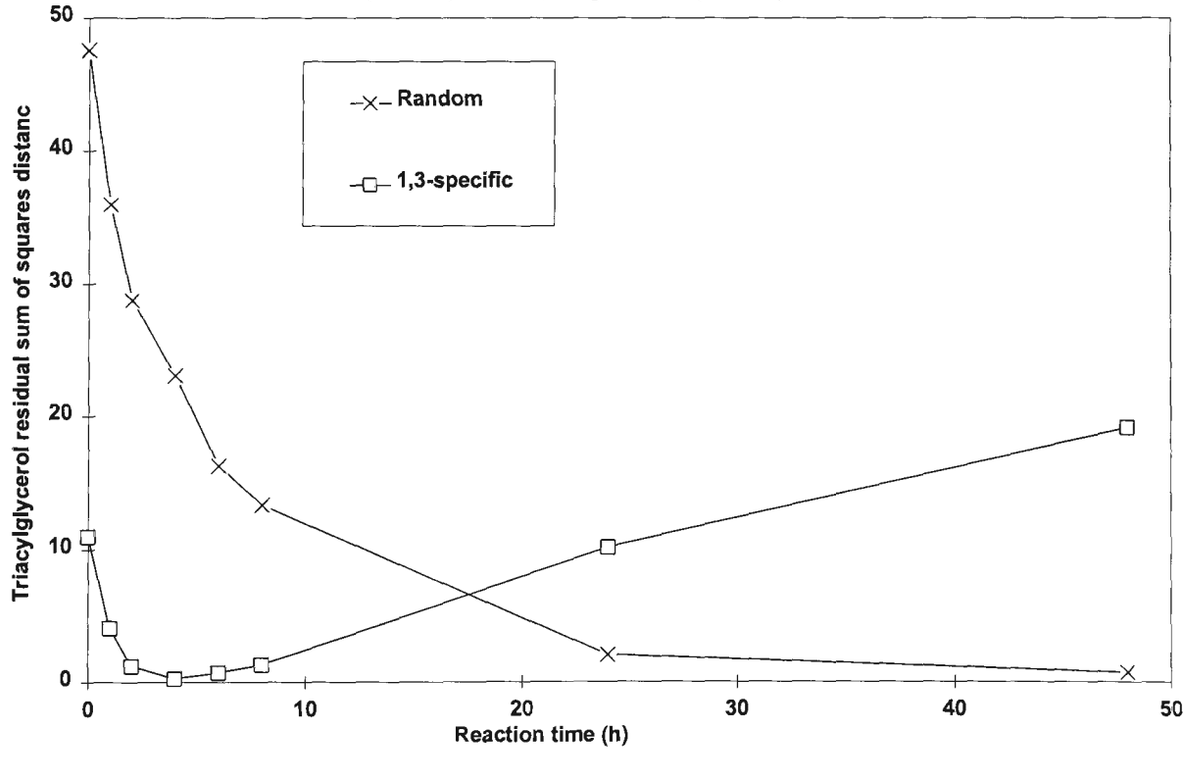


Figure 9.1.12 Triacylglycerol reaction profile for Brazilian cocoa butter interesterified using Lipozyme (4.0%) and water content of 0.48% (Treatment 12).
Standard deviations: Random (±0.27) and 1,3-specific (±0.11).



Discussion:

In general, the results of the enzymatic interesterification of Brazilian cocoa butter were comparable to those observed for Malaysian cocoa butter previously reported in Chapters 7 and 8. The Lipozyme content for the studies reported in this chapter ranged from 1.25 to 4%, while the water content ranged from 0.12 to 0.48%. These levels are intermediate to those studied previously Chapter 7 (Lipozyme 1.6-10.6%, water 0.22 – 1.03%) and Chapter 8 (Lipozyme 0.65-5.48%, water 0.08-0.66%).

The solid fat content results are given in Table 9.1.4A and B. These results show that the higher the initial enzyme and water contents, the larger and faster the changes occurred in the physical properties. In general, the solid fat content results for the treatments and the replicate treatments were similar, with some variation at 35°C, however, the changes fitted the same pattern. These points can be more easily seen in a subset of results given in Table 9.1.6. In this table the solid fat contents of samples taken after 24 hours are compared at the measuring temperatures of 5 and 35°C. All of the treatments decreased the solid fat content at 5°C and increased the solid fat content at 35°C compared to the original Brazilian cocoa butter. The different solid fat contents for the treatments with different initial reaction conditions indicated that the interesterification process was at a different stage for each of these treatments. The melting profiles of the samples identified as 1,3-specific and random interesterified were identified and are discussed in detail later in this section.

The lipid class results are given in Table 9.1.5A and B. These results showed that a large increase in the levels of by-products occurred in the first hour for all treatments (Tables 9.1.5A and B). For particular treatments, the levels of by-products continued to increase between 1 and 2 hours, however after 2 hours of reaction most treatments had reached hydrolysis equilibrium, and showed only very slight increases in the levels of by-products with continued reaction time. A comparison of the lipid class levels for samples taken after 24 hours is given in Table 9.1.7. The results in this table show that the lipid class levels for treatments and the replicated treatments were very similar, and that as the water

content increased for a particular enzyme content, the yield of triacylglycerol was decreased.

Table 9.1.6 Comparison of the solid fat contents (%) at measuring temperatures of 5 and 35°C of interesterified Brazilian cocoa butter, taken after 24 hours of reaction using the Lipozyme and water contents specified for each treatment.

Trt	%Lipozyme	%Water	SFC @ 5°C	SFC @ 35°C
Brazilian cocoa butter	-	-	89.63	1.15
1	1.25	0.12	83.17	6.18
2	1.25	0.20	81.02	10.19
3	1.25	0.20	81.53	7.96
4	1.25	0.40	74.68	20.2
5	2.5	0.17	80.23	15.3
6	2.5	0.30	79.31	18.21
7	2.5	0.30	79.56	18.02
8	2.5	0.48	73.3	22.81
9	4.0	0.24	79.81	17.03
10	4.0	0.24	78.5	20.82
11	4.0	0.48	71.53	22.47
12	4.0	0.48	71.24	22.04

Table 9.1.7 Comparison of the lipid class results (wt%) for interesterified Brazilian cocoa butter samples taken after 24 hours of reaction at the Lipozyme and water contents specified for each treatment.

Trt	%Lipozyme	%Water	FFA	DG	TG
Brazilian cocoa butter	-	-	1.3	2.6	95.9
1	1.25	0.12	2.7	5.4	91.9
2	1.25	0.20	4.1	7.3	88.7
3	1.25	0.20	3.3	7.3	89.4
4	1.25	0.40	8.4	15.2	76.4
5	2.5	0.17	4.6	9.4	86.0
6	2.5	0.30	5.0	9.5	85.5
7	2.5	0.30	4.9	9.1	85.9
8	2.5	0.48	7.6	13.5	78.7
9	4.0	0.24	4.0	8.1	87.9
10	4.0	0.24	4.1	7.8	88.0
11	4.0	0.48	8.2	13.4	78.4
12	4.0	0.48	7.9	13.5	78.1

The triacylglycerol reaction profiles for interesterified Brazilian cocoa butter are shown in Figures 9.1.1-9.1.12. The extra data points, particularly in the initial stages of the interesterification reaction, have enabled the curve of the triacylglycerol reaction profiles to be defined in more detail compared to those in Chapter 8. The triacylglycerol reaction profiles of the treatments compared to the replicated treatments appeared to be similar in shape, however there was some variation in the actual 'distance' values.

Increasing the enzyme and water contents increased the rate of interesterification, particularly for the random triacylglycerol reaction profile. For a given enzyme content, increasing the water content resulted in a much faster randomisation reaction, which could be seen by comparing the random triacylglycerol reaction profiles for treatments with the same enzyme content, for example treatments 1 to 4. The initial rate of 1,3-specific interesterification appeared to be similar for most treatments.

The triacylglycerol reaction profile distance value calculated by comparing the theoretical triacylglycerol compositions for the 1,3-specific and random interesterified Brazilian cocoa butter is 20.94. Therefore, the triacylglycerol composition of a sample is most like the 1,3-specific interesterified triacylglycerol composition when the 1,3-specific distance value is close to zero and the random distance value is around 20.94 and vice versa for the random interesterified triacylglycerol composition. This information and the triacylglycerol reaction profiles were used to identify the reaction times at which the interesterified samples were considered to be 1,3-specific and random interesterified. The solid fat contents of samples closest to the times identified are given in Table 9.1.8 for 1,3-specific interesterification and Table 9.1.9 for random interesterification.

The solid fat content values at each temperature were averaged and plotted in Figure 9.1.13, which clearly demonstrates the different melting profile of a 1,3-specific interesterified product compared to a random interesterified product. These results were very similar to those found in Chapter 7 and illustrated in Figure 7.22. The standard deviations for the solid fat contents at each measuring temperature are relatively small considering the number of treatments involved and the limited data available, as only

samples taken at 2, 8, 24 and 48 hours were analysed for solid fat content. This re-affirmed the finding in Chapter 7 that the presence of lipid by-products has only a minor effect on the melting curve of the interesterified cocoa butter.

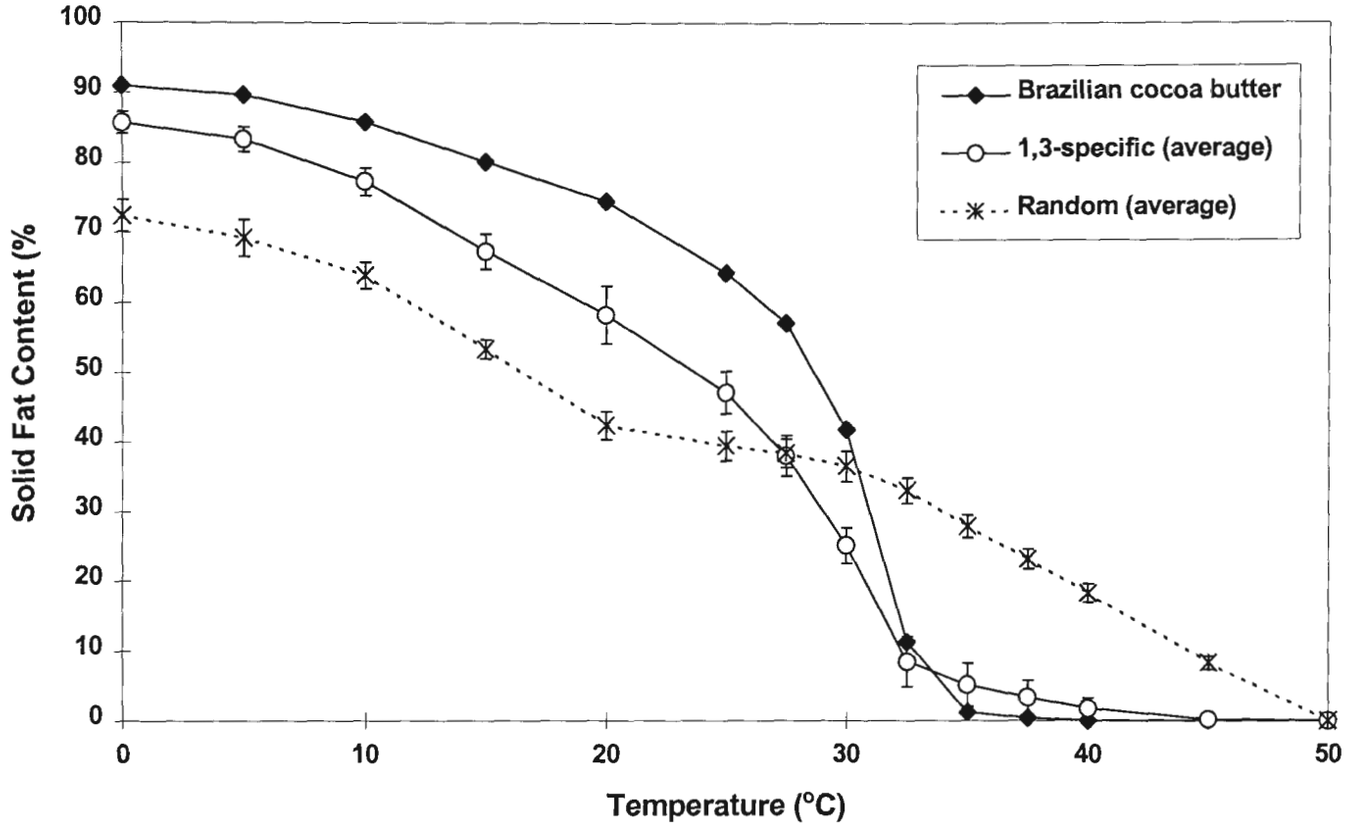
Table 9.1.8 The solid fat content (%) results for the treatments specified at the reaction time closest to where a 1,3-specific interesterified product was identified.

		Temperature (°C)												
Trt	**Time (h)**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**	**40**	**45**
1	**24**	85.95	83.17	77.34	68.47	61.24	49.15	40.46	27.84	10.36	6.18	4.17	2.12	0
2	**24**	83.84	81.02	74.82	64.15	53.77	45.46	37.48	26.87	13.83	10.19	6.61	4.07	0
3	**8**	86.36	84.39	78.63	70.95	63.78	50.42	41.23	26.37	7.36	3.72	1.9	0.76	0.24
4	**2**	88.52	86.29	80.49	67.65	52.23	43.18	33.56	21.64	3.87	1.59	0.6	0	0
5	**8**	85.74	83.58	77.98	70.53	63.99	52.16	42.78	28.5	10.05	5.91	3.57	1.61	0
6	**8**	83.95	81.42	74.87	65.22	56.31	46.19	37.51	25.85	11.47	7.97	5.22	2.3	0.59
7	**2**	87.69	85.64	79.97	71.8	64.28	51.93	41.93	26.06	5.15	1.63	0.44	0	0
8	**2**	86.87	84.67	78.55	66.61	56.66	46.3	36.6	22	4.06	1.88	0.41	0	0
9	**8**	83.9	81.63	75.24	66.1	58.13	45.73	37.13	26.13	11.41	7.15	6.9	3.59	0.63
10	**8**	84	81.29	74.97	65.63	56.75	46.68	38.34	26.73	12.93	9.39	6.1	3.71	0
11	**2**	85.6	83.65	77.26	65.72	56.17	44.28	35.37	21.82	5.09	2.53	1.55	0.93	0
12	**2**	86.28	84.01	77.83	65.49	55.74	44.39	35.12	22.09	5.93	3.33	2.28	1.77	0.38
Average		**85.73**	**83.40**	**77.33**	**67.36**	**58.25**	**47.16**	**38.13**	**25.16**	**8.46**	**5.12**	**3.31**	**1.74**	**0.15**
Std deviation		**1.56**	**1.75**	**1.98**	**2.53**	**4.09**	**3.03**	**2.91**	**2.53**	**3.61**	**3.09**	**2.45**	**1.47**	**0.25**

Table 9.1.9 The solid fat content (%) results for the treatments specified at the reaction time closest to where a random interesterified product was identified.

		Temperature (°C)												
Trt	**Time (h)**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**	**40**	**45**
4	**48**	70.57	66.36	60.71	50.35	41.15	38.42	37.12	35.29	32.41	28.15	22.71	17.53	7.24
6	**48**	74.67	71.68	65.35	53.78	40.33	37.23	35.98	34.15	30.97	26.92	21.62	16.78	7.37
7	**48**	74.33	71.79	65.26	53.7	40.34	37.05	37.11	34.16	31.25	27	21.9	16.87	7.32
8	**48**	70.34	66.43	61.92	52.67	43.78	40.98	39.36	38.07	34.69	30.31	24.9	19.05	8.61
9	**48**	75.09	72.25	66.11	54.85	41.04	37.94	36.8	35.28	31.39	24.9	21.62	17.69	7.53
10	**48**	73.96	71.13	65.17	54.27	42.91	40.05	38.78	37.19	33.7	28.89	23.64	18.54	9.08
11	**48**	70.08	67.51	63.31	53.74	45.11	42.26	41.25	39.42	35.49	28.75	24.62	20.15	9.38
12	**48**	70.15	67.15	63.29	53.93	45	42.16	41.33	39.42	35.21	28.52	24.7	19.92	9.33
Average		**72.40**	**69.29**	**63.89**	**53.41**	**42.46**	**39.51**	**38.47**	**36.62**	**33.14**	**27.93**	**23.21**	**18.32**	**8.23**
Std deviation		**2.29**	**2.64**	**1.90**	**1.38**	**2.01**	**2.14**	**2.05**	**2.20**	**1.87**	**1.64**	**1.43**	**1.31**	**0.96**

Figure 9.1.13 Solid Fat Content curves for Brazilian cocoa butter and the average data for samples taken during the interesterification of Brazilian cocoa butter under various initial reaction conditions at reaction times where the samples were identified as 1,3-specific interesterified and random interesterified.



In the following sections, the results were examined further and equations were developed that describe the lipid class levels and the random and 1,3-specific triacylglycerol reaction profiles based on the initial water and enzyme contents.

9.2 Lipid class equation development

In chapter 8, preliminary linear equations were developed for the lipid classes based on the results of interesterifications by regression analysis using Genstat software. The same approach was used in this section, with the linear equations developed using the lipid class data from all 12 treatments. The lipid class results from 2 hours onwards were used, because after 2 hours of reaction the lipid class levels had mostly reached hydrolysis equilibrium and were relatively stable with only minor changes over time, and therefore a linear equation would be suitable. The Genstat programs are given in Appendix 6.

Lipid class regression equations:

Where **L** = Lipozyme content (%), **W** = water content (%), **H** = Time (h)

Free fatty acids:

$$\%FFA = 1.12 - 0.23L + 14.72W + 0.013H \quad \text{.....9.2.1}$$

$R^2 = 89.7$ Standard error of observation (s.e) = 0.59

Diacylglycerols:

$$\%DG = 3.57 - 0.42L + 21.55W + 0.033H \quad \text{.....9.2.2}$$

$R^2 = 82.4$ s.e = 1.18

Triacylglycerols:

$$\%TG = 95.36 + 0.66L - 36.80W - 0.046H \quad \text{.....9.2.3}$$

$R^2 = 86.2$ s.e = 1.74

The developed linear equations for lipid classes were similar to those developed in Chapter 8 for Malaysian cocoa butter. The R^2 values for the equations developed in this chapter were slightly lower and the standard errors slightly higher than those calculated for the equations developed in Chapter 8. The terms of the equation were also slightly different compared to those in Chapter 8. For example, the equations 8.2.1 and 8.2.2 for FFA and DG had a small positive term to the enzyme concentration, whereas here they have a small negative term. This could mean that the enzyme concentration has a minor effect on the overall lipid class levels and the calculated results are within the standard error range. In this chapter, more data points and less treatment combinations were included in the data set for the regression analysis and this may account for some of the minor differences.

The equations are valid for reaction times of 2 hours and greater. The factor of time has a minimal effect on the lipid class levels, due to the very small coefficient of the time variable. For example, the triacylglycerol yields for the initial reaction conditions of

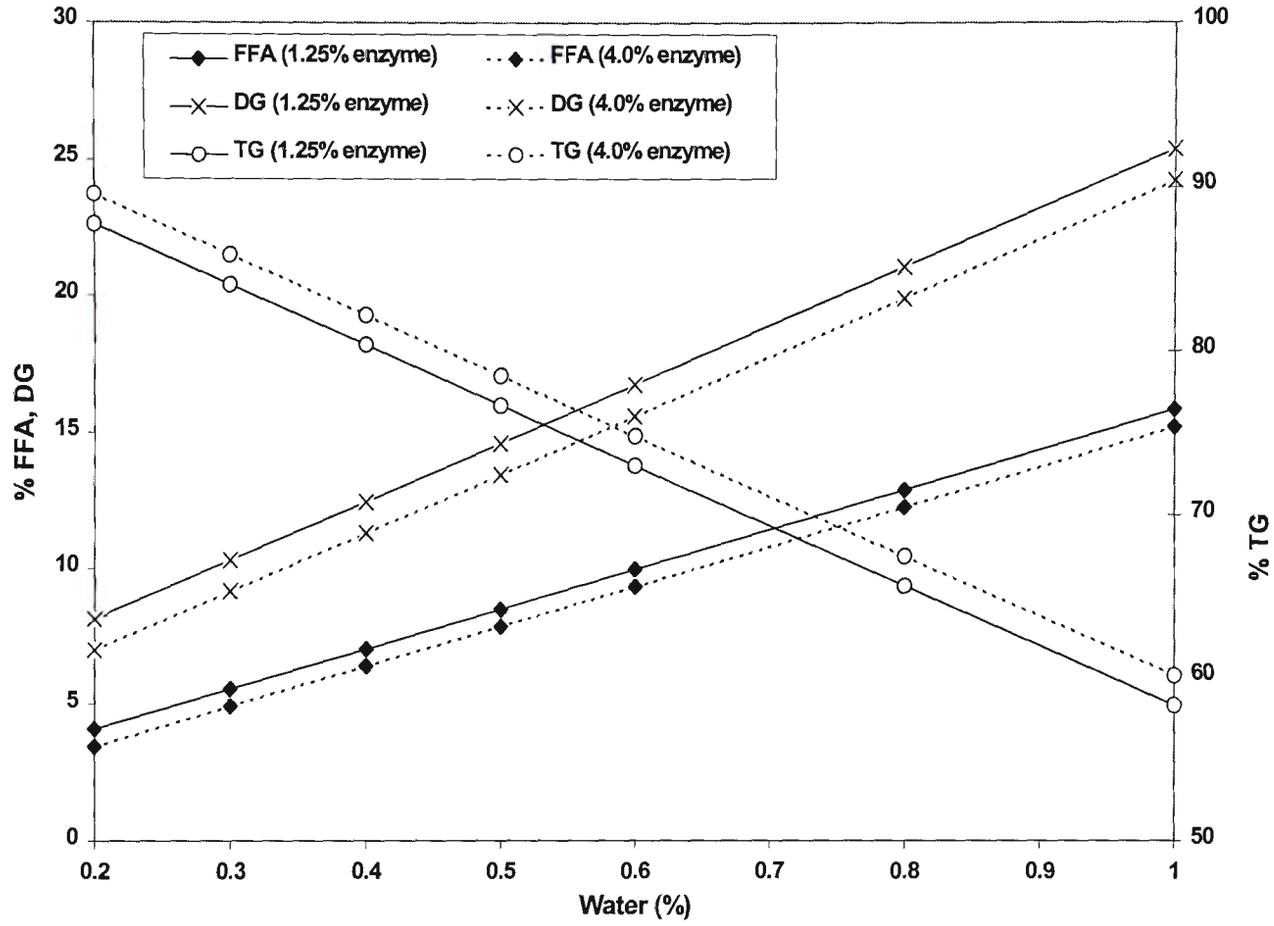
2.5% enzyme and 0.5% water at 2, 8 and 24 hours are 78.5, 78.2 and 77.5% respectively. A fixed reaction time could be used when comparing lipid class levels calculated using these equations under different initial enzyme and water contents.

In order to understand the effects of changing initial enzyme and water contents on equilibrium lipid class levels, a number of combinations of initial enzyme and water contents were calculated, using the developed equations at a reaction time of 24 hours; the results are given in Table 9.2.1. The results in Table 9.2.1 show that for a given enzyme content, the levels of FFA and DG increase, consequently the yield of TG decreases, as the water content is increased. These results are further illustrated in Figure 9.2.1, which plots the lipid class levels over a range of water contents for a fixed enzyme content of 1.25 and 4.0%. This figure shows that the enzyme content does not have a major influence on the lipid class levels, for a given water content when increased from 1.25% to 4.0%, however water content has a large effect. At overall water contents of 0.5% and higher, the calculated yield of triacylglycerol is reduced to below 80%, with a water content of 1% yielding around 60% triacylglycerol.

Table 9.2.1 Lipid class levels (wt%) calculated for interesterified Brazilian cocoa butter at specified initial Lipozyme and water contents at a fixed reaction time of 24 hours using the developed linear equations.

Lipozyme (%)	Water (%)	FFA	DG	TG
1.25	0.2	4.1	8.1	87.7
1.25	0.3	5.6	10.3	84.0
1.25	0.4	7.0	12.5	80.4
1.25	0.5	8.5	14.6	76.7
1.25	0.6	10.0	16.8	73.0
1.25	0.8	12.9	21.1	65.6
1.25	1	15.9	25.4	58.3
2.0	0.2	3.9	7.8	88.2
2.0	0.3	5.4	10.0	84.5
2.0	0.4	6.9	12.1	80.9
2.0	0.5	8.3	14.3	77.2
2.0	0.6	9.8	16.5	73.5
2.0	0.8	12.7	20.8	66.1
2.0	1	15.7	25.1	58.8
2.5	0.2	3.8	7.6	88.5
2.5	0.3	5.3	9.8	84.9
2.5	0.4	6.7	11.9	81.2
2.5	0.5	8.2	14.1	77.5
2.5	0.6	9.7	16.2	73.8
2.5	0.8	12.6	20.6	66.5
2.5	1	15.6	24.9	59.1
3.25	0.2	3.6	7.3	89.0
3.25	0.3	5.1	9.5	85.4
3.25	0.4	6.6	11.6	81.7
3.25	0.5	8.0	13.8	78.0
3.25	0.6	9.5	15.9	74.3
3.25	0.8	12.5	20.2	67.0
3.25	1	15.4	24.5	59.6
4.0	0.2	3.5	7.0	89.5
4.0	0.3	4.9	9.1	85.9
4.0	0.4	6.4	11.3	82.2
4.0	0.5	7.9	13.5	78.5
4.0	0.6	9.3	15.6	74.8
4.0	0.8	12.3	19.9	67.5
4.0	1	15.2	24.2	60.1

Figure 9.2.1 Calculated lipid class levels (wt%) for interesterified Brazilian cocoa butter at fixed enzyme contents of 1.25 and 4.0%, and a fixed reaction time of 24 hours.



These equations were further examined for their ability to predict the lipid class levels of enzymatic interesterifications carried out under specific initial reaction conditions and this work is reported in Chapter 10. However as a check of the calculated versus actual data for the treatments used to develop the equations, the lipid class levels for the treatments in this chapter were calculated and compared. An example of the results for treatment 6, which had an initial Lipozyme content of 2.5% and a water content of 0.30%, is given in Table 9.2.2. This data shows that the calculated and actual values were quite similar.

Table 9.2.2 The lipid class levels for the enzymatic interesterification of Brazilian cocoa butter using treatment 6 conditions of 2.5% Lipozyme and 0.30% water and the calculated lipid class levels using equations 9.2.1 to 9.2.3 at a reaction time of 24 hours.

Lipid class	Experimental	Calculated
TG	85.5	84.9
DG	9.5	9.8
FFA	5.0	5.3

In this section, linear equations that describe the lipid class levels based on the initial reaction conditions and the reaction time were developed. The triacylglycerol reaction profile data was also examined in further detail then equations were developed. These investigations are presented in the following sections.

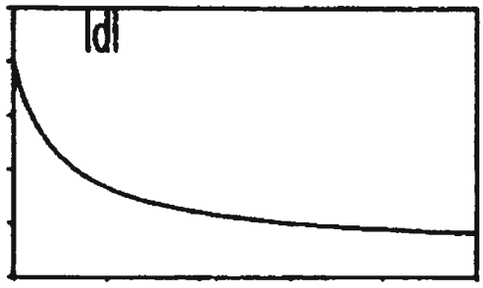
9.3 Triacylglycerol reaction profile equation development

The triacylglycerol reaction profiles for the interesterification of cocoa butter were not linear in shape and required more complex equations than those for the lipid classes to describe them. It was established in Chapter 8 that more than 4 data points would be necessary to do this and therefore in this chapter, each treatment had a total of 8 data points, including more in the important initial reaction stages. The approach in this section was to screen several types of curves to determine which one could best describe the triacylglycerol reaction profiles individually, and include terms relating to the reaction time. In the next section, the individual equations are combined through regression analysis to incorporate the levels of enzyme and water content, to develop one overall equation to estimate the triacylglycerol reaction profiles for the random and 1,3-specific interesterification of cocoa butter.

The types of curves that could be used to describe the 1,3-specific and random triacylglycerol reaction profiles were sourced from the standard non-linear curves library of the Genstat software program. These curves could be fitted to the data using the FITCURVE function of Genstat. An initial screening process eliminated the use of equations involving exponential terms (R^X) due to the complexity of the terms with no clear benefit regarding fitting of the data. The equations that were most suitable for the random or 1,3-specific triacylglycerol reaction profiles were:

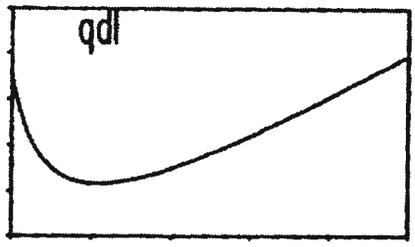
Linear divided by linear: CURVE = **LDL**

$$Y = A + B/(1+DX) \quad \text{.....9.3.1}$$



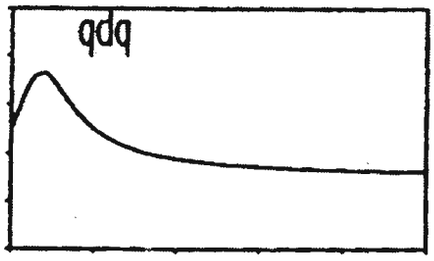
Quadratic divided by linear: CURVE = **QDL**

$$Y = A + B/(1 + DX) + CX \quad \text{.....9.3.2}$$



Quadratic divided by quadratic: CURVE = **QDQ**

$$Y = A + (B + CX)/(1 + DX + EX^2) \quad \text{.....9.3.3}$$



The equation types of LDL, QDL and QDQ are rational functions that are ratios of polynomials. LDL is a rectangular hyperbola, QDL is a hyperbola with a non-horizontal asymptote, while QDQ is a cubic curve having an asymmetric maximum falling to an asymptote.

The equation types of LDL and QDL were fitted to the random triacylglycerol reaction profile data for all treatments individually and the results are presented in Tables 9.3.1 and 9.3.2. The equation types of QDL and QDQ were fitted to the 1,3-specific triacylglycerol reaction profile data for all treatments individually and the results are presented in Tables 9.3.3 and 9.3.4. The Genstat programs are given in Appendix 7.

Table 9.3.1 Comparison of Fitcurve equation data, using the equation type LDL, for random triacylglycerol reaction profiles of interesterified Brazilian cocoa butter at the initial Lipozyme and water contents specified for each treatment.

	Treatments		**Equation information for Y = A + B/(1+DX), where Y = random triacylglycerol reaction profile distance and X = reaction time (h)**				
	%Lipozyme	**%Water**	**A**	**B**	**D**	**R^2**	**Std error**
1	1.25	0.12	1.90	32.08	0.0353	94.3	1.73
2	1.25	0.20	-1.92	44.56	0.0480	97.6	1.70
3	1.25	0.20	-2.90	31.52	0.0514	96.7	1.45
4	1.25	0.40	-1.96	37.77	0.2721	99.6	0.76
5	2.5	0.17	-4.41	51.47	0.0575	98.1	1.85
6	2.5	0.30	-6.82	49.26	0.1051	99.4	1.15
7	2.5	0.30	-4.32	35.42	0.1386	99.2	0.99
8	2.5	0.48	-4.42	49.41	0.2280	99.6	0.94
9	4.0	0.24	-7.13	48.51	0.0843	99.2	1.20
10	4.0	0.24	-6.02	45.49	0.1239	99.0	1.36
11	4.0	0.48	-3.92	45.85	0.2629	99.5	1.02
12	4.0	0.48	-3.79	50.81	0.2545	99.5	1.20
Average			**-3.81**	**43.51**	**-**	**98.5**	**1.28**
Standard deviation			**2.46**	**7.34**	**-**	**1.6**	**0.34**

Table 9.3.2 Comparison of Fitcurve equation data, using the equation type QDL, for random triacylglycerol reaction profiles of interesterified Brazilian cocoa butter at the initial Lipozyme and water contents specified for each treatment.

	Treatments		Equation information where Y = A + B/(1 + DX) + CX where Y = random triacylglycerol reaction profile distance and X = reaction time (h)					
	Lipozyme %	Water %	A	B	C	D	R^2	Std error
1	1.25	0.12	29.39	7.22	-0.3472	1.5716	99.9	0.28
2	1.25	0.20	31.08	13.76	-0.4474	0.4541	99.5	0.77
3	1.25	0.20	19.68	10.42	-0.3139	0.4115	99.3	0.66
4	1.25	0.40	-1.62	37.47	-0.0068	0.2775	99.5	0.85
5	2.5	0.17	21.48	26.62	-0.3400	0.1525	98.2	1.82
6	2.5	0.30	1.73	41.13	-0.1303	0.1403	99.3	1.18
7	2.5	0.30	-0.87	32.12	-0.0580	0.1651	99.0	1.08
8	2.5	0.48	-4.37	49.37	-0.0009	0.2284	99.6	1.05
9	4.0	0.24	-12.65	53.90	0.0727	0.0737	99.1	1.32
10	4.0	0.24	-0.53	40.32	-0.0872	0.1523	98.8	1.48
11	4.0	0.48	-6.51	48.22	0.0511	0.2358	99.4	1.09
12	4.0	0.48	-5.49	52.37	0.0335	0.2384	99.3	1.33
Average			**5.94**	**34.41**	-	-	**99.2**	**1.08**
Standard deviation			**15.11**	**16.55**	-	-	**0.4**	**0.40**

Table 9.3.3 Comparison of Fitcurve equation data, using the equation type QDL, for 1,3-specific triacylglycerol reaction profiles of interesterified Brazilian cocoa butter at the initial Lipozyme and water contents specified for each treatment.

	Treatments		Equation information where Y = A + B/(1 + DX) + CX, where Y = 1,3-specific triacylglycerol reaction profile distance and X = reaction time (h)					
	%Lipozyme	%Water	A	B	D	C	R^2	Std error
1	1.25	0.12	-0.35	7.40	0.360	0.0226	94.6	0.50
2	1.25	0.20	-2.58	12.28	0.296	0.0723	98.7	0.35
3	1.25	0.20	-4.56	10.45	0.223	0.1994	94.8	0.47
4	1.25	0.40	-1.72	8.89	1.428	0.6529	97.2	1.62
5	2.5	0.17	-3.80	15.79	0.314	0.1377	96.1	0.74
6	2.5	0.30	-6.85	15.94	0.396	0.3897	99.7	0.24
7	2.5	0.30	-4.45	10.61	0.574	0.5495	99.9	0.19
8	2.5	0.48	-3.71	13.49	1.032	0.4999	97.9	1.00
9	4.0	0.24	-9.35	17.92	0.263	0.4376	97.3	0.74
10	4.0	0.24	-5.53	14.03	0.586	0.4340	99.9	0.16
11	4.0	0.48	-1.94	10.68	2.136	0.5401	96.4	1.52
12	4.0	0.48	-3.56	14.59	1.252	0.4822	97.6	1.05
Average			**-4.03**	**12.67**	-	-	**97.5**	**0.72**
Standard deviation			**2.43**	**3.16**	-	-	**1.8**	**0.50**

Table 9.3.4 Comparison of Fitcurve equation data, using the equation type QDQ, for 1,3-specific triacylglycerol reaction profiles of interesterified Brazilian cocoa butter at the initial Lipozyme and water contents specified for each treatment.

Treatments			Equation information where $Y = A + (B + CX)/(1 + DX + EX^2)$ where Y = 1,3-specific triacylglycerol reaction profile distance and X = reaction time (h)						
	Lipozyme %	Water %	A	B	C	D	E	R^2	Std error
1	1.25	0.12	0.90	6.26	-0.30	0.4856	-0.0177	96.3	0.42
2	1.25	0.20	0.07	8.59	-0.38	0.4199	-0.0112	99.9	0.12
3	1.25	0.20	-2.41	8.59	1.17	0.5742	-0.0091	98.4	0.27
4	1.25	0.40	58.76	-51.69	-22.79	0.3349	0.0091	100	0.07
5	2.5	0.17	0.11	12.16	-0.35	0.5188	-0.0117	97.5	0.59
6	2.5	0.30	-77.17	86.33	34.42	0.4746	-0.0019	99.9	0.14
7	2.5	0.30	243.9	-237.8	-117.5	0.4734	0.0012	100	0.09
8	2.5	0.48	51.30	-41.62	-30.56	0.5153	0.0097	100	0.15
9	4.0	0.24	-18.80	27.73	8.48	0.5433	-0.0059	98.7	0.52
10	4.0	0.24	-289.6	298.1	179.4	0.6289	-0.0009	99.9	0.16
11	4.0	0.48	46.57	-37.92	-32.79	0.5976	0.0169	99.9	0.22
12	4.0	0.48	47.58	-36.62	-35.66	0.6441	0.0128	99.9	0.20
Average			**5.10**	**3.51**	-	-	-	**99.2**	**0.25**
Standard deviation			**120.6**	**121.0**	-	-	-	**1.2**	**0.17**

The difference between the equations LDL and QDL, used for the random triacylglycerol reaction profile data, is the extra term, CX, in the QDL equation. The coefficients for this term are small, indicating that this term does not have a major influence on the outcome of the equation. The average R-squared values are very similar and quite high for both equation types, with the QDL type equation value, 99.2, slightly higher than the LDL type equation, 98.5. The average standard errors are also similar, being 1.28 for the LDL type equation and 1.08 for the QDL type equation. For both equation types, the fitted curves were quite close to the actual data points for most treatments. As an example, the fitted curves for the two equation types versus actual data from treatment 6 results for comparison is shown in Figure 9.3.1. Treatment 6 was chosen for comparison because it was representative and the Lipozyme and water contents were mid-range.

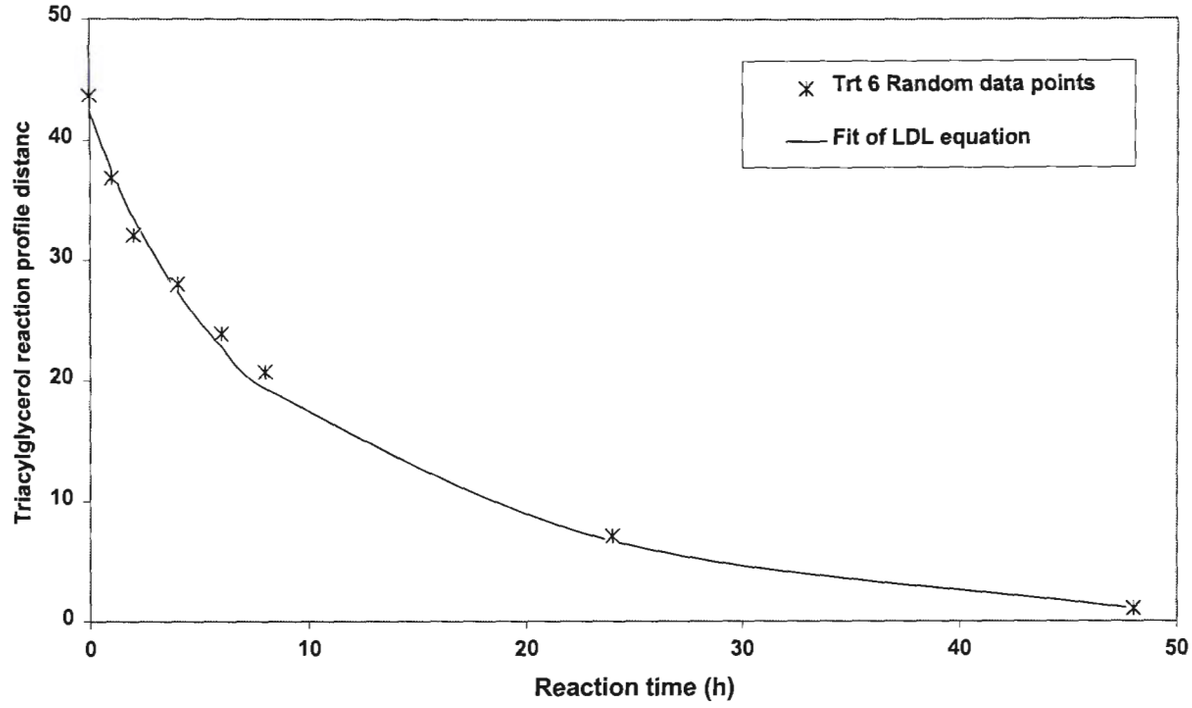
At time zero, when X=0, the initial value for the random triacylglycerol reaction profile distance is equal to A + B for the LDL and QDL equation types. From Tables 9.3.1 and 9.3.2, using the average values for A and B for each equation type, the initial value is

39.70 for the LDL type equation and 40.35 for the QDL type equation. The initial values are similar, which is to be expected because they were generated from the same data. However, the standard deviations for the A and B terms are much larger for the QDL type equation, 15.11 and 16.55, than for the LDL type equation, 2.46 and 7.34. Given this, the simpler equation type of linear divided by linear (LDL) was selected for use as a basis for an overall equation to describe the random triacylglycerol reaction profile including terms for the initial enzyme and water contents.

For the equations used to describe the 1,3-specific triacylglycerol reaction profiles, the QDQ equation has an extra quadratic term, EX^2, than the QDL equation. The values of the coefficient for the X^2 term for the individual equations are extremely small, suggesting that this term is of minor importance. The average R-squared values are very high for both equation types, with the QDQ type equation value, 99.2, slightly higher than the QDL type equation, 97.5. The average standard errors are similar, being 0.72 for the QDL type equation and 0.25 for the QDL type equation. For both equation types the fitted curves were close to the actual data points for most treatments, with a comparison for the treatment 6 data shown in Figure 9.3.2.

Figure 9.3.1. Comparison of the actual versus fitted values for the LDL type equation (**A**) and the QDL type equation (**B**) to describe the random triacylglycerol reaction profile for the interesterification of Brazilian cocoa butter using the initial reaction conditions of 2.5% Lipozyme and 0.30% water (treatment 6).

A



B

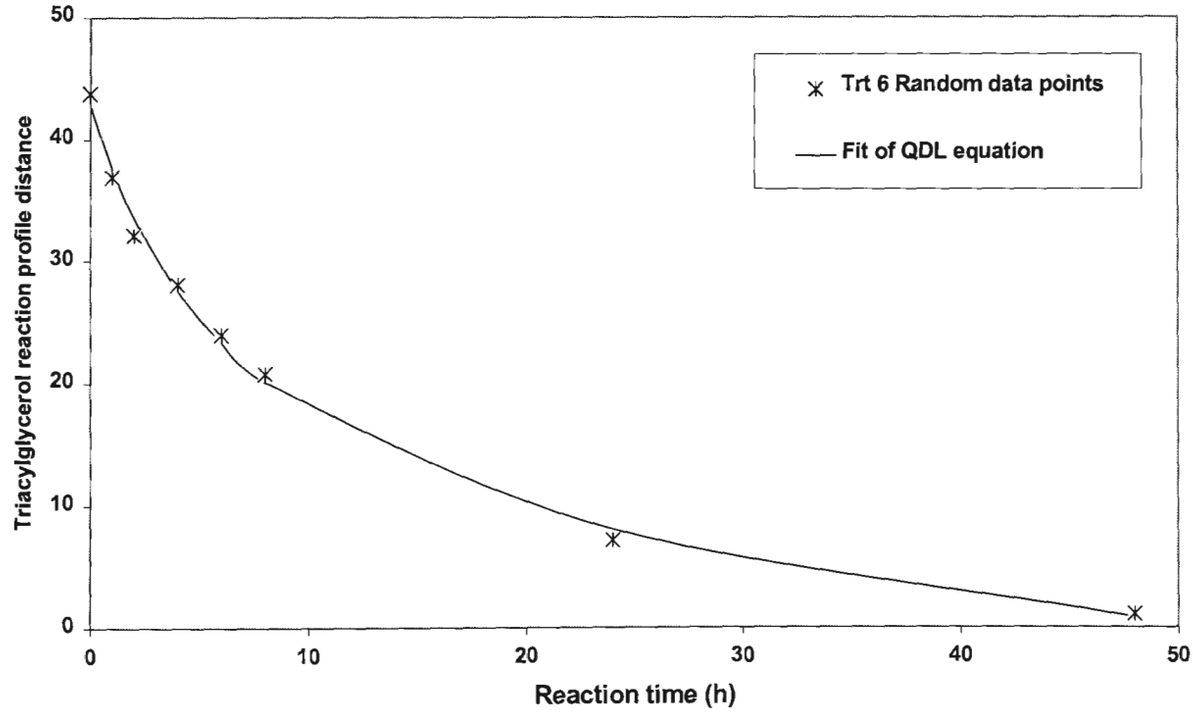
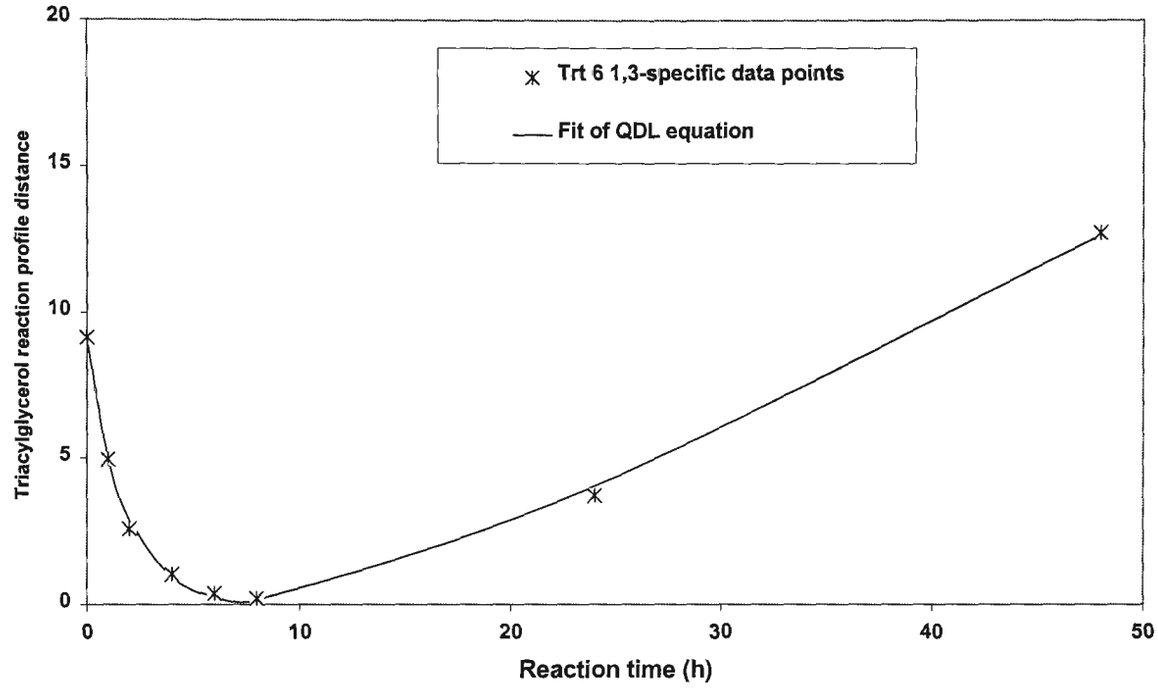
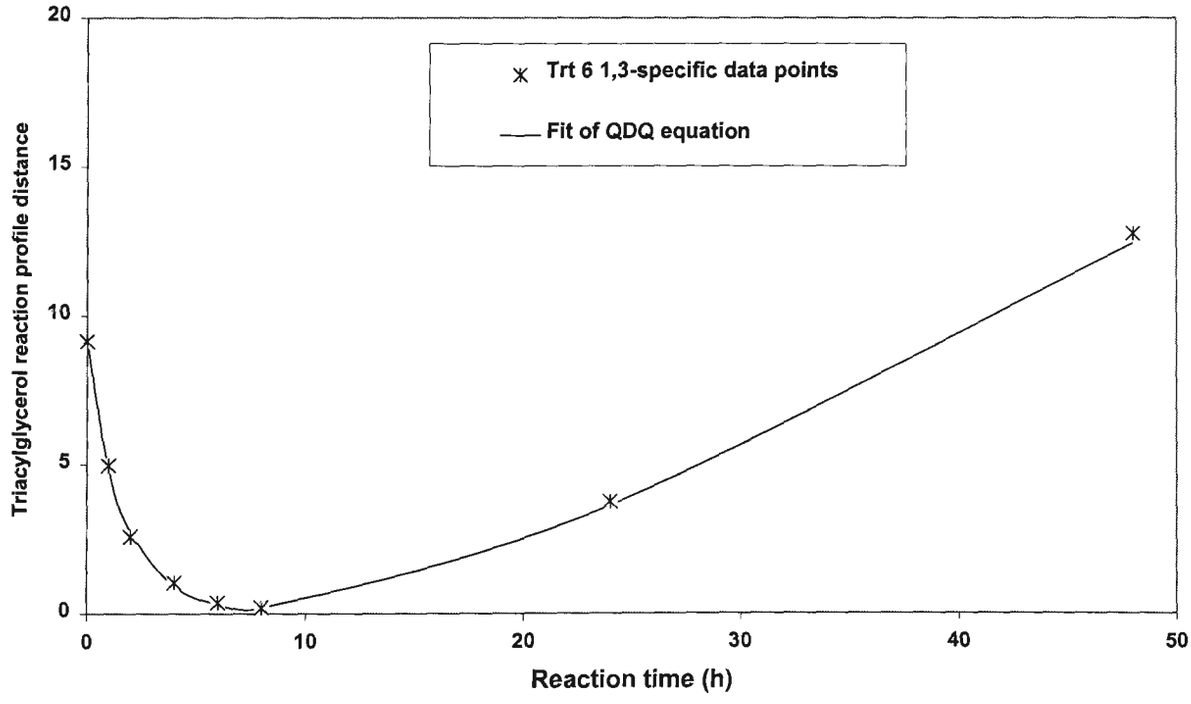


Figure 9.3.2. Comparison of the actual versus fitted values for the QDL type equation (**A**) and the QDQ type equation (**B**) to describe the 1,3-specific triacylglycerol reaction profile for the interesterification of Brazilian cocoa butter using the initial reaction conditions of 2.5% Lipozyme and 0.30% water (treatment 6).

A.



B.



At time zero, when X=0, the initial value for the 1,3-specific triacylglycerol reaction profile distance is equal to A + B for the QDQ and QDL type equations. Using the average values for A and B for each equation type from Tables 9.3.3 and 9.3.4, the initial value is 8.64 for the QDL type equation and 8.61 for the QDQ type equation. Again, the initial values are practically the same. However, the standard deviations for the A and B terms for the QDQ type equation, 120.6, 121.0, are very much larger than for the QDL type equation, 2.43, 3.16, and the values of A and B fluctuate considerably between each individual equation. Therefore, the relatively simpler equation type of quadratic divided by linear (QDL) was selected for use as a basis for an overall equation to describe the 1,3-specific triacylglycerol reaction profile including terms for the initial enzyme and water contents.

These results have demonstrated that it was possible to describe the 1,3-specific and random triacylglycerol reaction profiles individually using curve types sourced from the standard non-linear curve library of the Genstat software program. Equations were developed for each treatment individually that incorporated terms for the reaction time. In the next section, terms for the initial water and enzyme contents are also incorporated, to develop one overall equation for each triacylglycerol reaction profile type.

9.4 Overall triacylglycerol reaction profile equation development.

The aim of this section was to develop a single overall equation to describe the random and 1,3-specific triacylglycerol reaction profiles that is linked to the initial water and enzyme contents as well as reaction time. The approach was to use the basic equation type for each triacylglycerol reaction profile selected in section 9.3 and regress the coefficients of the time variables, X, with the water and enzyme contents used for each treatment. The triacylglycerol reaction profile distance values could then be calculated for given enzyme and water contents and at specified times. The Genstat programs used are given in Appendix 8.

Overall random triacylglycerol reaction profile equation:

The equation type LDL was selected for the random triacylglycerol reaction profile data. The average values from the individual equations for the constant terms, A and B, were used in the overall equation. The D coefficient was not constant for all treatments, and appeared to increase with increasing water and enzyme content. The D coefficient was regressed on the individual treatment information for the initial water and enzyme contents, creating a linear equation for that term. The overall equation now has terms for the initial water and enzyme contents as well as reaction time.

Equation type (LDL): $\mathbf{Y = A + B/(1 + DX)}$

Where **Y** = Random triacylglycerol reaction profile distance

A = -3.81 **B** = 43.51 **X** = Reaction time (h)

D = -0.055 – 0.005**L** + 0.681**W** **(R^2 = 89.1, s.e = 0.03)**

L = Lipozyme (%)

W = Water (%)

Overall equation for random triacylglycerol reaction profiles

$$\mathbf{Y = -3.81 + 43.51/(1+(-0.055-0.005L+0.681W)X)} \quad \text{.....9.4.1}$$

In order to assess the effect of varying the initial enzyme and water contents, random triacylglycerol reaction profile distance values were calculated over time using the developed equation for several combinations of enzyme and water contents. The calculated values are given in Table 9.4.1. From these results it was evident that the developed overall equation was slightly flawed in that the calculated values could be negative, when in fact the sum of squares calculation used to create the random triacylglycerol reaction profile distance values cannot be negative. However, for the examples given in Table 9.4.1, small negative distance values were calculated after 48 hours for water contents of 0.5% and 0.6%, and after 24 hours for water contents of 0.8% and above. In terms of monitoring the interesterification process, the initial stages of reaction are considerably more important, with most interesterification reactions

eventually producing a completely randomised product given a reaction time of 24 hours or longer, resulting in a distance value of zero or close to zero.

The calculated results indicated that for a given enzyme content, the triacylglycerol reaction profile distance values decreased, and hence the rate of randomisation increased, as the water content was increased. This effect is further illustrated in Figure 9.4.1, which shows the calculated random triacylglycerol reaction profiles for a fixed Lipozyme content of 2.5% and a range of water contents. For a fixed water content, the effect of increasing the enzyme content on the calculated distance values was minimal.

In section 9.3, the fitted random triacylglycerol reaction profiles for the individual treatments were compared to the actual data points and this was illustrated in Figure 9.3.1 for treatment 6 data. The random triacylglycerol reaction profile was calculated using the overall equation developed and the initial reaction conditions of treatment 6. This calculated random triacylglycerol reaction profile is compared to the actual data points for treatment 6 in Figure 9.4.2. This figure shows that the random triacylglycerol reaction profiles are similar in shape, however the distance values are higher for the calculated profile and this difference in the values increases over time. This suggests that the overall equation for the random triacylglycerol reaction profile may be limited in its ability to accurately predict the random triacylglycerol reaction profile distance values. The suitability of the developed overall equation for the random triacylglycerol reaction profiles for predicting the interesterification process was further studied and an evaluation is presented in Chapter 10.

Table 9.4.1 Random triacylglycerol reaction profile distance values for the interesterification of Brazilian cocoa butter at specified initial Lipozyme and water contents for reaction times up to 48 hours calculated using equation 9.4.1.

		Time (h)							
Lipozyme (%)	**Water (%)**	**0**	**1**	**2**	**4**	**6**	**8**	**24**	**48**
1.25	0.2	39.7	36.7	34.0	29.7	26.2	23.4	11.7	5.7
1.25	0.3	39.7	34.3	30.0	23.9	19.6	16.5	6.0	1.7
1.25	0.4	39.7	32.1	26.8	19.8	15.4	12.4	3.4	0.1
1.25	0.5	39.7	30.2	24.1	16.7	12.5	9.6	1.8	-0.8
1.25	0.6	39.7	28.5	21.9	14.4	10.3	7.7	0.9	-1.3
1.25	0.8	39.7	25.5	18.3	11.0	7.3	5.1	-0.4	-2.0
1.25	1	39.7	23.1	15.6	8.7	5.4	3.5	-1.1	-2.4
2	0.2	39.7	36.8	34.3	30.1	26.7	23.9	12.3	6.0
2	0.3	39.7	34.4	30.2	24.1	19.9	16.8	6.2	1.9
2	0.4	39.7	32.2	26.9	20.0	15.6	12.6	3.5	0.2
2	0.5	39.7	30.3	24.2	16.9	12.6	9.8	1.9	-0.8
2	0.6	39.7	28.6	22.0	14.5	10.4	7.8	0.9	-1.3
2	0.8	39.7	25.6	18.4	11.1	7.4	5.2	-0.3	-2.0
2	1	39.7	23.1	15.7	8.8	5.5	3.5	-1.1	-2.4
2.5	0.2	39.7	36.9	34.4	30.3	27.0	24.3	12.6	6.3
2.5	0.3	39.7	34.5	30.4	24.3	20.1	17.0	6.3	1.9
2.5	0.4	39.7	32.3	27.1	20.1	15.7	12.7	3.5	0.2
2.5	0.5	39.7	30.4	24.3	17.0	12.7	9.9	2.0	-0.7
2.5	0.6	39.7	28.6	22.1	14.6	10.5	7.9	0.9	-1.3
2.5	0.8	39.7	25.6	18.5	11.1	7.5	5.2	-0.3	-2.0
2.5	1	39.7	23.2	15.7	8.8	5.5	3.6	-1.0	-2.4
3.25	0.2	39.7	37.0	34.7	30.7	27.5	24.8	13.2	6.8
3.25	0.3	39.7	34.6	30.6	24.6	20.4	17.3	6.6	2.1
3.25	0.4	39.7	32.4	27.2	20.3	15.9	12.9	3.7	0.3
3.25	0.5	39.7	30.5	24.5	17.1	12.8	10.0	2.0	-0.7
3.25	0.6	39.7	28.7	22.2	14.7	10.6	8.0	1.0	-1.3
3.25	0.8	39.7	25.7	18.5	11.2	7.5	5.3	-0.3	-2.0
3.25	1	39.7	23.2	15.8	8.8	5.5	3.6	-1.0	-2.4
4	0.2	39.7	37.2	35.0	31.1	28.0	25.4	13.8	7.2
4	0.3	39.7	34.7	30.8	24.9	20.7	17.6	6.8	2.2
4	0.4	39.7	32.5	27.4	20.5	16.1	13.1	3.8	0.3
4	0.5	39.7	30.6	24.6	17.3	13.0	10.1	2.1	-0.6
4	0.6	39.7	28.8	22.3	14.8	10.7	8.0	1.0	-1.3
4	0.8	39.7	25.8	18.6	11.3	7.6	5.3	-0.3	-2.0
4	1	39.7	23.3	15.9	8.9	5.6	3.6	-1.0	-2.4

Figure 9.4.1 Calculated random triacylglycerol reaction profiles for the interesterification of Brazilian cocoa butter at various water contents with a fixed Lipozyme content of 2.5% using equation 9.4.1.

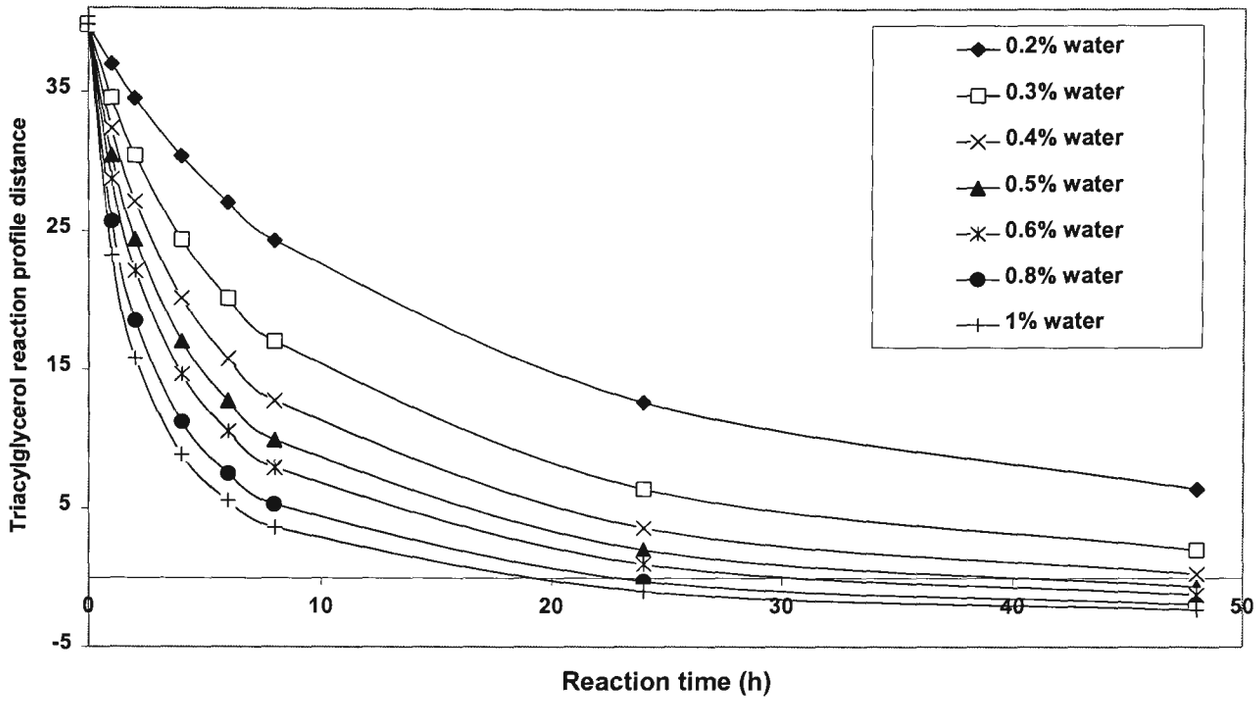
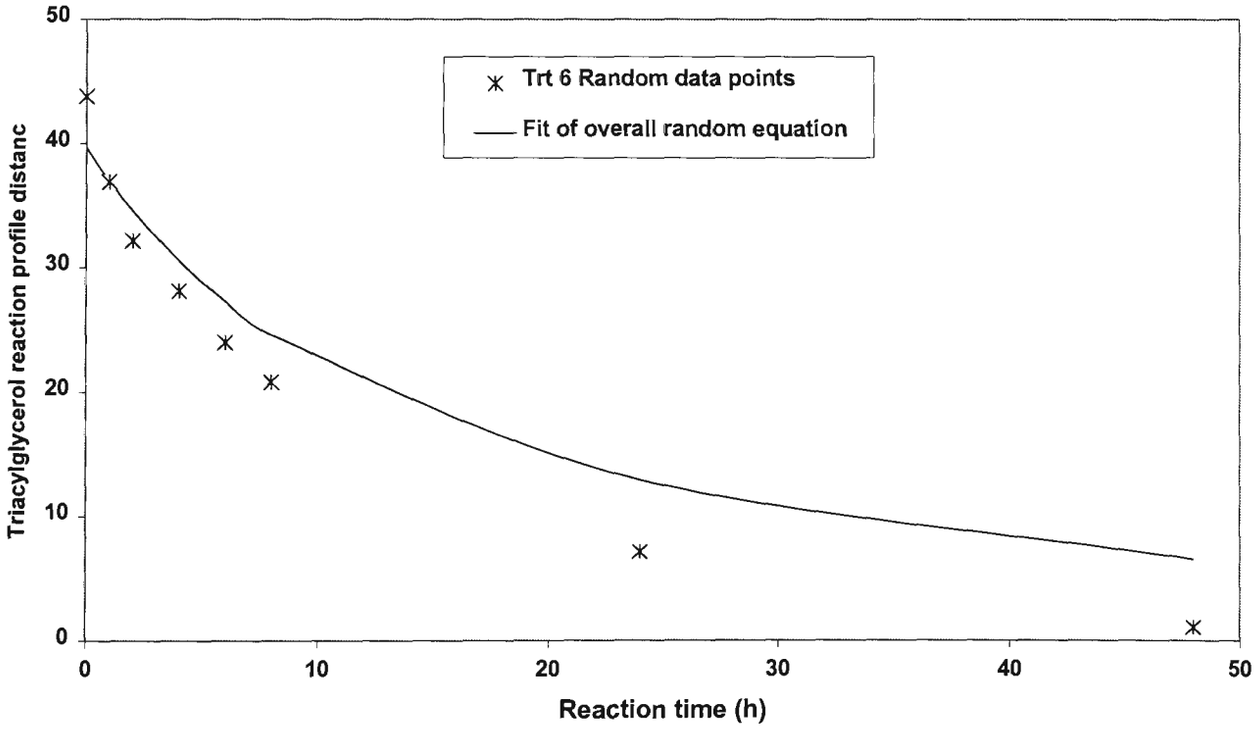


Figure 9.4.2 Comparison of the calculated random triacylglycerol reaction profile using equation 9.4.1 to the actual data points for the interesterification of Brazilian cocoa butter using the initial reaction conditions of 2.5% Lipozyme and 0.30% water (treatment 6).



1,3-specific triacylglycerol reaction profile equation:

The same approach was used for the 1,3-specific triacylglycerol reaction profile equation, using the selected equation type QDL and average values from the individual equations for the constant terms, A and B. The coefficients C and D were regressed on the treatment information for the initial water and enzyme contents, creating linear equations for those terms. Therefore, an overall equation was developed with terms for the initial enzyme and water contents as well as reaction time.

Equation type (QDL): $\mathbf{Y = A + B/(1 + DX) + CX}$

Where **Y** = 1,3-specific triacylglycerol reaction profile distance

A = -4.03 **B** = 12.67 **X** = Reaction time (h)

D = -0.440 – 0.005**L** + 3.954**W** **(R^2 = 65.3, s.e = 0.35)**

C = 0.060 + 0.031**L** + 1.153**W** **(R^2 = 58.1, s.e = 0.13)**

L = Lipozyme (%)

W = Water (%)

Overall equation for 1,3-specific triacylglycerol reaction profiles

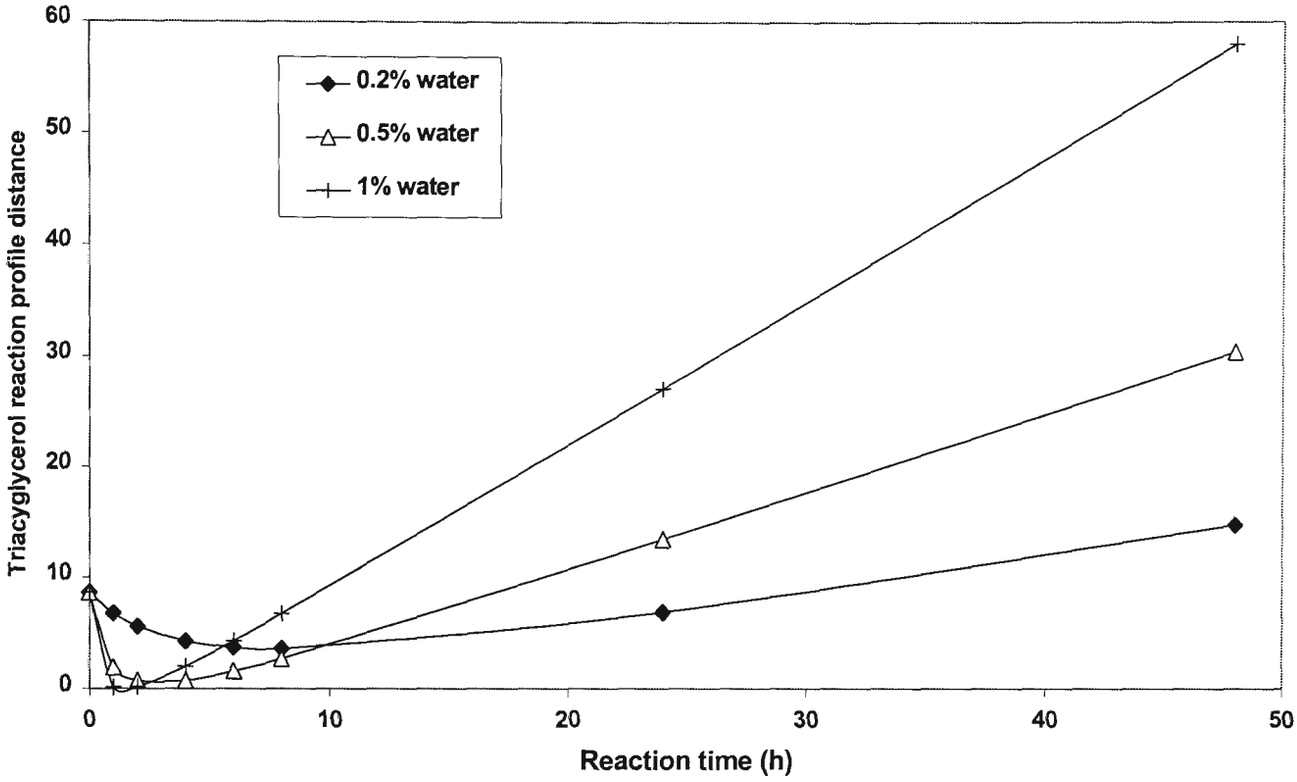
Y = -4.03+12.67/(1+(-.44-0.005L+3.954W)X)+ (0.06+0.031L+1.153W)X 9.4.2

As for the random triacylglycerol reaction profile equation, in order to assess the effect of various initial enzyme and water contents, 1,3-specific interesterification distance values were calculated using the developed overall equation for several combinations of enzyme and water contents. The calculated results are presented in Table 9.4.2. The calculated results indicated that for a given enzyme content, the calculated 'distance' values decreased and therefore the rate of initial 1,3-specific interesterification increased, as the water content was increased. This effect is illustrated in Figure 9.4.3, which shows the calculated 1,3-specific triacylglycerol reaction profiles for a fixed Lipozyme content of 2.5% and a range of water contents. For a fixed water content, as for the calculated random triacylglycerol reaction profile distances, the effect of increasing enzyme content on the calculated 1,3-specific distance values was minimal.

Table 9.4.2 1,3-Specific triacylglycerol reaction profile distance values for the interesterification of Brazilian cocoa butter at specified initial Lipozyme and water contents for reaction times up to 48 hours calculated using equation 9.4.2.

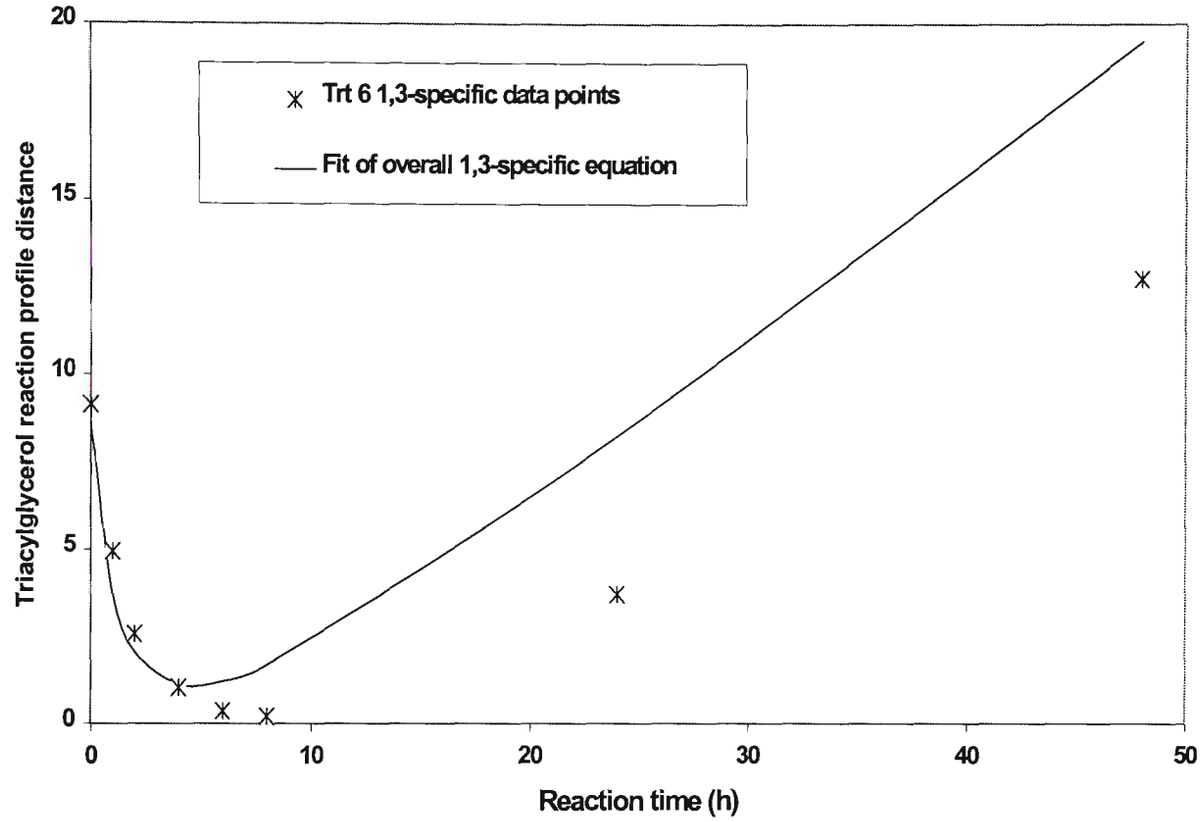
		Time (h)							
Lipozyme (%)	**Water (%)**	**0**	**1**	**2**	**4**	**6**	**8**	**24**	**48**
1.25	0.2	8.6	6.2	4.7	3.2	2.7	2.5	5.5	12.7
1.25	0.3	8.6	4.0	2.2	1.2	1.1	1.5	7.4	17.7
1.25	0.4	8.6	2.6	1.1	0.6	1.0	1.8	9.9	23.1
1.25	0.5	8.6	1.8	0.5	0.5	1.3	2.4	12.5	28.6
1.25	0.6	8.6	1.2	0.2	0.6	1.8	3.1	15.2	34.1
1.25	0.8	8.6	0.5	0.0	1.1	2.8	4.7	20.7	45.1
1.25	1	8.6	0.1	0.1	1.8	4.1	6.4	26.2	56.1
2	0.2	8.6	6.5	5.2	3.8	3.3	3.1	6.3	13.9
2	0.3	8.6	4.2	2.5	1.4	1.4	1.8	8.0	18.8
2	0.4	8.6	2.8	1.3	0.8	1.2	2.0	10.5	24.2
2	0.5	8.6	1.9	0.7	0.7	1.5	2.6	13.1	29.7
2	0.6	8.6	1.3	0.3	0.8	1.9	3.3	15.8	35.2
2	0.8	8.6	0.5	0.1	1.3	3.0	4.9	21.2	46.2
2	1	8.6	0.1	0.1	1.9	4.2	6.6	26.7	57.2
2.5	0.2	8.6	6.8	5.6	4.3	3.7	3.6	6.9	14.8
2.5	0.3	8.6	4.3	2.7	1.6	1.6	2.0	8.4	19.6
2.5	0.4	8.6	2.9	1.4	0.9	1.4	2.2	10.8	25.0
2.5	0.5	8.6	2.0	0.7	0.7	1.6	2.7	13.5	30.4
2.5	0.6	8.6	1.3	0.4	0.8	2.0	3.4	16.2	35.9
2.5	0.8	8.6	0.6	0.1	1.3	3.1	5.0	21.6	46.9
2.5	1	8.6	0.2	0.2	2.0	4.3	6.7	27.1	58.0
3.25	0.2	8.6	7.2	6.2	5.0	4.6	4.4	7.8	16.1
3.25	0.3	8.6	4.6	2.9	1.9	1.9	2.3	9.0	20.7
3.25	0.4	8.6	3.0	1.5	1.1	1.6	2.4	11.4	26.1
3.25	0.5	8.6	2.1	0.9	0.9	1.8	2.9	14.0	31.5
3.25	0.6	8.6	1.4	0.5	1.0	2.2	3.6	16.7	37.0
3.25	0.8	8.6	0.6	0.2	1.4	3.2	5.2	22.2	48.1
3.25	1	8.6	0.2	0.2	2.1	4.5	6.9	27.7	59.1
4	0.2	8.6	7.6	6.8	5.9	5.6	5.5	9.0	17.6
4	0.3	8.6	4.8	3.2	2.2	2.2	2.6	9.6	21.9
4	0.4	8.6	3.2	1.7	1.3	1.8	2.6	12.0	27.2
4	0.5	8.6	2.2	1.0	1.0	2.0	3.2	14.6	32.7
4	0.6	8.6	1.5	0.6	1.1	2.3	3.8	17.3	38.2
4	0.8	8.6	0.7	0.3	1.5	3.4	5.4	22.7	49.2
4	1	8.6	0.3	0.3	2.2	4.6	7.1	28.2	60.2

Figure 9.4.3 Calculated 1,3-specific triacylglycerol reaction profiles for the interesterification of Brazilian cocoa butter at various water contents and a fixed Lipozyme content of 2.5% using equation 9.4.2.



The 1,3-specific triacylglycerol reaction profile was calculated using the overall equation developed and the initial reaction conditions of treatment 6 for comparison to the actual data points, as was carried out for the random triacylglycerol reaction profiles. This comparison is shown in Figure 9.4.4, where it can be seen that the calculated 1,3-specific profile is similar initially to the actual data points, but over time the calculated values are much higher than the actual data points in this example. This suggests that the overall equation for the 1,3-specific triacylglycerol reaction profile was also limited in its ability to accurately predict the 1,3-specific triacylglycerol reaction profile distance values. In Chapter 10, the suitability of the developed overall equation for the 1,3-specific triacylglycerol reaction profiles for predicting the process of 1,3-specific interesterification was further studied and an evaluation is presented.

Figure 9.4.4 Comparison of the calculated 1,3-specific triacylglycerol reaction profile using equation 9.4.2 to the actual data points for the interesterification of Brazilian cocoa butter using the initial reaction conditions of 2.5% Lipozyme and 0.30% water (treatment 6).



9.5 Chapter conclusions

In these studies, Brazilian cocoa butter was used as a substrate instead of Malaysian cocoa butter. It was found that the two fats had similar properties and were equivalent for studying the interesterification process. Brazilian cocoa butter was interesterified under several different treatment conditions with partial replication, using 3 levels of enzyme content (1.25, 2.5 and 4.0%) and water contents in the range of 0.12-0.48%. Samples were taken during the interesterification reaction at 0, 1, 2, 4, 8, 24 and 48 hours and analysed for solid fat content, lipid class, triacylglycerol composition and triacylglycerol reaction profiles. These data were then used to develop several equations that described the outcomes of the interesterification process based on the initial reaction conditions.

The same trends in the results were evident for the Brazilian cocoa butter as was found previously for Malaysian cocoa butter, in that with increasing water and enzyme content, the rate of interesterification was increased and the levels of lipid by-products were higher at hydrolysis equilibrium.

For each treatment, the reaction times at which the samples were considered 1,3-specific and random interesterified were identified. The solid fat profiles of the samples closest to these reaction times from each treatment were averaged and compared. It was found that the melting profiles for the 1,3-specific interesterified samples were distinct from the random interesterified samples. The curves were similar to those identified for Malaysian cocoa butter in Chapter 7.

Linear equations were developed for the lipid classes of TG, DG and FFA and included terms for Lipozyme and water content as well as reaction time. These equations were developed from the individual treatments using data from 2 hours onwards. The equations were slightly different to those developed in the preliminary investigations using Malaysian cocoa butter in Chapter 8. The lipid class levels were calculated using the developed equations for a range of enzyme and water contents at a fixed reaction time to examine the expected effect of altering the reaction conditions on the lipid class levels. The main trend evident was the water content was the dominant factor determining the levels of lipid classes at equilibrium.

For the triacylglycerol reaction profiles, several standard non-linear equations were selected from the reference curve library of the Genstat software program that could described the triacylglycerol reaction profiles for each treatment individually and used only terms for the reaction time. The most suitable equation for the random triacylglycerol reaction profiles was the 'linear divided by linear (LDL)' type, while for the 1,3-specific triacylglycerol reaction profiles the 'quadratic divided by linear (QDL)' type equation was the most suitable. The individual equation data was then used to develop an overall equation for the random and 1,3-specific triacylglycerol reaction profiles. To achieve this, several of the reaction time coefficients were regressed on the

individual treatment data to incorporate terms for the enzyme and water contents. The constant terms were averaged from the individual treatment data. The final equations to describe the triacylglycerol reaction profile distance from the initial reactions conditions were as follows:

Equation 9.4.1: Overall equation for random triacylglycerol reaction profiles

$$Y = -3.81 + 43.51/(1+(-0.055-0.005L+0.681W)X)$$

Equation 9.4.2 Overall equation for 1,3-specific triacylglycerol reaction profiles

$$Y = -4.03+12.67/(1+(-.44-0.005L+3.954W)X)+ (0.06+0.031L+1.153W)X$$

Where **Y** = triacylglycerol reaction profile distance **X** = Reaction time (h)
L = Lipozyme (%) **W** = Water (%)

The triacylglycerol reaction profile distances were calculated using the developed equations for a range of enzyme and water contents at certain reaction times to examine the expected effect of altering the reaction conditions on the triacylglycerol reaction profiles. The water content was found to have a major influence on the interesterification rate and specificity of the interesterified products. On comparison to the actual data from an individual treatment, it was found that the calculated distance values varied increasingly as the reaction time increased.

The ability of the equations developed to predict the outcomes of enzymatic interesterification were assessed through further studies using cocoa butter and other fats. This work is reported in the following chapter.

Chapter 10

An evaluation of the ability of the developed model equations to predict the outcomes of enzymatic interesterification

In Chapter 9, several model equations were developed to describe the outcomes of the enzymatic interesterification of cocoa butter based on the initial enzyme and water contents. The aim of the work in this chapter was to evaluate the specific and wider applications of the developed equations by comparing the experimental results of interesterifications of cocoa butter and other fats under particular reaction conditions to the results calculated using the developed equations. Brazilian cocoa butter, cottonseed oil and a blend of the two fats were interesterified under several initial reaction conditions. The first section in this chapter details the experimental design and results of the enzymatic interesterifications of the various starting fats. The actual experimental results were then compared to the calculated results for the lipid classes (section 10.2) and the triacylglycerol reaction profiles (section 10.3).

10.1 Experimental interesterifications

In this chapter fats other than cocoa butter were studied in order to assess the potential wider applicability of the developed equations. Cottonseed oil was investigated in Chapter 5 as an alternative fat to cocoa butter for further study. It was found that although a reasonable triacylglycerol reaction profile could be generated for the interesterification of cottonseed oil. The changes in the physical properties, however, were considered to be too small to provide other meaningful data, with cottonseed oil being a liquid at room temperature. However, the developed equations for cocoa butter relate to changes in the chemical composition rather than the physical properties. Therefore cottonseed oil and a blend of cocoa butter and cottonseed oil (70:30 w/w) were used for comparisons with the outcomes of the enzymatic interesterification of cocoa butter and those calculated using the equations developed in Chapter 9.

Cottonseed oil (Eta Food Service, Meadow Lea Foods, Victoria) was analysed for overall and 2-positional fatty acid composition according to the procedures described in sections 3.8 and 3.9. The overall and 2-positional fatty acid compositions for Brazilian cocoa butter, cottonseed oil and the 70:30 blend of cocoa butter and cottonseed oil are given in Table 10.1.1. The theoretical triacylglycerol compositions for completely random and 1,3-specific interesterification for all three fats were calculated using the Tricalc program and normalised to 100% for the triacylglycerol carbon number groups of T48, T50, T52 and T54, and the results are presented in Table 10.1.2.

Table 10.1.1 Overall and 2-positional fatty acid composition of Brazilian cocoa butter, cottonseed oil and a blend (70/30 cocoa butter/cottonseed oil).

	Fatty acid (wt%)								
	C14:0	**C16:0**	**C16:1**	**C17:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**
Cocoa butter									
overall	0.05	25.39	0.26	0.26	36.22	33.52	2.91	0.18	1.20
2-position	-	4.91	-	-	4.69	83.11	7.30	-	-
Cottonseed oil									
overall	0.67	23.46	0.52	-	2.64	17.30	55.41	-	-
2-position	-	6.92	-	-	1.65	26.26	65.16	-	-
Blend (70/30)									
overall	0.24	24.81	0.3	0.20	26.15	28.65	18.66	0.13	0.84
2-position	-	5.51	-	-	3.78	66.06	24.66	-	-

Table 10.1.2 Calculated theoretical triacylglycerol compositions (relative weight %) for completely 1,3-specific and random interesterified Brazilian cocoa butter, cottonseed oil and a blend (70/30 cocoa butter/cottonseed oil).

	Brazilian cocoa butter		**Cottonseed oil**		**Blend (70/30)**	
TG	**1,3-specific**	**Random**	**1,3-specific**	**Random**	**1,3-specific**	**Random**
T48	0.86	2.13	1.68	2.43	1.14	2.23
T50	16.77	16.32	15.39	15.30	16.32	16.03
T52	46.40	42.92	44.04	41.49	45.75	42.52
T54	35.98	38.63	38.89	40.78	36.79	39.22

The water contents of the Brazilian cocoa butter, cottonseed oil and Lipozyme were analysed according to the procedure described in section 3.5 and were 0.065% for Brazilian cocoa butter, 0.031% for cottonseed oil and 4.56% for Lipozyme.

Experimental Procedures:

The three fats, batch size 80g, were interesterified according to the procedure described in section 3.1, using the initial water and enzyme content treatments outlined in Table 10.1.3. A total of four treatments, at two enzyme and two water levels, with one replicate treatment, were carried out for each starting fat. A blank treatment, where no enzyme or water was added to the fat, was included for each of the three fats. When extra water was required, it was added directly to the fat prior to the addition of the Lipozyme, 7.0 BAUN/g. Samples were taken after 0, 1, 2, 4, 6, 8, 24, 30 and 48 hours. Selected samples (0, 2, 8, 24 and 48 hours) were analysed for solid fat content (section 3.7), while all samples were analysed for lipid class and triacylglycerol composition (section 3.12) and triacylglycerol reaction profiles were developed for each treatment (section 5.3).

Table 10.1.3 Outline of initial enzyme and water content treatments for interesterification of Brazilian cocoa butter, cottonseed oil and blend (70/30 cocoa butter/cottonseed oil).

Treatment	Lipozyme (%)	Overall water (%)	Replicate
1	2.0	0.20	Cocoa butter (1A)
2	2.0	0.40	Cottonseed oil (2A)
3	3.25	0.20	Blend (3A)
4	3.25	0.40	-
5	0	no additional	(Blank)

Results:

The results for the solid fat content are presented in Tables 10.1.4 to 10.1.6, the lipid class results are presented in Tables 10.1.7 to 10.1.9, and the triacylglycerol reaction profiles are presented in Figures 10.1.1 to 10.1.3.

Table 10.1.4 Solid fat content results (%) for the interesterification of cocoa butter under several treatment conditions (treatments 1 and 1A: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water; treatment 5: no added Lipozyme or water).

		Temperature (°C)													
Trt	**Time**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**	**40**	**45**	**50**
1	0	91.32	89.68	85.25	80.18	74.33	63.79	56.6	43.11	15.69	1.08	-	-	-	-
1	2	89.78	87.47	81.97	73.74	66.22	53.57	42.35	28.72	8.65	1.23	0.60	-	-	-
1	8	86.74	84.11	77.76	69.69	62.42	49.83	39.33	27.20	10.10	5.10	3.09	1.88	-	-
1	24	83.54	80.83	74.06	63.83	50.76	42.91	34.77	27.38	17.09	13.41	9.32	6.12	0.47	-
1	48	81.81	78.91	72.74	62.06	43.71	31.27	30.37	28.03	25.91	21.86	16.83	12.75	4.72	-
1A	0	91.81	90.15	86.00	81.12	75.77	65.14	57.38	43.4	16.28	1.03	-	-	-	-
1A	2	89.89	87.77	82.10	74.49	67.85	54.46	42.98	29.53	9.05	1.64	0.45	-	-	-
1A	8	87.42	84.81	78.71	70.61	62.97	47.17	40.06	27.98	9.93	5.60	3.05	1.05	-	-
1A	24	84.25	81.18	75.03	64.13	50.93	43.04	35.02	27.81	17.75	13.66	9.6	6.08	0.31	-
1A	48	81.78	78.67	72.24	62.01	44.00	32.26	31.17	29.03	26.36	22.55	17.09	12.9	4.49	-
2	0	91.65	89.86	86.13	80.89	75.31	64.52	56.96	43.33	16.04	1.05	-	-	-	-
2	2	88.30	85.69	79.93	69.01	59.11	47.36	36.89	25.13	6.58	2.96	1.54	0.80	0.30	-
2	8	86.27	83.95	77.83	65.80	50.29	41.23	31.56	20.89	11.88	8.52	5.47	3.23	1.46	-
2	24	80.28	77.36	70.65	58.10	37.97	28.75	27.53	26.00	23.25	19.62	15.18	10.61	2.94	-
2	48	73.04	69.41	63.20	51.54	40.86	37.35	36.59	34.99	32.43	28.20	22.9	18.10	8.68	-
3	0	91.68	90.16	86.20	80.92	75.78	64.68	57.47	43.57	16.74	1.25	-	-	-	-
3	2	89.33	86.51	81.49	74.07	66.72	53.61	42.89	29.59	9.07	2.50	0.90	0.45	-	-
3	8	85.98	83.69	77.52	68.93	60.99	48.39	38.31	27.55	11.70	7.22	4.25	2.07	-	-
3	24	84.52	81.69	75.90	65.74	48.58	36.43	32.74	26.79	21.48	17.77	13.04	9.57	1.93	-
3	48	78.03	75.38	68.69	57.80	41.16	35.67	34.81	33.51	30.78	26.42	21.02	16.30	7.77	-
4	0	91.45	90.41	85.97	81.04	75.48	65.03	57.64	43.61	16.50	1.43	-	-	-	-
4	2	87.77	85.2	79.05	68.96	60.23	47.77	37.38	24.66	6.35	2.59	1.08	0.65	-	-
4	8	86.03	83.14	77.55	65.88	48.67	38.72	31.46	21.54	13.49	10.18	7.04	3.82	1.34	-
4	24	78.31	75.33	68.24	56.01	37.4	31.78	30.85	29.03	26.23	22.44	17.76	13.40	4.58	-
4	48	71.18	67.77	62.5	53.41	44.02	40.39	39.73	37.95	34.77	30.39	25.35	20.31	10.10	-
5	0	91.98	90.24	86.08	81.04	75.77	65.29	57.62	43.93	16.46	1.58	-	-	-	-
5	2	91.83	90.41	86.64	81.21	76.16	65.32	57.57	43.97	16.32	1.64	0.71	-	-	-
5	8	92.01	90.44	86.42	81.13	75.98	65.05	57.40	43.69	16.08	1.37	-	-	-	-
5	24	91.84	90.31	86.25	81.09	75.96	65.06	57.5	44.14	15.78	1.49	-	-	-	-
5	48	91.72	90.3	86.40	81.02	75.9	65.35	57.92	43.96	16.35	1.19	-	-	-	-

Table 10.1.5 Solid fat content results (%) for the interesterification of cottonseed oil under several treatment conditions (treatment 1: 2.0% Lipozyme and 0.20% water; treatments 2 and 2A: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water; treatment 5: no added Lipozyme or water).

		Temperature (°C)										
Trt	**Time**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**
1	0	12.92	9.85	4.53	3.22	2.36	1.37	1.25	0.87	-	-	-
1	2	4.90	4.07	3.74	3.30	2.52	1.60	0.97	0.94	0.44	-	-
1	8	5.55	4.87	4.01	3.47	3.02	2.05	1.47	1.19	-	-	-
1	24	6.49	5.80	5.14	4.4	3.65	2.13	1.98	0.97	0.43	-	-
1	48	9.00	9.81	9.17	7.12	5.25	3.42	2.64	1.87	1.23	0.83	-
2	0	13.91	9.91	4.88	3.00	2.13	1.57	1.16	0.74	0.37	0.57	-
2	2	6.53	4.95	3.99	3.69	3.03	1.82	1.49	0.90	0.60	0.78	-
2	8	7.34	6.65	5.04	3.89	3.18	2.32	1.82	1.84	1.04	0.40	-
2	24	8.93	8.13	5.96	5.61	4.41	2.85	2.17	1.75	1.06	1.00	-
2	48	10.75	10.20	9.26	7.23	5.1	3.57	3.14	2.50	1.12	-	-
2A	0	14.69	10.78	4.58	3.19	2.24	1.68	1.60	1.46	0.90	-	-
2A	2	6.56	5.50	4.17	4.05	2.88	1.77	1.36	1.28	0.75	0.76	-
2A	8	7.55	6.73	5.07	4.57	3.38	2.44	1.76	1.41	1.02	0.36	-
2A	24	9.23	8.00	6.60	5.82	5.00	2.99	2.95	2.38	1.04	0.77	-
2A	48	11.04	10.09	8.66	7.49	4.94	4.01	3.52	2.05	1.22	0.87	
3	0	14.51	10.53	4.68	3.07	2.38	1.71	1.69	0.82	0.29	0.38	-
3	2	5.17	4.43	3.21	3.13	2.07	1.47	1.11	1.02	0.79	0.34	-
3	8	5.36	5.04	4.26	3.32	2.60	1.98	1.35	0.84	0.55	-	-
3	24	6.83	6.42	6.05	5.08	3.45	2.30	1.57	0.96	0.51	-	-
3	48	15.70	15.05	11.71	7.88	5.19	3.6	2.72	1.70	1.29	0.45	-
4	0	14.63	10.63	4.97	3.33	2.42	1.93	1.50	1.59	0.70	0.87	-
4	2	6.17	6.01	4.01	3.26	2.86	2.34	1.67	1.63	1.18	0.70	-
4	8	8.08	6.95	5.21	4.64	3.74	2.88	2.15	1.62	1.2	1.01	-
4	24	9.69	8.78	7.53	6.48	5.32	3.47	2.7	2.8	1.39	0.62	-
4	48	12.69	11.59	9.94	7.11	5.43	3.53	2.79	2.49	1.54	0.79	-
5	0	14.42	9.94	3.78	2.23	1.61	0.80	0.32	0.42	-	-	-
5	2	14.60	10.78	4.93	3.51	2.60	1.54	1.04	0.9	0.69	-	-
5	8	14.64	11.03	5.06	3.11	2.36	1.57	1.40	0.63	0.66	0.51	-
5	24	14.32	10.42	4.72	2.78	2.60	1.16	0.93	0.48	0.70	-	-
5	48	14.35	9.77	4.13	2.81	1.71	0.94	1.01	0.35	-	-	-

Table 10.1.6 Solid fat content results (%) for the interesterification of Blend A (70/30 cocoa butter/cottonseed oil) under several treatment conditions (treatment 1: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatments 3 and 3A: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water; treatment 5: no added Lipozyme or water).

		Temperature (°C)												
Trt	**Time**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**	**40**	**45**
1	0	64.00	59.43	55.53	50.38	41.54	28.94	22.82	13.95	2.22	-	-	-	-
1	2	65.34	59.74	52.76	42.39	30.80	19.00	12.07	7.28	0.97	0.30	-	-	-
1	8	64.64	59.41	51.86	37.13	14.74	5.28	4.83	3.36	2.53	1.38	0.24	-	-
1	24	59.6	54.23	44.14	27.53	10.73	9.46	8.89	8.02	6.66	4.62	2.94	1.25	-
1	48	54.55	48.89	37.29	21.48	15.77	14.59	13.84	12.9	11.28	9.00	6.62	3.96	-
2	0	65.15	60.57	56.6	51.38	42.18	29.27	23.32	14.27	2.28	-	-	-	-
2	2	66.37	61.82	52.54	39.15	18.23	9.36	7.00	2.22	0.81	0.56	0.56	-	-
2	8	59.81	55.67	45.67	28.33	9.94	7.65	6.87	5.77	4.26	2.99	1.65	1.18	-
2	24	52.93	46.01	37.12	21.90	15.61	13.52	12.54	11.63	9.92	7.74	5.42	2.87	-
2	48	49.99	42.05	33.72	26.22	20.39	18.69	17.62	16.41	14.47	11.80	9.12	6.22	-
3	0	65.69	61.43	57.26	52.17	43.2	30.57	23.72	14.99	3.32	0.48	0.37	-	-
3	2	67.50	63.66	56.04	44.60	27.77	17.30	10.34	5.71	1.41	0.87	-	-	-
3	8	63.54	59.74	51.17	37.18	14.00	6.68	5.63	4.85	3.67	2.15	0.52	-	-
3	24	58.27	53.37	42.99	26.78	13.36	12.32	11.26	10.72	8.86	6.68	4.18	1.92	-
3	48	52.51	45.21	37.13	23.88	19.54	17.57	16.69	15.72	13.6	11.24	8.51	5.33	
3A	0	64.92	61.05	57.01	52.26	43.39	30.89	23.85	14.89	3.12	0.54	-	-	-
3A	2	67.06	63.45	56.24	44.87	27.86	17.60	10.90	5.85	1.93	1.16	-	-	-
3A	8	63.69	59.51	49.55	36.87	13.96	6.39	6.04	5.32	4.06	2.59	0.96	0.33	-
3A	24	58.48	53.51	42.98	26.98	14.07	12.76	11.71	11.12	9.23	7.03	5.11	2.41	-
3A	48	52.03	44.83	36.69	24.33	19.29	17.66	17.27	16.24	14.20	11.08	8.48	6.03	-
4	0	65.62	61.41	57.51	52.36	43.56	30.72	24.14	15.31	3.39	0.93	0.43	-	-
4	2	65.94	61.15	53.02	38.64	17.06	7.43	6.02	2.57	1.21	0.69	0.25	-	-
4	8	58.97	54.24	44.68	27.53	11.65	9.17	8.23	7.02	5.98	4.30	2.55	1.08	-
4	24	51.15	43.88	35.34	23.45	17.74	15.27	14.81	13.79	11.78	9.54	6.97	3.96	-
4	48	49.98	43.29	37.21	28.91	22.61	20.56	20.13	19.13	16.32	13.68	10.61	7.04	0.68
5	0	64.98	61.57	57.25	51.73	43.36	30.43	23.69	14.90	2.97	0.4	-	-	-
5	2	65.31	61.20	57.66	52.08	43.01	30.88	24.21	15.45	3.29	0.61	-	-	-
5	8	65.26	61.26	57.39	51.72	43.27	30.75	23.67	15.22	2.75	0.58	-	-	-
5	24	64.92	60.96	57.02	51.78	43.23	30.85	23.89	15.59	3.13	0.30	-	-	-
5	48	65.38	60.57	57.44	52.32	43.79	31.08	24.15	15.37	3.80	1.15	-	-	-

Table 10.1.7 Lipid class results (wt%) for samples taken during the interesterification of Brazilian cocoa butter under several initial reaction conditions, as specified for the treatments used. Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2.

	Treatment 1: 2.0% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.0	3.1	3.3	3.5	3.4	3.4	3.5	3.6	3.6
DG	1.9	6.0	6.4	6.8	6.8	6.9	7.6	7.4	7.3
TG	97.1	90.9	90.3	89.6	89.8	89.6	88.9	89.0	89.1
	Treatment 2: 2.0% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.9	5.3	5.6	5.7	5.8	5.9	6.1	6.0	6.1
DG	1.5	9.4	10.1	10.2	10.7	10.8	11.0	10.8	11.2
TG	97.6	85.1	84.2	84.1	83.3	83.1	82.8	83.1	82.6
	Treatment 2A: 2.0% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.3	3.2	3.3	3.4	3.5	3.5	3.8	3.6	3.7
DG	2.6	6.3	6.4	6.8	6.8	7.4	8.1	7.3	7.4
TG	96.1	90.5	90.3	89.7	89.8	89.2	88.1	89.1	89.0
	Treatment 3: 3.25% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.1	3.2	3.4	3.5	3.6	3.5	3.6	3.7	3.7
DG	2.3	6.0	6.6	7.0	7.2	7.2	7.4	7.7	7.7
TG	96.6	90.8	90.1	89.5	89.3	89.2	88.9	88.6	88.5
	Treatment 4: 3.25% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.4	5.5	5.8	5.9	5.9	6.0	6.2	6.3	6.4
DG	2.8	9.8	10.1	10.6	10.5	11.0	11.2	11.3	11.4
TG	95.8	84.6	84.0	83.4	83.4	82.9	82.5	82.3	82.1
	Treatment 5: no added Lipozyme or water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.9	-	-	-	-	1.7	1.4	-	1.3
DG	1.9	-	-	-	-	3.0	2.6	-	2.5
TG	97.3	-	-	-	-	95.3	96.1	-	96.2

Table 10.1.8 Lipid class results (wt%) for samples taken during the interesterification of cottonseed oil under several initial reaction conditions, as specified for the treatments used. Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2.

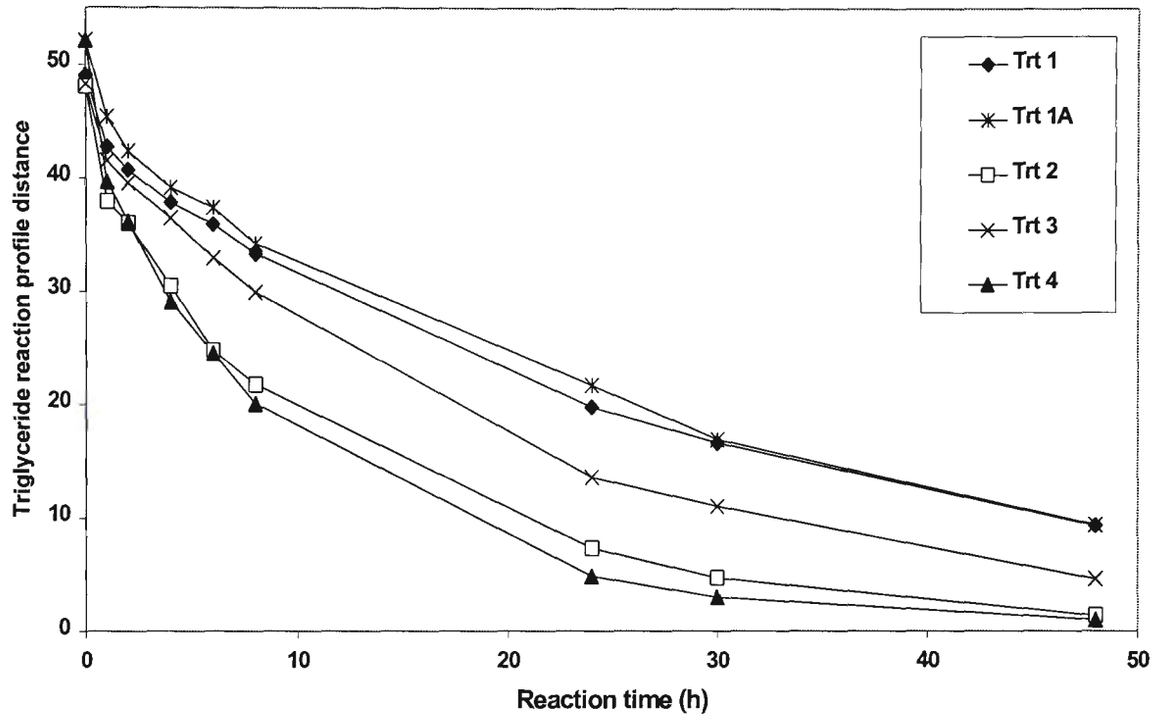
	Treatment 1: 2.0% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.5	2.4	2.8	2.7	3.1	2.8	2.8	3.1	3.0
DG	3.2	7.1	7.4	7.7	7.9	7.8	8.0	8.3	8.3
TG	96.3	90.5	89.8	89.7	89.0	89.4	89.3	88.6	88.7
	Treatment 2: 2.0% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.7		6.1	5.6	6.0	5.9	6.1	6.0	-
DG	3.5		12.7	12.9	13.4	13.3	13.9	13.4	-
TG	95.8		81.0	81.2	80.3	80.5	79.7	80.4	-
	Treatment 2A: 2.0% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.1	4.4	5.2	5.9	5.4	5.5	5.7	6.2	5.5
DG	2.9	10.9	11.9	12.7	12.3	12.9	13.7	14.1	13.1
TG	97.0	84.5	82.6	81.1	82.0	81.3	80.4	79.5	81.2
	Treatment 3: 3.25% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.0	2.2	2.4	2.4	2.6	2.6	2.7	2.7	2.7
DG	2.8	6.7	7.2	7.4	7.5	7.9	8.1	8.3	8.1
TG	97.2	91.1	90.3	90.1	89.9	89.3	89.2	88.9	89.1
	Treatment 4: 3.25% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.0	4.2	4.6	4.9	4.8	4.9	5.0	5.1	5.1
DG	2.1	10.4	10.9	10.8	11.3	11.0	11.4	11.4	12.2
TG	97.9	85.2	84.2	84.1	83.6	83.9	83.4	83.3	82.5
	Treatment 5: no added Lipozyme or water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.1	-	-	-	-	0.1	0.2	-	0.1
DG	3.2	-	-	-	-	3.0	3.2	-	3.2
TG	96.8	-	-	-	-	96.8	96.7	-	96.8

Table 10.1.9 Lipid class results (wt%) for samples taken during the interesterification of a blend of Brazilian cocoa butter and cottonseed oil (70:30) under several initial reaction conditions, as specified for the treatments used. Average standard deviations: FFA ± 0.1, DG ± 0.2, TG ± 0.2.

	Treatment 1: 2.0% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.0	3.3	3.3	3.4	3.5	3.5	3.6	3.7	3.7
DG	2.2	6.9	7.5	7.6	7.5	7.6	8.2	7.8	8.2
TG	96.8	89.9	89.2	89.0	89.0	89.0	88.2	88.6	88.1
	Treatment 2: 2.0% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.0	5.3	5.8	5.8	5.9	6.0	6.1	6.2	6.2
DG	2.6	9.9	11.0	11.2	11.3	11.7	12.2	12.8	12.8
TG	96.3	84.8	83.3	83.0	82.9	82.3	81.7	81.0	80.9
	Treatment 3: 3.25% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.6	2.5	2.7	2.7	2.8	2.9	3.0	3.0	3.1
DG	1.8	5.9	5.8	6.2	6.4	6.8	6.8	7.2	6.9
TG	97.7	91.6	91.5	91.0	90.8	90.4	90.2	89.8	90.0
	Treatment 3A: 3.25% Lipozyme, 0.20% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	0.6	2.6	2.9	3.3	3.1	3.1	3.4	3.4	3.3
DG	2.0	5.9	6.4	7.1	6.6	6.6	7.2	7.7	7.3
TG	97.4	91.5	90.7	89.6	90.3	90.3	89.5	89.0	89.4
	Treatment 4: 3.25% Lipozyme, 0.40% water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.0	5.1	5.3	5.8	5.6	5.8	5.9	6.0	5.9
DG	2.4	9.8	10.3	11.2	11.1	11.2	11.6	11.6	12.1
TG	96.6	85.1	84.3	82.9	83.2	82.9	82.6	82.4	82.0
	Treatment 5: no added Lipozyme or water								
	0 h	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**	**30h**	**48 h**
FFA	1.3	-	-	-	-	0.7	-	-	1.0
DG	2.8	-	-	-	-	2.5	-	-	2.4
TG	96.0	-	-	-	-	96.8	-	-	96.6

Figure 10.1.1 Random (**A**) and 1,3-specific (**B**) triacylglycerol reaction profiles for Brazilian cocoa butter interesterified under several initial reaction condition treatments (treatments 1 and 1A: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water).

A.



B.

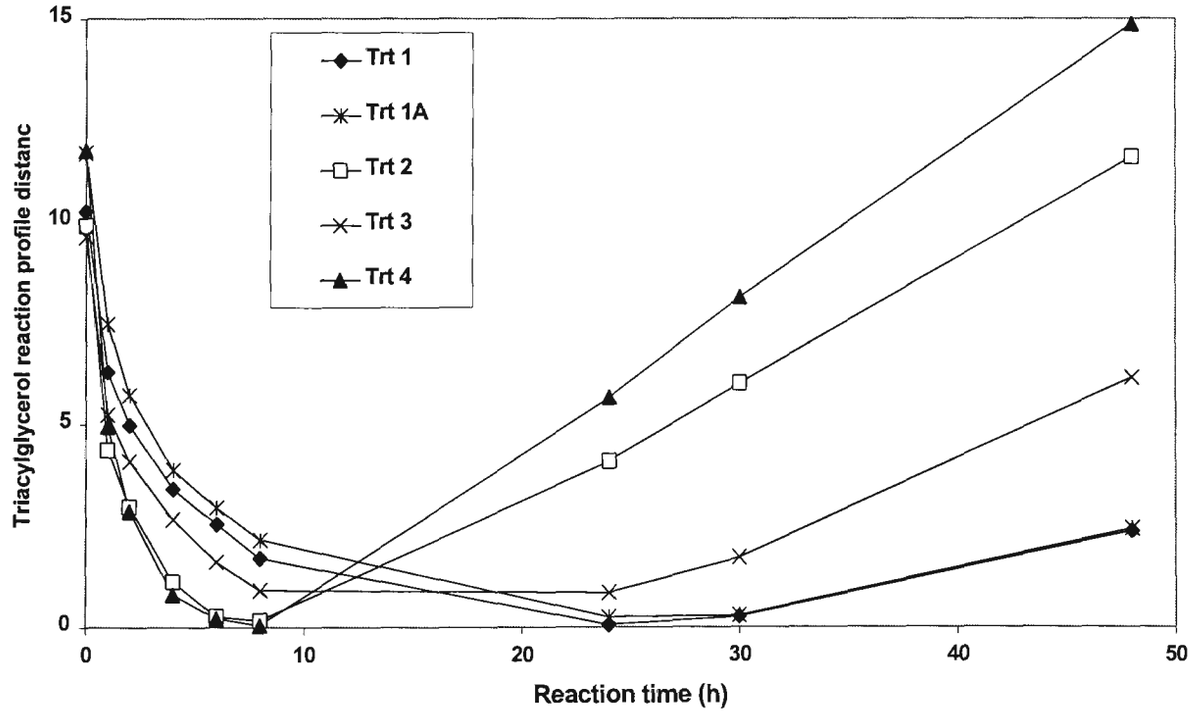
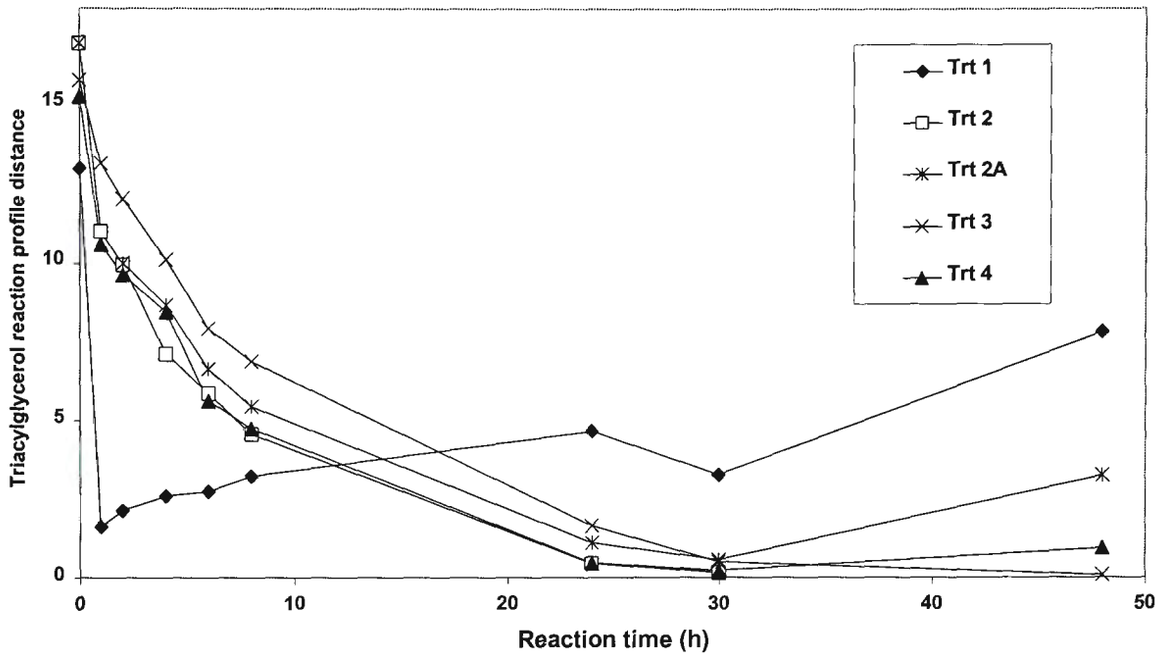


Figure 10.1.2 Random (**A**) and 1,3-specific (**B**) triacylglycerol reaction profiles for cottonseed oil interesterified under several initial reaction condition treatments (treatment 1: 2.0% Lipozyme and 0.20% water; treatments 2 and 2A: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water).

A.



B

B.

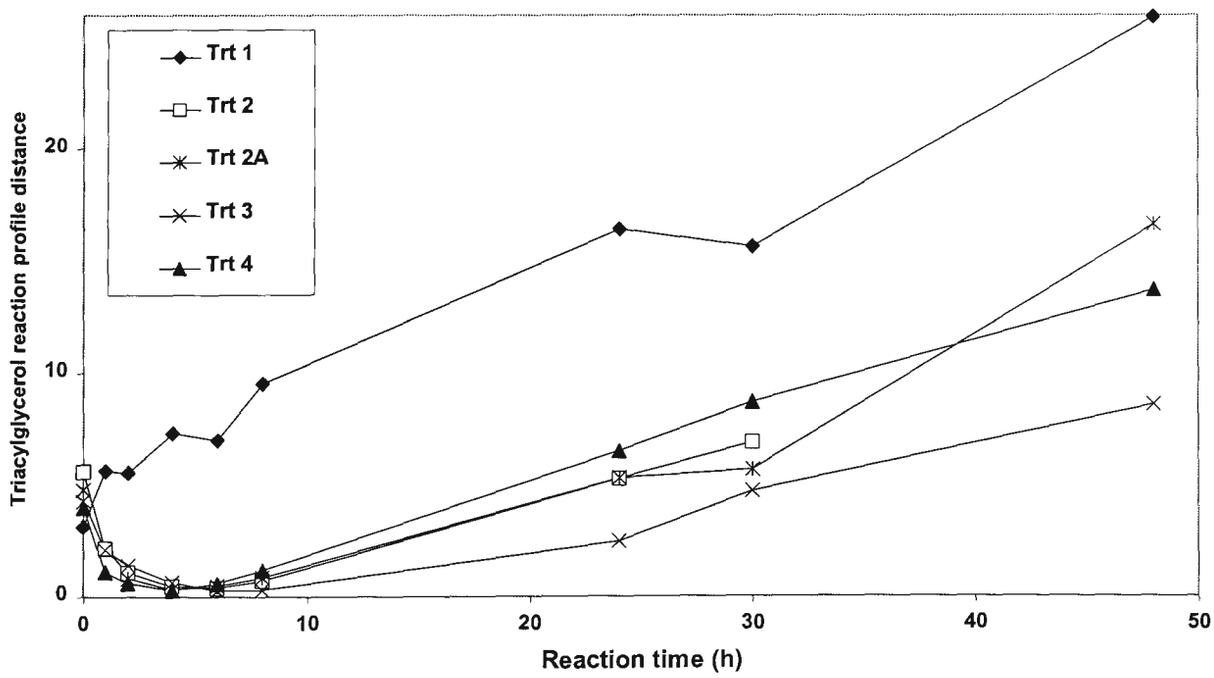
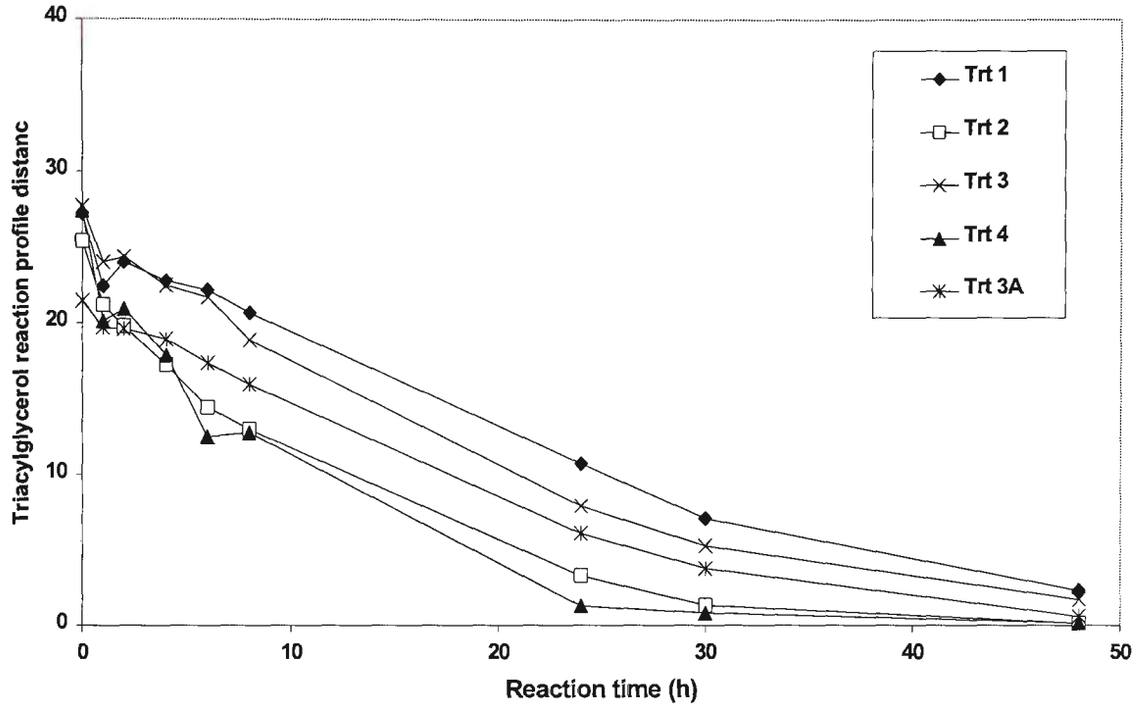
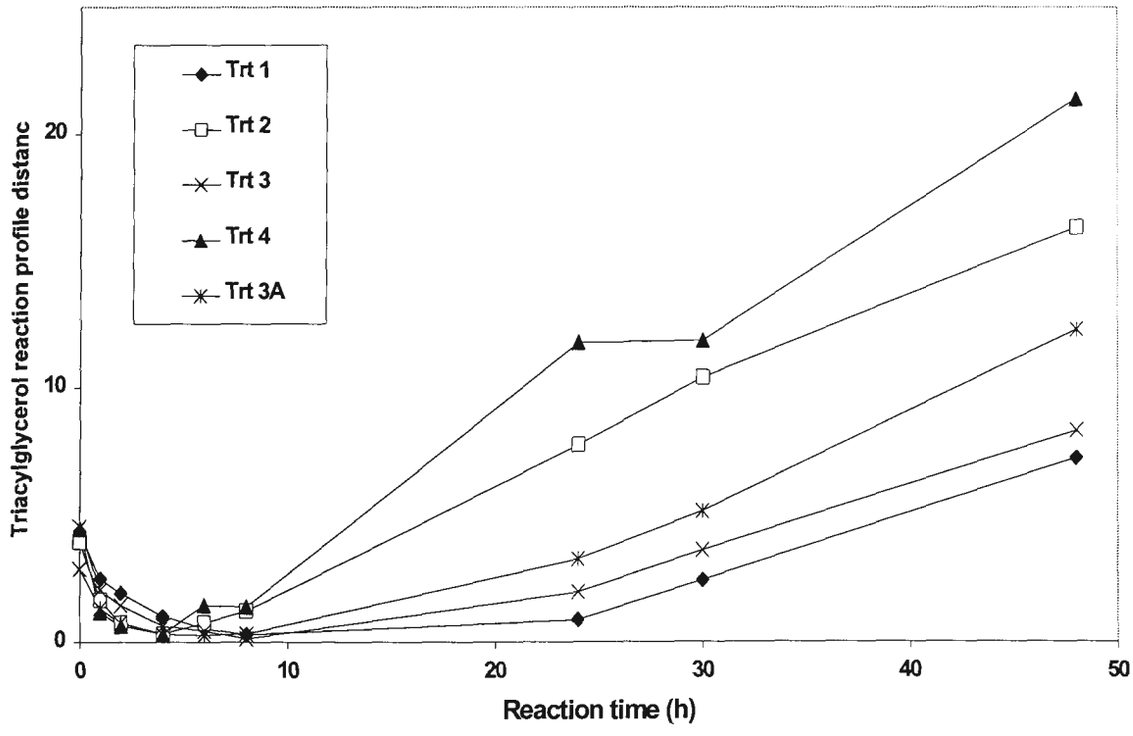


Figure 10.1.3 Random (**A**) and 1,3-specific (**B**) triacylglycerol reaction profiles for a blend of Brazilian cocoa butter and cottonseed oil (70:30) interesterified under several initial reaction condition treatments (treatment 1: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatments 3 and 3A: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water).

A.



B.



Discussion:

The overall and 2-positional fatty acid compositions of the cottonseed oil used for these interesterifications were slightly different to that of the cottonseed oil studied in chapter 5, which may be due to natural variations in the composition of cottonseed oils from different sources. The current cottonseed oil had more palmitic acid (C16:0) and less linolenic acid (C18:2) in the overall fatty acid composition, while in the 2-position there was more palmitic and oleic acid but less linolenic acid. Consequently, the calculated triacylglycerol compositions for completely random and 1,3-specific interesterified cottonseed oil were also slightly different, with the calculated triacylglycerol compositions for the current cottonseed oil having a slightly higher percentage for the T50 group and a slightly lower percentage for the T54 group.

The initial reaction conditions for the treatments used were selected to be within the range of the water and enzyme contents that were used in Chapter 9 to generate the equations for calculating the outcomes of interesterification in terms of lipid class levels and triacylglycerol reaction profiles. In a practical situation, the economics of the interesterification process along with the yield of interesterified triacylglycerol are factors to be considered. With these factors in mind, the conditions were also selected with the aim of achieving a high yield of interesterified triacylglycerol using a minimal amount of enzyme. Therefore the water content was kept below 0.5%, as it was indicated in chapter 9 that water contents above this level could result in a triacylglycerol yield of less than 80%.

In this section, the results are discussed in general with the main points highlighted using several tables and figures. The experimental results were compared to calculated results for interesterification under the selected treatment conditions and the results are presented in sections 10.2 and 10.3.

Generally, the solid fat content results, which were given in Tables 10.1.4 – 10.1.6, show that the more water and enzyme present, the more extensive and rapid were the changes that occurred in the melting profiles. To illustrate the difference between the melting profiles for the three fats and highlight the changes during interesterification, the solid fat content results for treatment 2 (2% Lipozyme and 0.4% water) are shown in Figures 10.1.4-10.1.6. The cocoa butter melting profiles (Figure 10.1.4) show a gradual decrease in the solid fat content at temperatures below 20°C and an increase in the solid fat contents at temperatures greater than 35°C, with a flattening of the melting profile of the original cocoa butter.

The results for cottonseed oil, shown in Figure 10.1.5, demonstrate that there were different types and relatively large changes in the physical properties during interesterification. The absolute changes were not on the same scale as cocoa butter, with the overall change in solid fat content at most measuring temperatures after 48 hours being less than 5%. One interesting aspect to the changes in the cottonseed oil melting profile was an initial drop in the solid fat content at 0 and 5°C, followed by a steady increase in the solid fat content at all temperatures over time. The changes in the solid fat contents during interesterification for the blend of cocoa butter and cottonseed oil (70/30) for treatment 2, shown in Figure 10.1.6, were more like those of cocoa butter.

Figure 10.1.4 Solid Fat Content curves for samples taken during the interesterification of Brazilian cocoa butter using Lipozyme at 2.0% and an overall initial water content of 0.4% (Treatment 2).

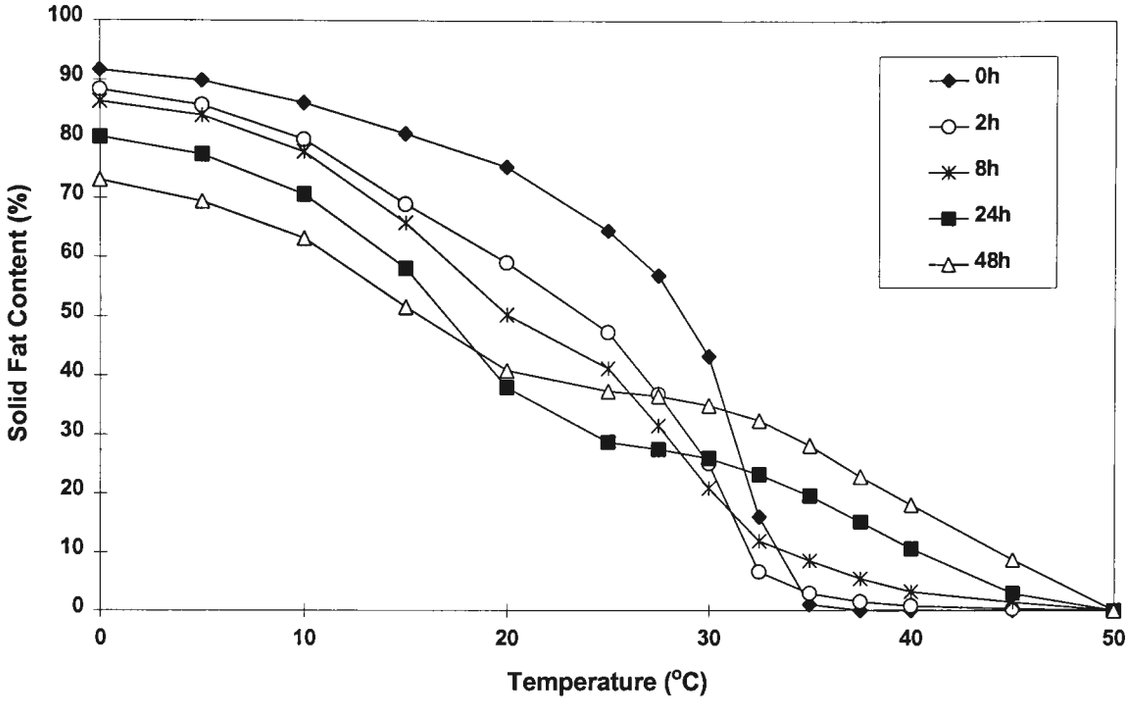


Figure 10.1.5 Solid Fat Content curves for samples taken during the interesterification of cottonseed oil using Lipozyme at 2.0% and an overall initial water content of 0.4% (Treatment 2).

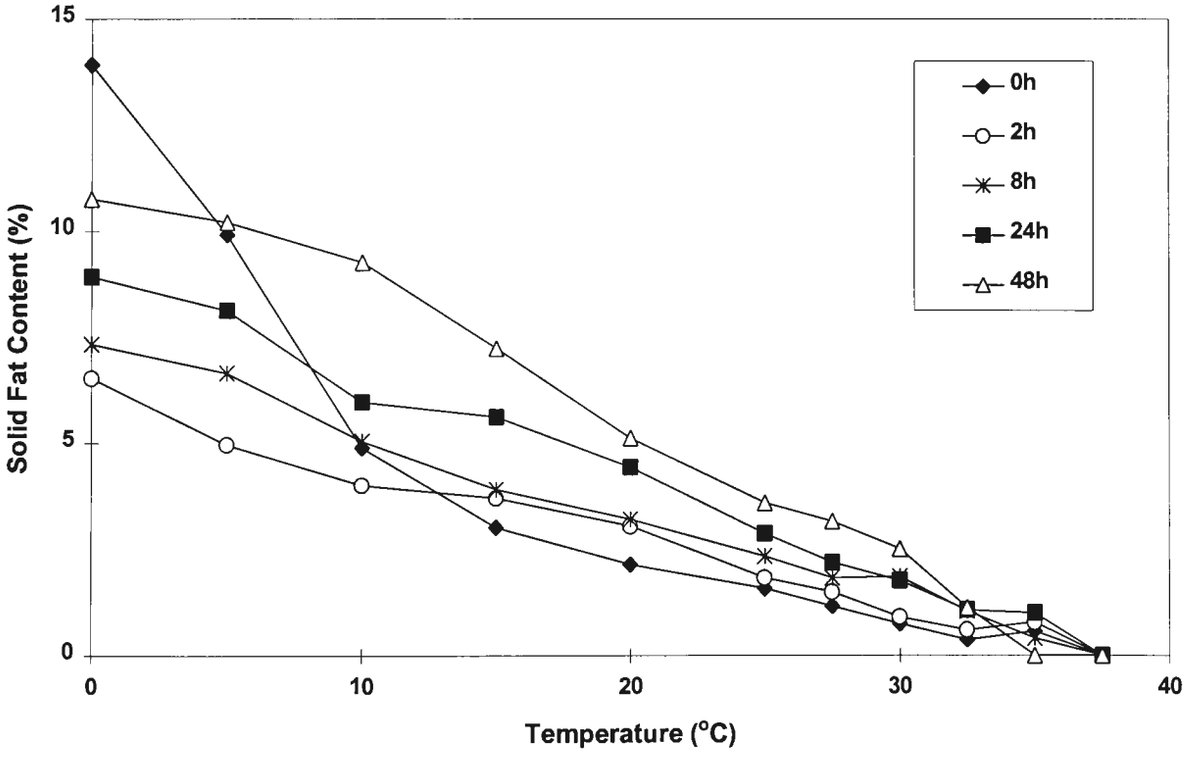
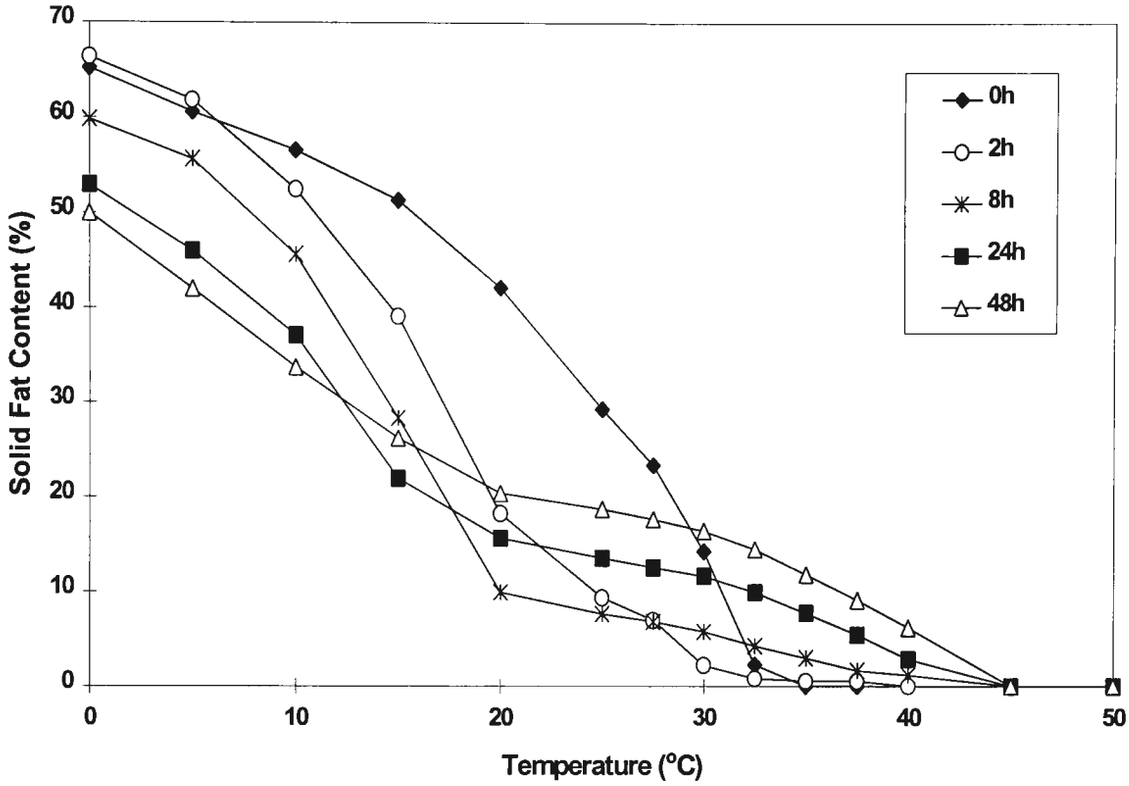


Figure 10.1.6 Solid Fat Content curves for samples taken during the interesterification of a blend of Brazilian cocoa butter and cottonseed oil (70:30) using Lipozyme at 2.0% and an overall initial water content of 0.4% (Treatment 2).



The lipid class method was suitable to use for cottonseed oil as the fatty acid and triacylglycerol composition of cottonseed oil was such that there was no overlap between the peaks for the different lipid classes. The initial triacylglycerol content of cottonseed oil was very similar to the Brazilian cocoa butter, approximately 97%. There were some differences in the average levels of DG and FFA however, with cottonseed oil having a higher level of DG at 3% compared to 2.1% for cocoa butter and a lower level of FFA at 0.2% compared to 1.1% for cocoa butter.

The lipid class results, given in Tables 10.1.7-10.1.9, show that hydrolysis equilibrium was reached within 1 hour for most treatments, with small changes in lipid class levels occurring after this time. The trends for all three fats indicate that the more water present, the higher the levels of by-products and the lower the yield of triacylglycerol. The triacylglycerol yields at 24 hours for all treatments and fats are compared in Table 10.1.10. This table highlights the similarities in the equilibrium triacylglycerol levels

between treatments and the replicated treatments and between all three fats interesterified under the same treatment conditions.

Table 10.1.10 Comparison of the triacylglycerol yield (wt%) for interesterified samples taken after 24 hours of reaction at the Lipozyme and water contents specified for each treatment.

Treatment	Triacylglycerol yield (wt%)		
	Cocoa butter	**Cottonseed oil**	**Blend (70/30 cocoa butter/ cottonseed oil)**
1. 2.0% Lipozyme 0.2% Water	88.9, 88.1	89.3	88.2
2. 2.0% Lipozyme 0.4% Water	82.8	79.7, 80.4	81.7
3. 3.25% Lipozyme 0.2% Water	88.9	89.2	90.2, 89.5
4. 3.25% Lipozyme 0.4% Water	82.5	83.4	82.6

The triacylglycerol reaction profile results for interesterified Brazilian cocoa butter were shown in Figure 10.1.1. The triacylglycerol composition of the blank treatments did not change and no triacylglycerol reaction profiles were presented. The triacylglycerol reaction profiles show that the interesterifications can be grouped mainly according to their water contents, with treatments 1 and 3 and treatments 2 and 4 having reasonably similar triacylglycerol reaction profiles. Treatments 2 and 4, with higher water content, show faster reaction rates for random and 1,3-specific interesterification. Treatment 3 results show that increasing enzyme is also increasing the rate of interesterification. A 1,3-specific product was obtained after 6-8 hours for treatments 2 and 4, compared to between 8 and 24 hours for treatments 1 and 3. The triacylglycerol reaction profiles for the treatment that was replicated (1 and 1A), were very similar.

The triacylglycerol reaction profile results for interesterified cottonseed oil, which were shown in Figure 10.1.2, do not indicate major differences between the treatments, except for treatment 1 where the results could be described as atypical. The reason for this atypical result is not known. It was known from the preliminary work on cottonseed oil in

Chapter 5 that the triacylglycerol reaction profiles would be on a smaller scale and not as 'ideal' as those of cocoa butter to assess the progress of the interesterification reaction.

Cottonseed oil, although having an asymmetric fatty acid distribution, does not have the more structured triacylglycerol composition of cocoa butter and changes in the fatty acid distribution were more difficult to monitor. The triacylglycerol reaction profile technique appears not to be sensitive enough to differentiate clearly between the rates of reaction for different initial reaction conditions using cottonseed oil. Some trends were evident however, for instance treatments 2 and 4, which had the higher water contents, move faster away from the 1,3-specific interesterified triacylglycerol composition than treatment 3.

The triacylglycerol reaction profiles for the interesterified blend of Brazilian cocoa butter and cottonseed oil (70/30) were shown in Figure 10.1.3. These triacylglycerol reaction profiles showed similar trends to interesterified cocoa butter, with the rate of reaction being mainly associated with the initial water content, but also increasing slightly with increasing enzyme content. The scale of the triacylglycerol reaction profiles was smaller than cocoa butter, which was to be expected.

In Chapter 7 and 9, the melting profiles for samples at the same stage of interesterification, either 1,3-specific or random interesterified, were compared and found to be very similar. The reaction times at which the triacylglycerol composition of the interesterified cocoa butter was considered to be 1,3-specific and random were identified and the closest solid fat profiles compared. The results are presented in Tables 10.1.11 and 10.1.12 along with the averaged data from Chapter 9 for comparison. These results were plotted in Figure 10.1.7 for the 1,3-specific interesterified samples and Figure 10.1.8 for the random interesterified samples.

Table 10.1.11 The solid fat content (%) results for the interesterification of Brazilian cocoa butter using different treatments at the reaction time closest to where a 1,3-specific interesterified product was identified.

		Temperature (°C)												
Trt	**Time (h)**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**	**40**	**45**
1	**24**	85.95	83.17	77.34	68.47	61.24	49.15	40.46	27.84	10.36	6.18	4.17	2.12	0
2	**24**	83.84	81.02	74.82	64.15	53.77	45.46	37.48	26.87	13.83	10.19	6.61	4.07	0
3	**8**	86.36	84.39	78.63	70.95	63.78	50.42	41.23	26.37	7.36	3.72	1.9	0.76	0.24
4	**2**	88.52	86.29	80.49	67.65	52.23	43.18	33.56	21.64	3.87	1.59	0.6	0	0
Chapter 9 data (Table 9.1.8):														
Average		85.73	83.40	77.33	67.36	58.25	47.16	38.13	25.16	8.46	5.12	3.31	1.74	0.15
Std deviation		1.56	1.75	1.98	2.53	4.09	3.03	2.91	2.53	3.61	3.09	2.45	1.47	0.25

Table 10.1.12 The solid fat content (%) results for the interesterification of Brazilian cocoa butter using different treatments at the reaction time closest to where a random interesterified product was identified.

		Temperature (°C)												
Trt	**Time (h)**	**0**	**5**	**10**	**15**	**20**	**25**	**27.5**	**30**	**32.5**	**35**	**37.5**	**40**	**45**
1	**24**	85.95	83.17	77.34	68.47	61.24	49.15	40.46	27.84	10.36	6.18	4.17	2.12	0
2	**24**	83.84	81.02	74.82	64.15	53.77	45.46	37.48	26.87	13.83	10.19	6.61	4.07	0
3	**8**	86.36	84.39	78.63	70.95	63.78	50.42	41.23	26.37	7.36	3.72	1.9	0.76	0.24
4	**2**	88.52	86.29	80.49	67.65	52.23	43.18	33.56	21.64	3.87	1.59	0.6	0	0
Chapter 9 data (Table 9.1.9):														
Average		72.40	69.29	63.89	53.41	42.46	39.51	38.47	36.62	33.14	27.93	23.21	18.32	8.23
Std deviation		2.29	2.64	1.90	1.38	2.01	2.14	2.05	2.20	1.87	1.64	1.43	1.31	0.96

Figure 10.1.7 Solid Fat Content curves for Brazilian cocoa butter, the averaged data from Chapter 9, and for samples taken during the interesterification of Brazilian cocoa butter under various initial reaction conditions at reaction times where the samples were identified as 1,3-specific interesterified.

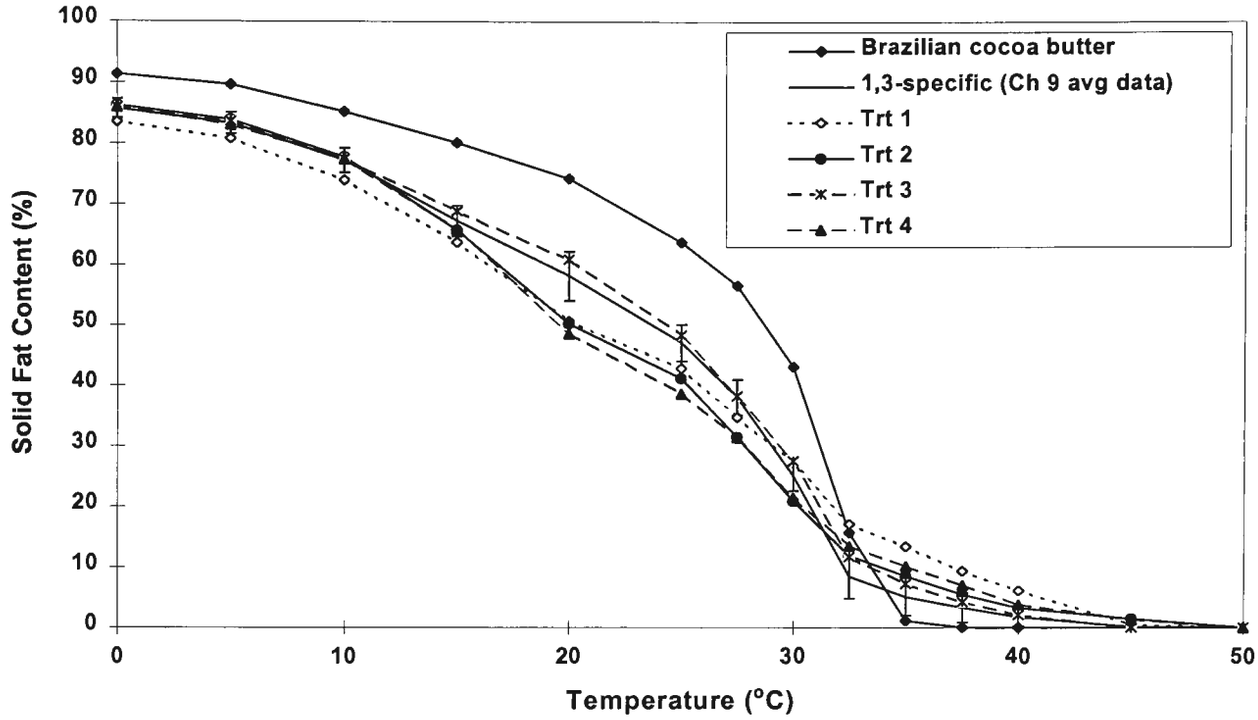
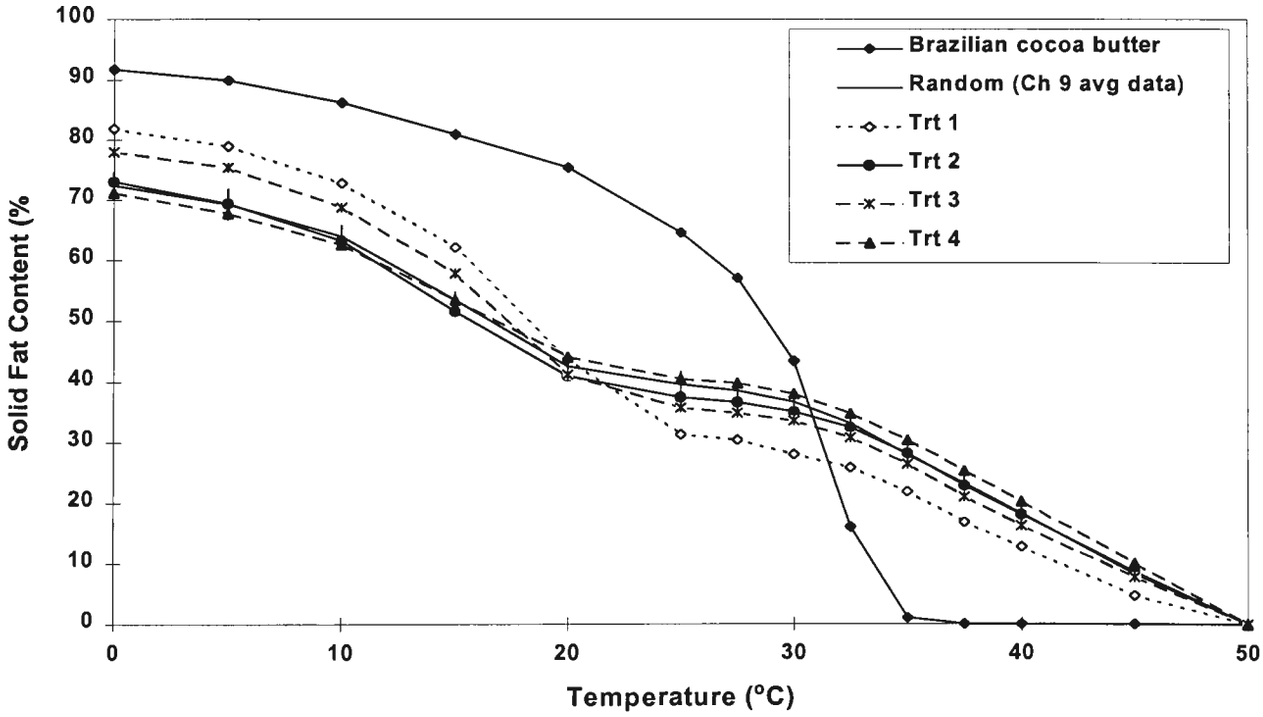


Figure 10.1.8 Solid Fat Content curves for Brazilian cocoa butter, the averaged data from Chapter 9, and for samples taken during the interesterification of Brazilian cocoa butter under various initial reaction conditions at reaction times where the samples were identified as random interesterified.



Figures 10.1.7 and 10.1.8 show that the melting profiles for the 1,3-specific and random interesterified samples from these interesterifications were very similar to the averaged melting profiles for 1,3-specific and random interesterified samples from Chapter 9. For the 1,3-specific interesterified melting profiles, this was particularly true for the measuring temperatures between 0 and 15°C. For the random interesterified melting profiles, those for treatments 2 and 4 were very close to the random melting profile from the Chapter 9 data. From the triacylglycerol reaction profiles, Figure 10.1.1, it can be seen that the random triacylglycerol reaction profile distances were closer to zero for treatments 2 and 4 than for treatments 1 and 3. This suggests that the interesterification reaction is not quite at equilibrium for treatments 1 and 3 and that some further changes may have occurred in the melting profiles if the reaction time was prolonged.

10.2 Comparison between experimental and predicted lipid class levels

The predicted lipid class levels for the interesterification of Brazilian cocoa butter under the treatment conditions used in these studies and at a reaction time of 24 hours were calculated using the developed equations for FFA, DG and TG. The results are presented in Table 10.2.1. As expected from discussions of the equations in Chapter 9, the water content had a major influence on the calculated results, with higher water contents generating lower calculated triacylglycerol yields and higher by-product levels at hydrolysis equilibrium.

Table 10.2.1 Predicted lipid class levels (wt%) for given initial enzyme and water contents for interesterification of Brazilian cocoa butter at a fixed reaction time of 24 hours, using equations developed in chapter 9.

Treatment	Lipozyme (%)	Water (%)	FFA	DG	TG
1	2.0	0.2	3.9	7.8	88.2
2	2.0	0.4	6.9	12.1	80.9
3	3.25	0.2	3.6	7.3	89.0
4	3.25	0.4	6.6	11.6	81.7

The standard errors associated with a calculated value using each equation were 0.6, 1.2 and 1.7 for FFA, DG and TG respectively (Chapter 9). These errors were much higher than the average standard deviations for the experimental results, which was less than 0.2. For each calculated value, a range of values was determined based on the standard errors associated with the calculated value, which the experimental lipid classes should fall

within. The calculated ranges of values for each lipid class under the treatments used are compared to the experimental values in Table 10.2.2.

Table 10.2.2 The predicted lipid class levels for given initial enzyme and water contents at a reaction time of 24 hours using the equations from Chapter 9 and the experimental results for the different interesterified fats where; treatment 1: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water.

Trt		Lipid class (wt%) Calculated	Std error range	Experimental lipid class results (%) Cocoa butter	Cottonseed oil	Blend (70/30)
1	FFA	3.9	3.3 - 4.5	3.5, 3.8	2.8	3.5
1	DG	7.8	6.6 - 9.0	7.6, 8.1	8.0	7.6
1	TG	88.2	86.5 – 89.9	88.9, 88.1	89.3	89.0
2	FFA	6.9	6.3 – 7.5	6.1	6.1, 5.7	6.0
2	DG	12.1	10.9 – 13.3	11.0	13.9, 13.7	10.8
2	TG	80.9	79.2 – 82.6	82.8	79.7, 80.4	83.1
3	FFA	3.6	3.0 – 4.2	3.6	2.7	3.7, 3.4
3	DG	7.3	6.1 – 8.5	7.4	8.1	7.7, 7.2
3	TG	89.0	87.3 – 90.7	88.9	89.2	88.6, 89.5
4	FFA	6.6	6.0 – 7.2	6.2	5.0	6.3
4	DG	11.6	10.4 – 12.8	11.2	11.4	11.3
4	TG	81.7	80.0 – 83.4	82.5	83.3	82.3

From this table it can be seen that all the experimental results for cocoa butter, apart from the FFA and TG levels of treatment 2 which are just outside the range, fall within the range of one standard error for the predicted lipid class values. The FFA experimental results for cottonseed oil were all below the calculated range, which suggests that the initial levels of FFA and DG do impact on the equilibrium levels of by-products. In an earlier discussion of the lipid class results, it was noted that the level of FFA in cottonseed oil was much lower than the level of FFA in cocoa butter. The triacylglycerol yields, however, were all within the calculated range. The results for the blend of Brazilian cocoa butter and cottonseed oil (70/30) were all within the expected ranges of predicted lipid class levels, apart from treatment 2.

These comparisons have demonstrated that the equations developed in chapter 9 could be used to predict the equilibrium lipid class levels for the interesterification of cocoa butter under these conditions. These equations may also be used to predict changes in the levels

of triacylglycerol in fats other than cocoa butter, however the initial lipid class composition of any starting fat should also be taken into consideration and the equations may require further refining to take this into account.

10.3 Comparison between experimental and predicted triacylglycerol reaction profiles

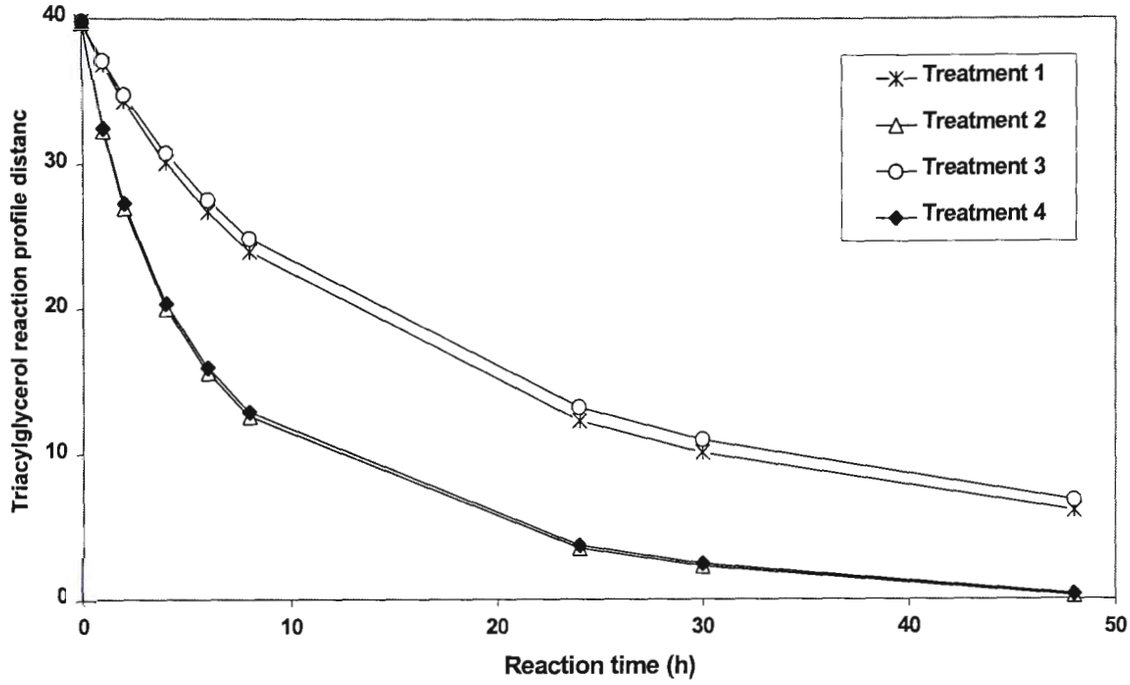
The random and 1,3-specific triacylglycerol reaction profiles describe the changes occurring in the triacylglycerol composition during the interesterification of cocoa butter. In Chapter 9 overall equations were developed to calculate the triacylglycerol reaction profile values that relate specifically to the interesterification of Brazilian cocoa butter, requiring the input of the initial Lipozyme and water contents as well as the reaction time (Equations 9.4.1 and 9.4.2). The predicted triacylglycerol reaction profile values for the different treatments used in these interesterifications were calculated at various reaction times, given in Table 10.3.1. This data is also presented in Figure 10.3.1 showing the random and 1,3-specific triacylglycerol reaction profiles on separate figures.

Table 10.3.1 Predicted random and 1.3-specific reaction profile values for Brazilian cocoa butter under the four treatment combinations used in the enzymatic interesterifications.

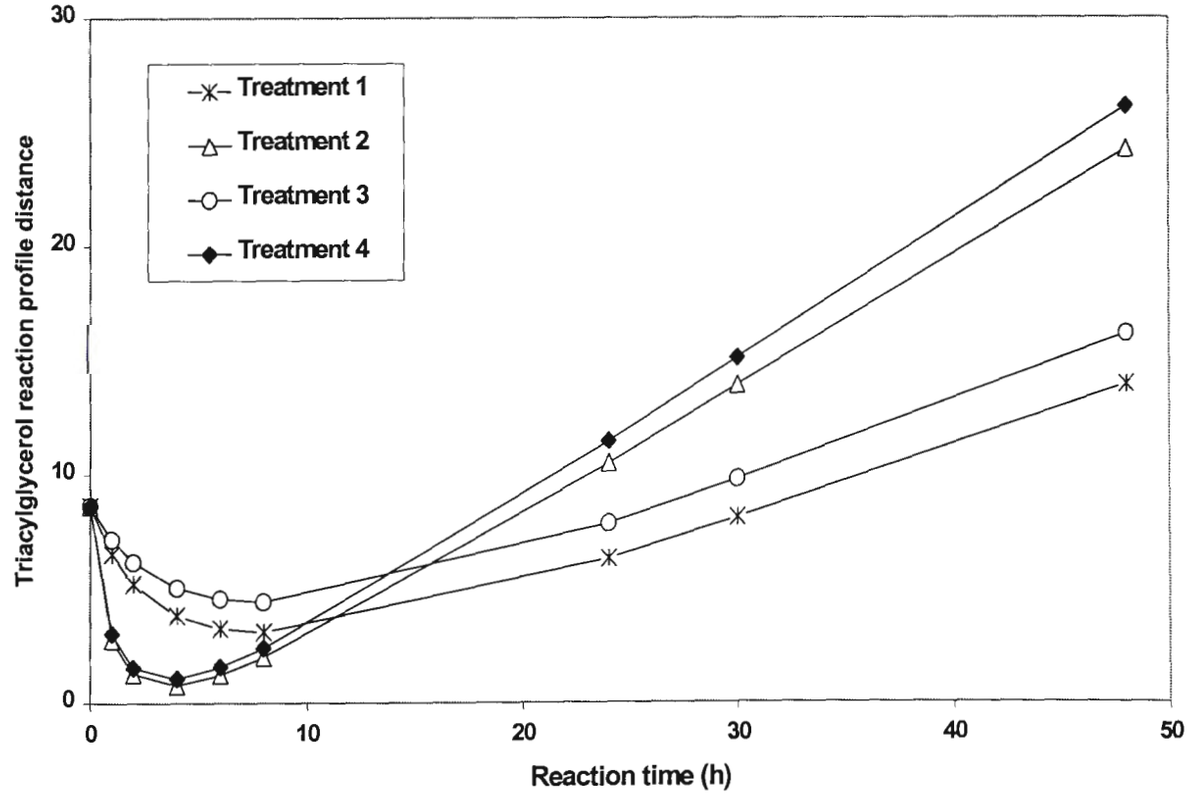
Random			**Time (h)**								
Trt	**%Lipozyme**	**%Water**	**0**	**1**	**2**	**4**	**6**	**8**	**24**	**30**	**48**
1	**2**	**0.2**	39.8	36.9	34.4	30.1	26.8	24.0	12.3	10.1	6.1
2	**2**	**0.4**	39.8	32.3	27.0	20.1	15.7	12.6	3.5	2.3	0.2
3	**3.25**	**0.2**	39.8	37.1	34.8	30.8	27.6	24.9	13.3	11.0	6.8
4	**3.25**	**0.4**	39.8	32.5	27.3	20.4	16.0	12.9	3.7	2.5	0.4
1,3-specific			**Time (h)**								
Trt	**%Lipozyme**	**%Water**	**0**	**1**	**2**	**4**	**6**	**8**	**24**	**30**	**48**
1	2	0.2	8.6	6.5	5.2	3.8	3.3	3.1	6.3	8.1	13.9
2	2	0.4	8.6	2.8	1.3	0.8	1.2	2.0	10.5	13.9	24.2
3	3.25	0.2	8.6	7.2	6.2	5.0	4.6	4.4	7.8	9.8	16.1
4	3.25	0.4	8.6	3.0	1.5	1.1	1.6	2.4	11.4	15.1	26.1

Figure 10.3.1 Predicted random (A) and 1,3-specific (B) triacylglycerol reaction profiles for cocoa butter interesterified under specified initial reaction conditions (treatment 1: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water).

A



B



From the comparison of the predicted random triacylglycerol reaction profiles in Figure 10.3.1A, it can be seen that the water content was expected to be the major influence on the rate of random interesterification. The treatments that have the higher initial water content, treatments 2 and 4, have a predicted faster reaction rate than treatments 1 and 3. From the calculated 1,3-specific triacylglycerol reaction profiles in Figure 10.3.1B, the initial water content is also a major factor increasing the rate of 1,3-specific interesterification with increasing water, although the enzyme content also has a minor influence with a higher enzyme content slightly increasing the reaction rate.

The equations used to calculate the triacylglycerol reaction profile values were based on averaged data from individual triacylglycerol reaction profile equations and also contained linear equations that substituted for some of the coefficients of the time (X) terms. Therefore, an estimation of the errors associated with each calculated value was difficult to determine. The average standard error from the individual equations could have been used as a starting point, and they were 1.28 for the random (Table 9.3.1) and 0.72 for the 1,3-specific (Table 9.3.3) interesterification equation. The standard errors of the linear equations incorporating the Lipozyme and enzyme contents that were included in the final equations would also add to the overall error associated with a calculated value. Although these errors were relatively small (<0.4), as these linear equations were substituted for the coefficients of certain reaction time terms, the degree of error increased as the reaction progressed. It is the early stages of interesterification which are of particular interest, where the errors in the calculated distance values would be comparatively lower.

It was not possible to calculate proper standard errors for the calculated distance values to determine a range of values that the experimental values should fall within. The experimental and calculated triacylglycerol reaction profiles for Brazilian cocoa butter were compared on an individual basis. For all three fats interesterified, the trends in the predicted calculated triacylglycerol reaction profiles were compared to the trends in the experimental triacylglycerol reaction profiles, such as the rates of reaction and the effect of increasing enzyme and water content.

The experimental triacylglycerol reaction profile results for the interesterification of Brazilian cocoa butter were given in section 10.1 and illustrated in Figure 10.1.1. The trends identified in the experimental profiles are in agreement with those of the predicted triacylglycerol reaction profiles (Figure 10.3.1). In particular, there were similarities between triacylglycerol reaction profiles for the treatments with the same water content, with treatments 2 and 4 having the higher water contents and demonstrating faster rates of interesterification.

The experimental and predicted triacylglycerol reaction profiles for the individual treatments are compared in Figures 10.3.2-10.3.9. In general, the experimental random triacylglycerol reaction profiles followed the same shape as the calculated triacylglycerol reaction profiles, however the experimental distance values were higher than those calculated (Figures 10.3.2-10.3.5). This difference was more evident at the initial reaction times (0-8h), with the calculated and experimental values becoming closer after a reaction time of 24 hours. The random triacylglycerol reaction profiles were better predicted for treatments 3 and 4, which had a higher initial enzyme content (3.25% Lipozyme), than for treatments 1 and 2, which had an initial enzyme content of 2.0% Lipozyme.

The experimental and predicted 1,3-specific triacylglycerol reaction profiles, shown in Figures 10.3.6-10.3.9, did not agree as well as those of the random triacylglycerol reaction profiles, after the initial decrease in distance values. Most of the initial 1,3-specific experimental triacylglycerol reaction profile values (0-8h) were closer to the calculated 1,3-specific triacylglycerol reaction profiles than after this time. The experimental profiles for treatments 1 and 3 had lower 1,3-specific triacylglycerol reaction profile distance values for a longer reaction time than those predicted, indicating that the reaction product was more 1,3-specific like at 24 hours than it was calculated to be under the given reaction conditions. There was better correlation for the treatments with a higher initial water content (0.4%), treatments 2 and 4, compared to treatments 1 and 3, which had a water content of 0.2% overall initially.

Figure 10.3.2 Predicted and experimental random triacylglycerol reaction profiles for cocoa butter interesterified under treatment 1 conditions of 0.2% water and 2% Lipozyme.

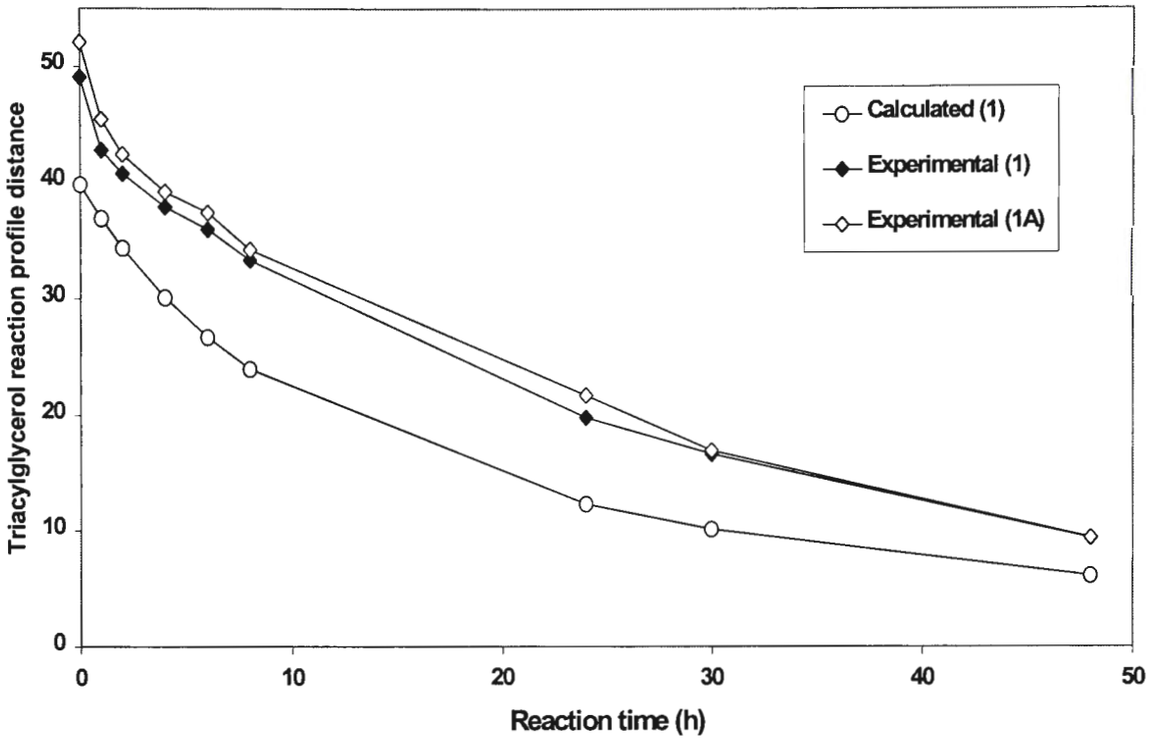


Figure 10.3.3 Predicted and experimental random triacylglycerol reaction profiles for cocoa butter interesterified under treatment 2 conditions of 0.4% water and 2% Lipozyme.

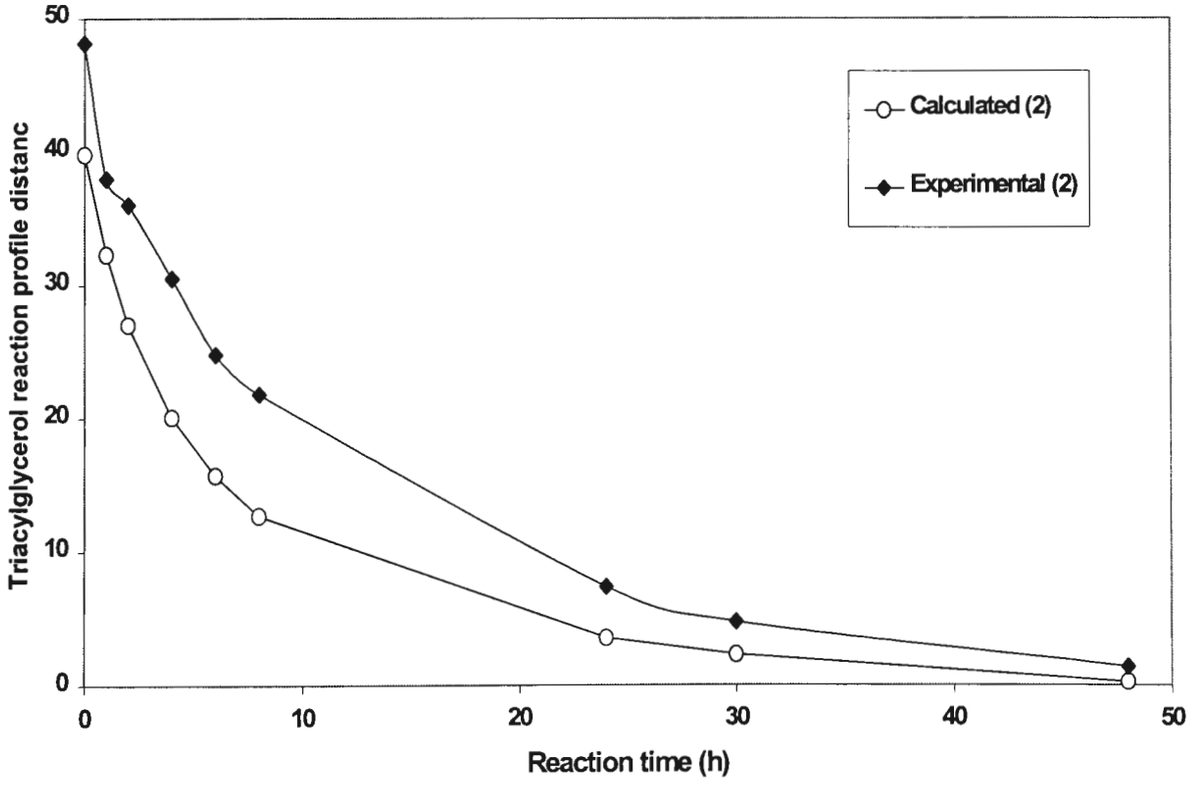


Figure 10.3.4 Predicted and experimental random triacylglycerol reaction profiles for cocoa butter interesterified under treatment 3 conditions of 0.2% water and 3.25% Lipozyme.

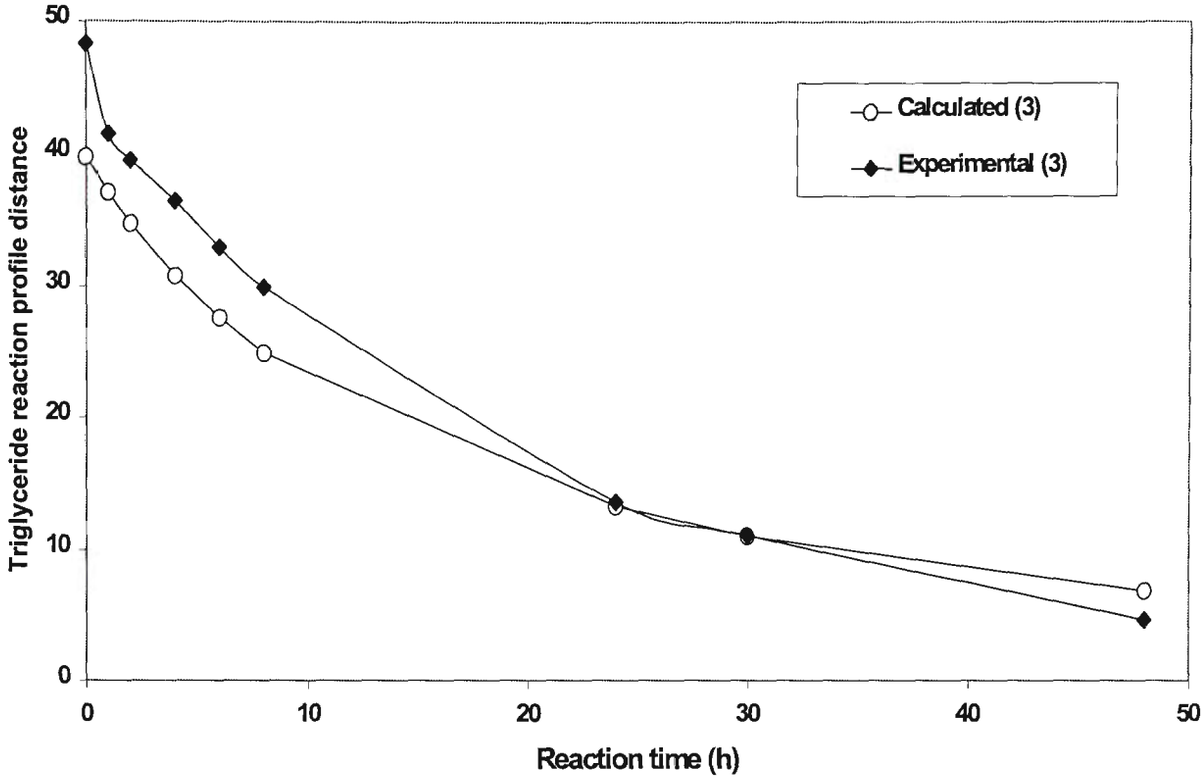


Figure 10.3.5 Predicted and experimental random triacylglycerol reaction profiles for cocoa butter interesterified under treatment 4 conditions of 0.4% water and 3.25% Lipozyme.

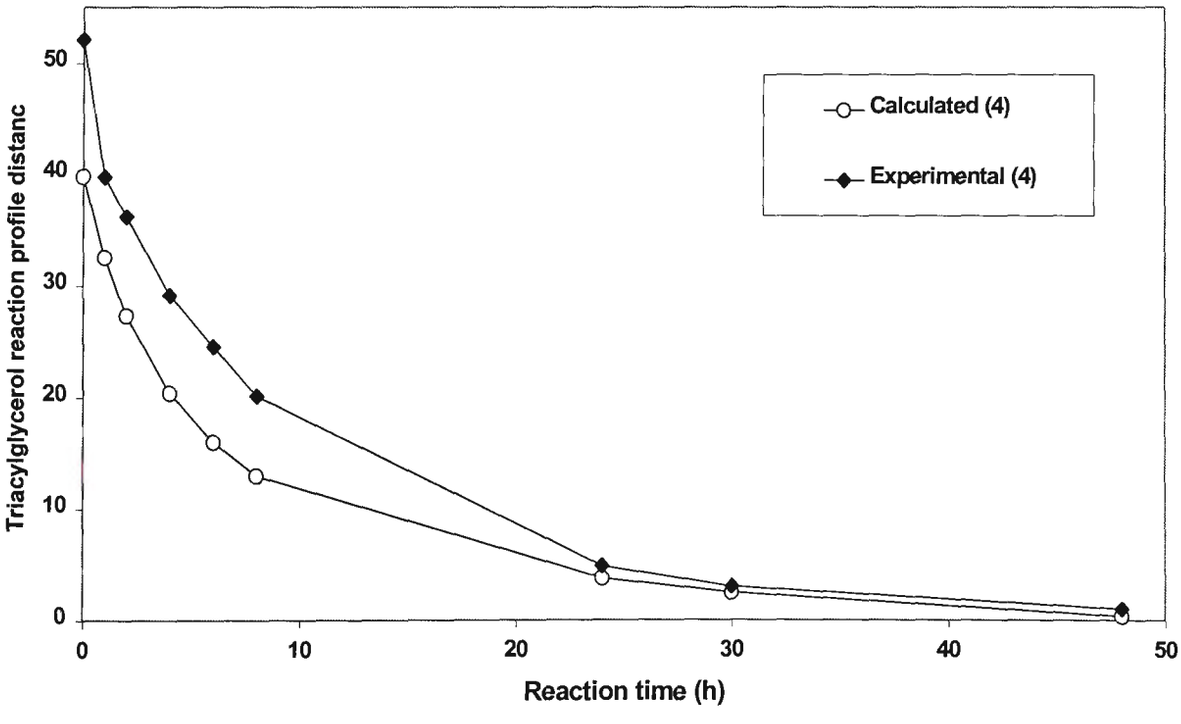


Figure 10.3.6 Predicted and experimental 1,3-specific triacylglycerol reaction profiles for cocoa butter interesterified under treatment 1 conditions of 0.2% water and 2% Lipozyme.

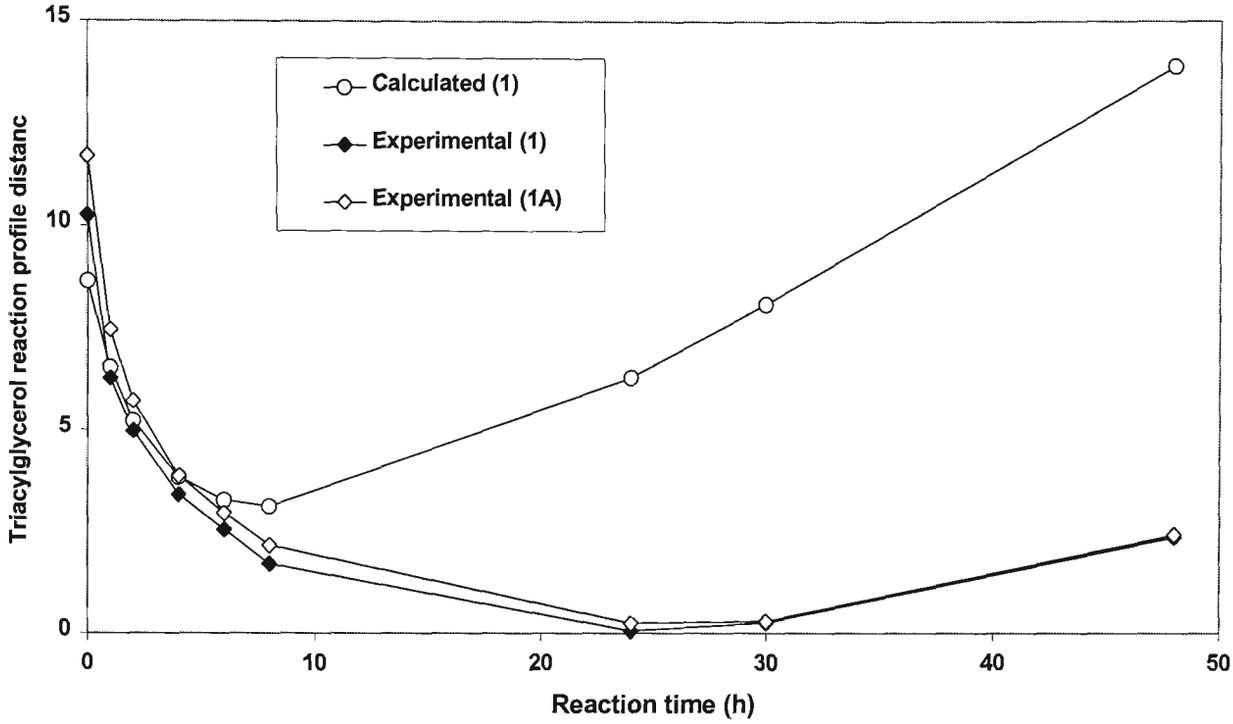


Figure 10.3.7 Predicted and experimental 1,3-specific triacylglycerol reaction profiles for cocoa butter interesterified under treatment 2 conditions of 0.4% water and 2% Lipozyme.

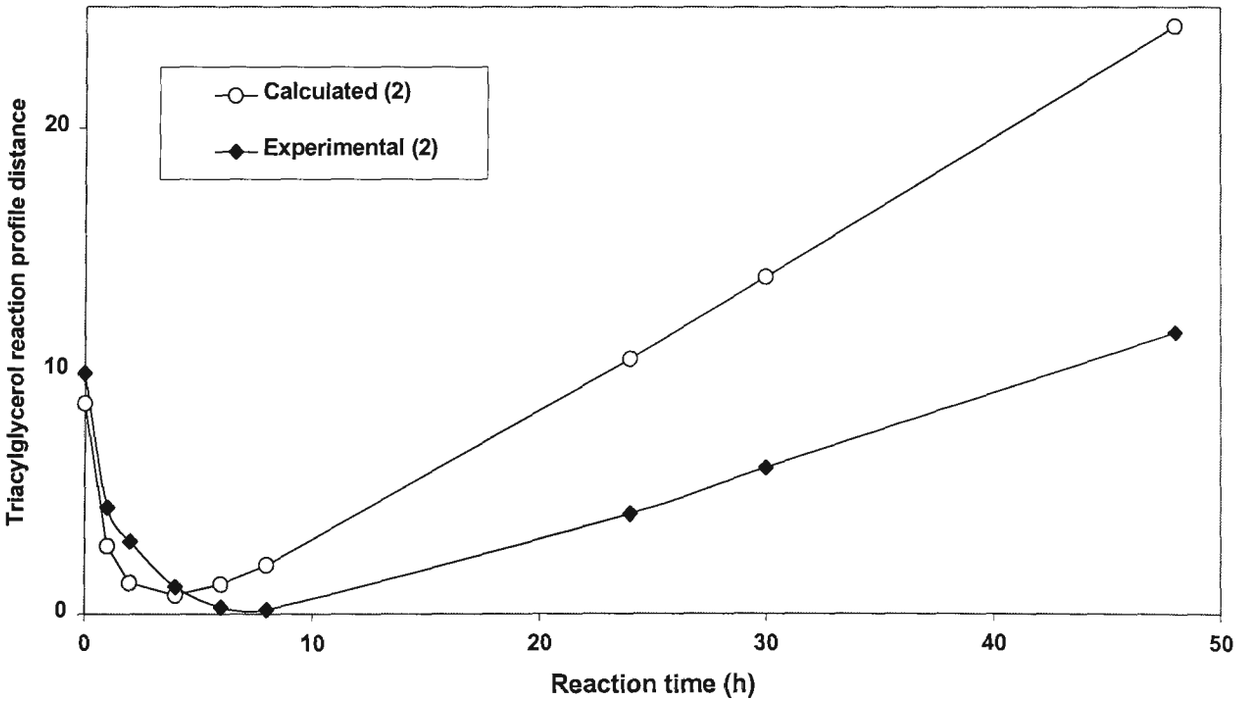


Figure 10.3.8 Predicted and experimental 1,3-specific triacylglycerol reaction profiles for cocoa butter interesterified under treatment 3 conditions of 0.2% water and 3.25% Lipozyme.

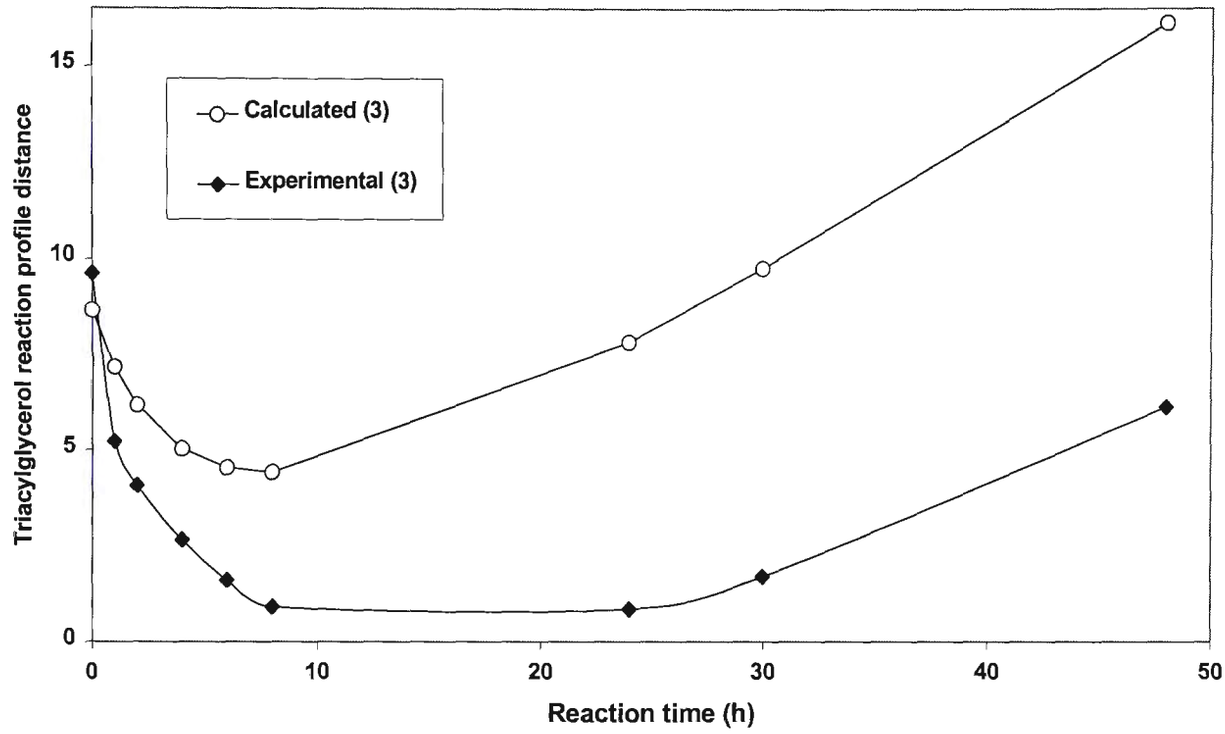
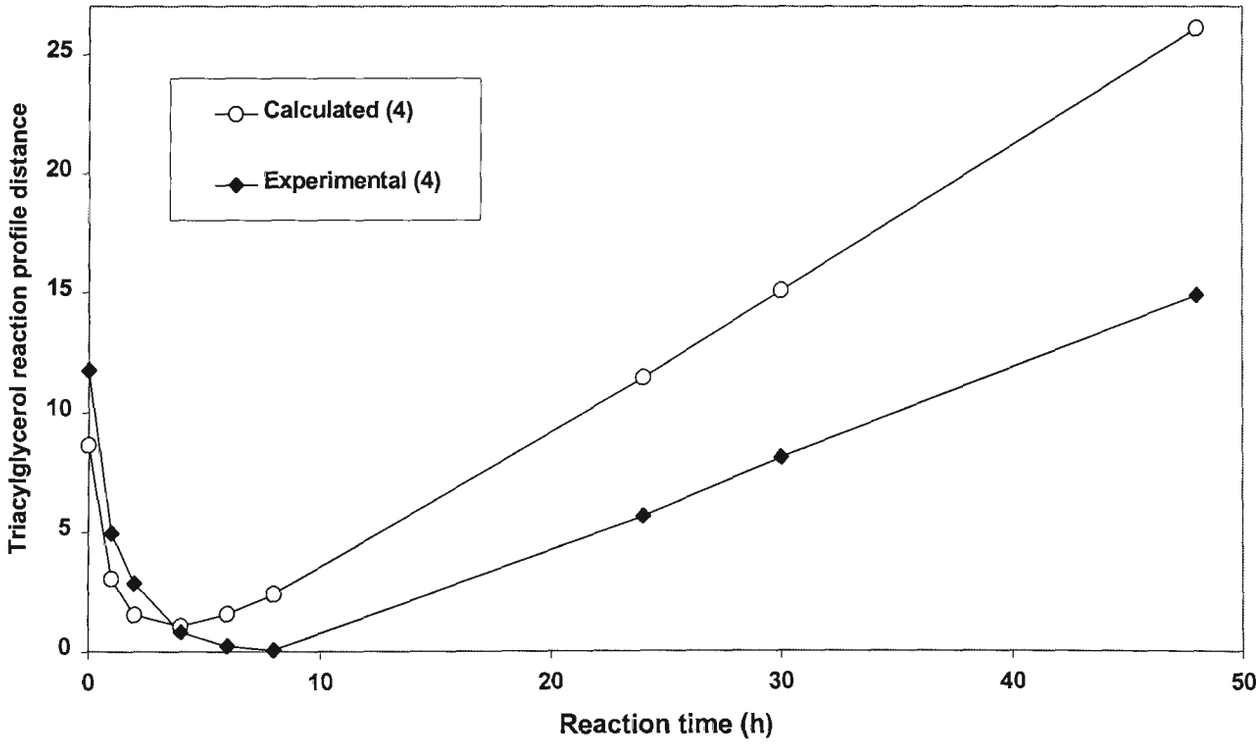


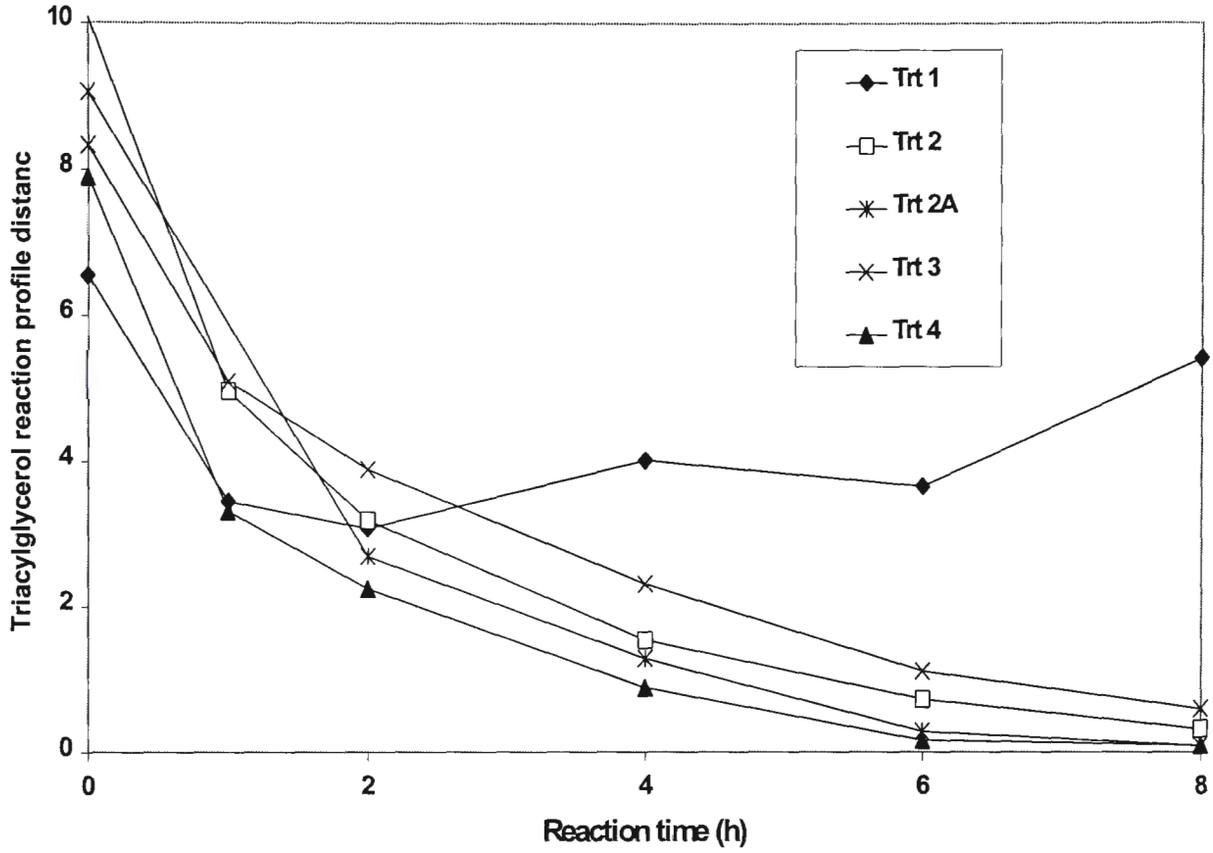
Figure 10.3.9 Predicted and experimental 1,3-specific triacylglycerol reaction profiles for cocoa butter interesterified under treatment 4 conditions of 0.4% water and 3.25% Lipozyme.



In Chapter 9, the actual triacylglycerol reaction profile data points for the interesterification of Brazilian cocoa butter with a Lipozyme content of 2.5% and a water content of 0.3% were compared to the calculated triacylglycerol reaction profile in Figures 9.4.2 and 9.4.4. These reaction conditions were similar to treatment 2 in these interesterification studies, with a Lipozyme content of 2% and a water content of 0.4%. In Chapter 9, the calculated random triacylglycerol reaction profile distance values were slightly higher than the actual data points (Figure 9.4.2), whereas in this chapter for similar initial reaction conditions the predicted, or calculated, triacylglycerol reaction profile distance values were lower than the actual distance values (Figure 10.3.3). These comparisons indicate that the equations may need further refining and optimising to more accurately describe the outcomes of 1,3-specific and random interesterification.

The triacylglycerol reaction profiles for the interesterification of cottonseed oil were given in Figure 10.1.2. For the cottonseed oil triacylglycerol reaction profiles, the results for treatment 1 were atypical and not considered further when identifying the trends in the results. For the random triacylglycerol reaction profiles, treatments 2 and 4 do have similar profiles and show a slightly faster reaction than for treatment 3. Figure 10.3.10 is a more detailed view of the first 8 hours of the 1,3-specific triacylglycerol reaction profiles for cottonseed oil. Although due to the scale of the earlier figure, the initial profiles appeared to be very similar, subtle distinctions can be made upon a closer inspection. The rate of 1,3-specific interesterification was higher for treatments 2 and 4 than for treatment 3, as predicted by the equations at higher water content.

Figure 10.3.10 The 1,3-specific triacylglycerol reaction profiles up to 8 hours of reaction for cottonseed oil interesterified under several initial reaction condition treatments. (treatment 1: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water).

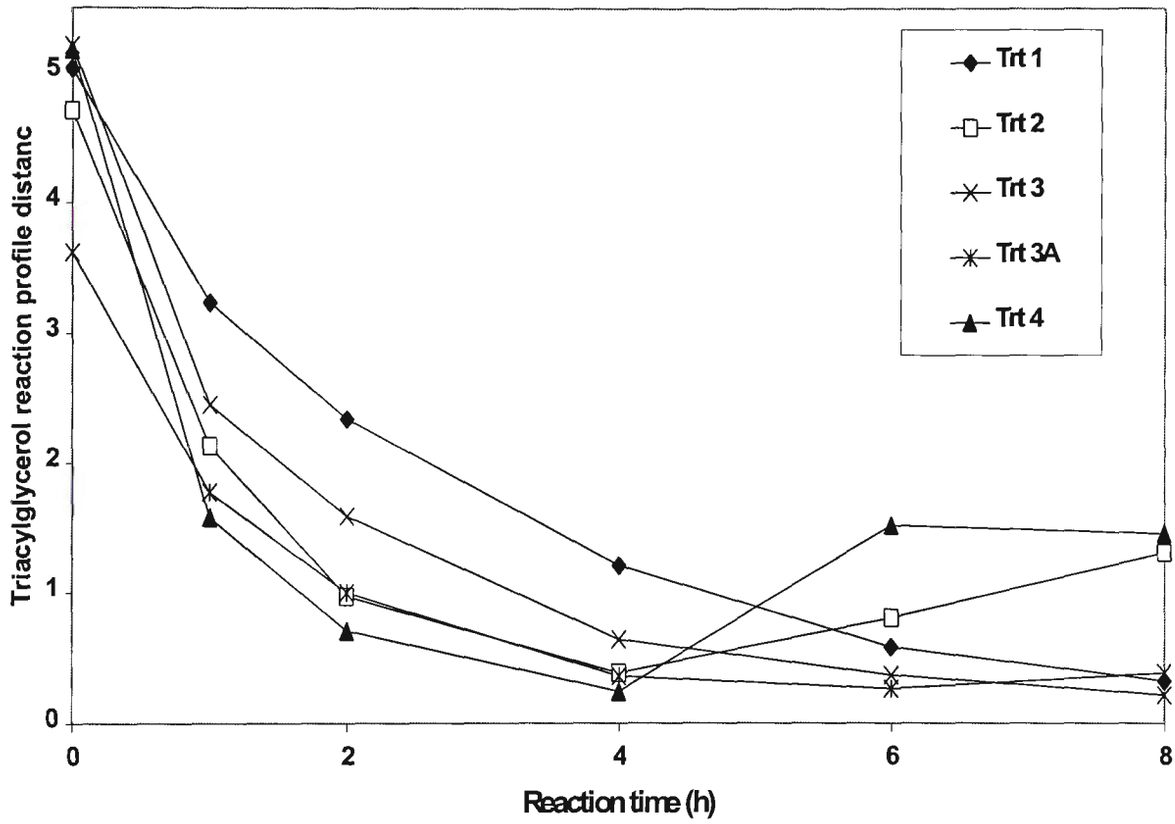


For the cocoa butter and cottonseed oil blend (70:30), the random triacylglycerol reaction profiles were given in an earlier section and illustrated in Figure 10.1.3. The random triacylglycerol reaction profile results showed that the rate of interesterification for treatments 2 and 4 was faster than the rate of interesterification for treatments 1 and 3. The random triacylglycerol reaction profiles for treatments 2 and 4 were very similar to each other and showed that the interesterification products were almost completely randomised after 24 hours.

The initial stages of the 1,3-specific triacylglycerol reaction profiles have been expanded in Figure 10.3.11. This figure shows that the 1,3-specific interesterification for treatment 1 occurred at a slower rate than that for treatments 2 and 4, while treatment 3 was in between. The interesterified products of treatments 2 and 4 were 1,3-specific after 4

hours, while for treatments 1 and 3 a 1,3-specific interesterified product was reached after 8 hours of reaction. In Figure 10.1.3, the triacylglycerol reaction profiles show a difference in the reaction rates more clearly after 24 hours, with treatments 2 and 4 having higher reaction profile distance values than treatments 1 and 3.

Figure 10.3.11 The 1,3-specific triacylglycerol reaction profiles up to 8 hours of reaction for a blend of Brazilian cocoa butter and cottonseed oil (70:30) interesterified under several initial reaction condition treatments. (treatment 1: 2.0% Lipozyme and 0.20% water; treatment 2: 2.0% Lipozyme and 0.40% water; treatment 3: 3.25% Lipozyme and 0.20% water; treatment 4: 3.25% Lipozyme and 0.40% water).



These comparisons have demonstrated that the overall equations developed in Chapter 9 based on the interesterification of Brazilian cocoa butter under various initial reaction conditions could, within limits, predict the progress and outcomes of enzymatic interesterification. The equations were developed from Brazilian cocoa butter and were specifically relevant to Brazilian cocoa butter. The predicted effects were also observed

with other fats. More extensive trials, however, using other fats could demonstrate their application to other fats and oils more clearly.

There were various factors that could have contributed to the triacylglycerol reaction profile results and therefore influenced the ability of the developed equations to predict the experimental results. The activity of the Lipozyme may have varied slightly for each interesterification due to inherent variation or been affected by storage. There may have been fatty acid or positional specific effects on the activity of the Lipozyme when fats other that cocoa butter were used. Small variations in the water contents, particular when using such low levels, may have impacted on the rate of interesterification as well as the levels of lipid by-products at equilibrium.

The aim of developing an overall equation for predicting the triacylglycerol reaction profile distance values has been achieved to a limited degree. The equations need further refining through more detailed interesterification studies with improved control over the reaction conditions and analyses.

10.4 Chapter conclusions:

The enzymatic batch interesterification of Brazilian cocoa butter, cottonseed oil and a blend was carried out under 4 initial reaction treatment conditions of two enzyme contents (2.0 and 3.24%) and 2 water contents (0.2 and 0.4%). Samples were taken during the interesterification reaction at 0, 1, 2, 4, 8, 24 and 48 hours and analysed for solid fat content, lipid class, triacylglycerol composition and triacylglycerol reaction profiles. The experimental results were compared to the results predicted using the equations developed in Chapter 9 for lipid classes and triacylglycerol reaction profiles to assess the ability of the equations to predict the outcomes of enzymatic interesterification.

It was found that the lipid classes could be predicted reasonably well for cocoa butter and the other fats. The actual results were within, or very close to, the predicted standard error range.

The overall triacylglycerol reaction profile equations could be used to predict the influence of altering the enzyme or water content on the interesterification of cocoa butter. They were not robust enough, however, to accurately predict the actual triacylglycerol reaction profile distance values. The triacylglycerol reaction profile equations need to be developed and refined further before wider adoption could be recommended.

It was found in studies reported earlier in this thesis that the solid fat content profiles could be related to the stage of the interesterification reaction as described by the triacylglycerol reaction profiles, with quite distinct melting profiles for a 1,3-specific interesterified cocoa butter compared to a random interesterified cocoa butter. The solid fat content results of these studies have affirmed this relationship. This information could have important practical value for control and monitoring of reactions when specific changes in the physical properties are required.

Chapter 11

Conclusions and recommendations

11.1 General Conclusions

The aim of developing a predictive model for the enzymatic interesterification of edible fats and oils under solvent-free conditions was achieved as a result of these studies.

The specific area of the triacylglycerol:triacylglycerol batch interesterification process in a solvent-free, natural fat system was investigated using a variety of fats, reaction conditions and analytical techniques. The interesterifications were characterised using an innovative approach, providing powerful tools to describe and follow reactions. The main findings/conclusions from this work are summarised below.

An effective process of immobilising an enzyme to a carrier was found to be very important in delivering consistent and productive interesterification results. After assessing a range of immobilised enzymes, a commercially available immobilised lipase, Lipozyme from Novo Nordisk, was found to be best suitable. This enzyme was used for the majority of the interesterification studies in this thesis.

The selection of an appropriate fat system to use for further investigations was also found to be very important. It was concluded quite early in the research that the assay used generally to assess the lipase interesterification activity, did not describe the behaviour of the lipase under 'real' application conditions in a natural fat matrix. Therefore, the fat and analytical system for study had to meet certain requirements in order to be a real fat matrix system, as well as allowing changes occurring in the triacylglycerol composition and physical properties to be monitored. Cocoa butter was selected as a suitable fat for further investigations due to its relatively ideal fatty acid composition and their distribution in the triacylglycerol composition.

A novel process for characterising changes occurring in the triacylglycerol composition of the cocoa butter due to interesterification was developed. The asymmetric fatty acid distribution of cocoa butter was used as a basis for determining

whether changes in the fatty acid composition due to interesterification were 1,3-specific or random in nature, during the various stages of the reaction. By comparison of calculated completely interesterified 1,3-specific and random triacylglycerol compositions, to triacylglycerol composition of samples taken during the interesterification, using a sum of squares calculation, a single figure was generated which was representative of the similarity of the two triacylglycerol compositions. The more alike the two triacylglycerol compositions were, the smaller the number generated or 'distance' between them. Therefore, if the two triacylglycerol compositions were identical, the 'distance' would be zero. Using a plot of these triacylglycerol reaction profile distance values over time, the extent and rate of the reaction as well as the reaction time at which the samples were 1,3-specific and random interesterified could be determined. This 'triacylglycerol reaction profile technique' was applied to other fats that had asymmetric fatty acid distributions, namely milkfat, cottonseed oil and egg yolk lipids. These fats, however, were not considered as suitable as cocoa butter.

The process of enzymatic interesterification was characterised using 1) the triacylglycerol reaction profile technique to monitor changes in the fatty acid distribution of the triacylglycerols, 2) lipid classes to provide information on the yield of the interesterified triacylglycerols and 3) the solid fat contents of the samples to monitor changes in the physical properties. The combination of these three analytical results was found to be essential and sufficient for assessing and monitoring the enzymatic interesterification process in a natural fat system.

The effect of the lipid classes on the physical properties of cocoa butter was examined. It was found that a 10% blend of palmitic, stearic and oleic acid had only a small effect on the solid fat profile of cocoa butter. Diacylglycerols were also considered to affect the physical properties, however, it was not considered resource effective to remove the lipid by-products in this study. Their effect on the solid fat content results was, however, taken into account when interpreting solid fat profiles.

The effects of varying the initial water and enzyme contents on the outcomes of enzymatic interesterification of cocoa butter were studied. It was found that increasing the water content increased the levels of minor lipids at hydrolysis

equilibrium as well as the rate of randomisation. Increasing the enzyme content increased the rate of the interesterification slightly, however, this was also dependent on the water content of the system. It was also found that when additional water was added to a reaction system, it made little difference to the interesterification process, whether the water was added to the enzyme or directly to the fat medium.

Changes in the physical properties of the cocoa butter could be related to the stage of the interesterification process. It was clearly demonstrated, when the solid fat contents for samples that were closest to a 1,3-specific and random triacylglycerol composition, were compared, that the solid fat profiles of 1,3-specific and random interesterified Malaysian cocoa butter were quite distinct from each other. Melting profiles for 1,3-specific or random interesterified cocoa butter samples from different reactions at the same stage of interesterification were very similar, regardless of the reaction conditions used, the speed of the reaction and the lipid class levels. The potential to control the solid fat profiles through measuring the stage and type of the interesterification reaction will be of commercial importance, particularly when changes in the physical properties are required.

There were clear relationships between the initial reaction conditions of water and enzyme content, and the changes over time in the lipid class levels as well as the 1,3-specific and random triacylglycerol reaction profiles. These relationships were quantified by the development of equations that linked the initial reaction conditions to the measured outcomes of lipid classes and triacylglycerol reaction profile distance values.

The lipid classes of triacylglycerols, diacylglycerols and free fatty acids were described by linear equations that included terms for the enzyme and water content as well as reaction time. The equations were developed from the outcomes of several interesterifications, using the data from 2 hours of reaction onwards. The water content was found to have the major influence on the levels of lipid classes, having the largest coefficient in the equations. These equations were tested for their ability to predict the lipid class levels for interesterified cocoa butter, cottonseed oil and a blend of the two. The results indicated that the lipid classes could be predicted quite well

for cocoa butter and the other fat systems that were tested. The actual results were within, or very close to, the standard error range of the predicted values.

Interesterifications of cocoa butter were also used as a basis for the development of equations for the triacylglycerol reaction profiles. The 1,3-specific and random triacylglycerol reaction profiles were described using several standard, non-linear equations for each treatment individually incorporating only terms for the reaction time. The data from the individual equations were subsequently used to develop overall equations for the random and 1,3-specific triacylglycerol reaction profiles that included terms for the enzyme content, water content as well as reaction time. On comparison to the actual data from an individual treatment, it was found that the calculated distance values varied increasingly, as the reaction time increased.

The ability of the overall equations to predict the triacylglycerol reaction profiles of interesterified cocoa butter under specified conditions was assessed. It was found that the overall triacylglycerol reaction profile equations could be used to predict the general effect of altering the enzyme or water content, however they were not robust enough to accurately predict the measured triacylglycerol reaction profile distance values. The triacylglycerol reaction profile equations and/or the analytical techniques need to be tested and refined further before wider applications could be adopted.

In conclusion, model equations were developed to predict the outcomes of the enzymatic interesterification of edible fats and oils under solvent-free conditions. The developed model equations were limited in their application, however they have allowed the process of solvent-free enzymatic interesterification to be studied and understood in a way that has not been done before. The developed triacylglycerol reaction profile method is an innovative way of assessing the type of interesterification process as well as quantifying its progress. These considered in conjunction with the lipid class levels and the solid fat content results, provided a very good insight into how changes in the initial reaction conditions resulted in changes in the chemical and physical properties over time, particularly when using cocoa butter as a natural fat system.

11.2 Recommendations for future work

This work has provided tools and new knowledge regarding the enzymatic interesterification of natural fats and oils under solvent-free conditions. The following recommendations for future work are based on these investigations.

- Investigations into the applicability of the triacylglycerol reaction profile to a wider range of fats, using cocoa butter as a model system to optimise the interesterification process and conditions. Particularly tailoring to specific chemical or physical properties of interesterified fat blends to improve their functionality for specific purposes, such as table spreads, could be aimed at.

- Investigations into the use of a continuous reaction system, by means of a column reactor then adapting sampling procedures to build up triacylglycerol reaction profiles that characterise a continuous process. Alternatively, investigations into the repeated use of immobilised lipases in batch reactors could provide further understanding of the interesterification process.

- Investigations into differentiating the process of acyl migration from catalysed random interesterification and by-product production in detail. This could elucidate more clearly the mechanisms for acyl migration as well as identifying factors controlling the rate and extent of randomisation. This may involve detailed analysis of the types of diacylglycerols at each stage in the reaction, comparing 1,2-diacylglycerols with 1,3-diacylglycerols.

- Investigations into using these tools for assessing the performance of a range of other immobilised lipases for their interesterification activity in a natural fat system as significant differences may be expected from these, especially if they are from a different source or immobilised in a different way.

- Investigations into the scaling up of selected reactions to produce interesterified fats for evaluation in targeted food products.

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Appendix 1

Fatty acid composition method data

Primary milkfat standard from the Bureau of European Communities CRM164 was analysed at the same time as samples. Response factors were calculated from comparing known concentrations to normalised concentrations of prepared CRM164.

Response factor calculation for each fatty acid (peak):

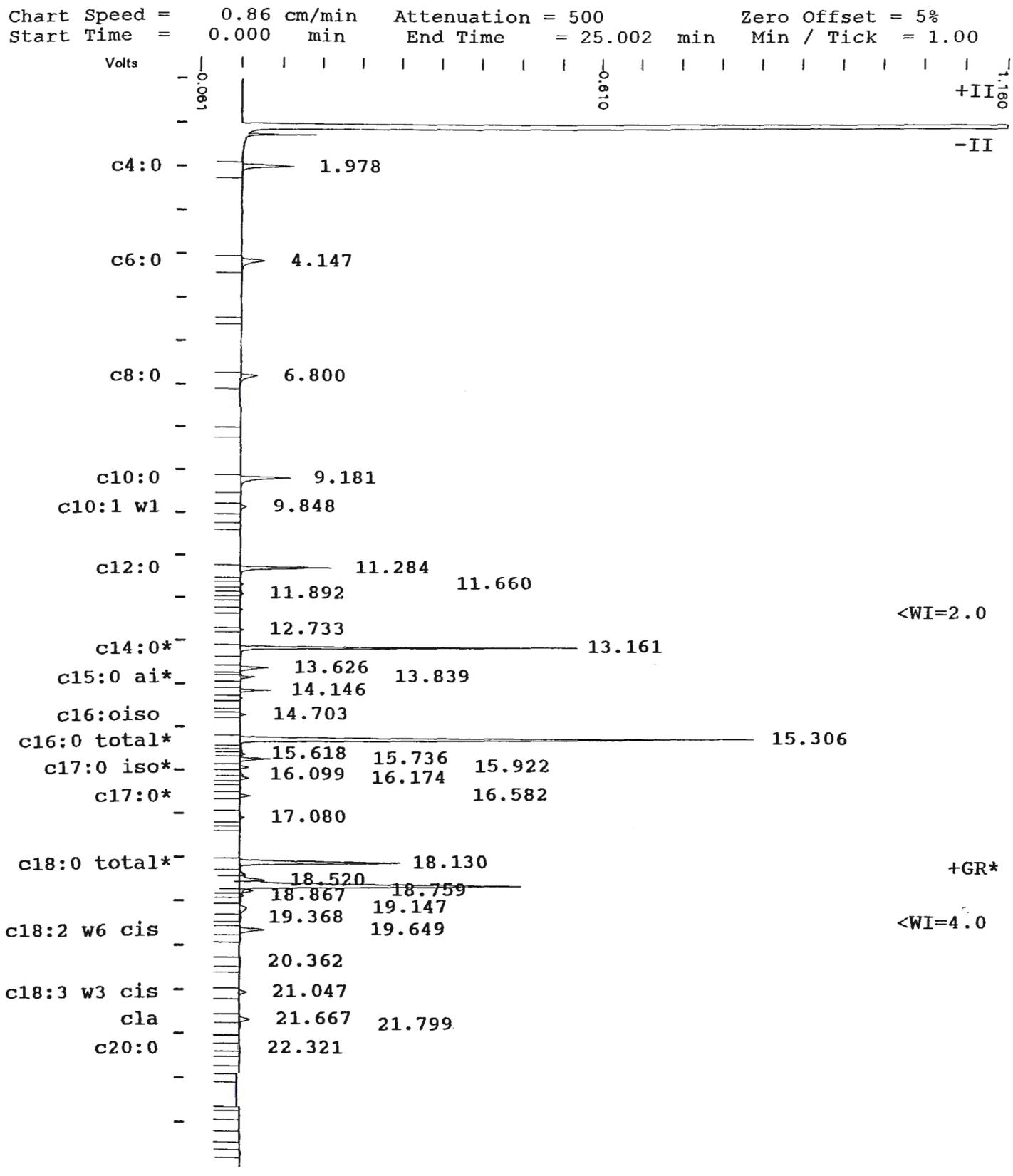
$$\text{Response factor} = \frac{\text{Certified value \% (CRM164)}}{\text{Peak Area \% (CRM164)}}$$

Sample fatty acid calculation: Fatty acid % = Response factor x Peak Area %

Table 1A Fatty acid composition data and typical response factors for standard reference milkfat CRM164.

Fatty acid	Certified Value (%) CRM164	Peak Area (%) CRM164	Response factor
4:0	4.3	3.09	1.39
6:0	2.36	2.05	1.15
8:0	1.36	1.33	1.02
10:0	2.89	3.17	0.91
10:1ω1	0.3	0.35	0.86
12:0	4.03	4.55	0.89
14:0	10.79	11.94	0.90
14:1ω5	1.1	1.42	0.77
15:0 ante iso	0.5	0.56	0.89
15:0	1.0	1.15	0.87
16:0 iso	0.2	0.24	0.83
16:0 total	26.91	27.86	0.97
16:1 total	1.5	1.36	1.10
17:0 iso	0.5	0.42	1.19
17:0 ante iso	0.4	0.43	0.93
17:0	0.5	0.49	1.02
17:1	0.3	0.31	0.97
18:0 total	10.51	10.29	1.02
18:1 total	24.82	23.38	1.06
18:2 ω6 cis	1.8	1.64	1.10
18:2 cla	0.9	0.79	1.14
18:3 ω3 cis	0.51	0.49	1.04
20:0	0.1	0.13	0.77
others	2.5	2.56	0.98

Figure 1A Chromatogram of reference milkfat CRM164



Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result (%)	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	c4:0	3.09	1.978	0.068	27563	BB	3.3	
2	c6:0	2.05	4.147	0.112	18266	BB	4.6	
3	c8:0	1.33	6.800	0.133	11830	BB	4.0	
4	c10:0	3.17	9.181	0.122	28314	BB	3.2	
5	c10:1 w1	0.35	9.848	0.154	3166	BB	3.3	
6	c12:0	4.55	11.284	0.119	40599	BB	2.7	
7		0.11	11.660	0.000	1011	BV	2.5	
8		0.14	11.892	0.000	1234	VB	2.5	
9		0.12	12.733	0.000	1075	BB	1.8	
10	c14:0	11.94	13.161	0.104	106544	BB	1.9	
11	c14:1	1.42	13.626	-0.001	12676	BV	2.6	
12	c15:0 ai	0.56	13.839	0.002	4964	VB	2.2	
13	c15:0	1.15	14.146	0.118	10296	BB	2.0	
14	c16:oiso	0.24	14.703	-0.007	2144	PB	2.1	
15	c16:0 total	27.86	15.306	-0.010	248534	BB	2.9	
16		0.22	15.618	0.000	1996	TF	0.0	
17	c16:1	1.36	15.736	-0.038	12118	TF	0.0	
18	c17:0 iso	0.42	15.922	-0.041	3750	TF	0.0	
19		0.17	16.099	0.000	1524	TF	0.0	
20	c17:0 ai	0.43	16.174	0.011	3876	TF	0.0	
21	c17:0	0.49	16.582	-0.112	4369	PB	2.6	
22	c17:1	0.31	17.080	-0.166	2731	BB	3.0	
23	c18:0 total	10.29	18.130	-0.026	91764	BV	3.4	
24	c18:1	23.38	18.520	-0.080	208618	GR	0.0	
25		0.40	18.759	0.000	3536	TS	0.0	
26		0.14	18.867	0.000	1213	TS	0.0	
27		0.79	19.147	0.000	7063	TF	0.0	
28		0.15	19.368	0.000	1322	TF	0.0	
29	c18:2 w6 cis	1.64	19.649	0.064	14641	BB	3.7	
30		0.14	20.362	0.000	1275	BV	4.8	
31	c18:3 w3 cis	0.49	21.047	-0.012	4377	BB	3.7	
32	cla	0.79	21.667	0.010	7064	BV	4.2	
33		0.17	21.799	0.000	1511	VB	5.4	
34	c20:0	0.13	22.321	0.013	1163	VV	5.2	
	Totals:	99.99		0.537	892127			

Total Unidentified Counts : 22760 counts

Detected Peaks: 60 Rejected Peaks: 24 Identified Peaks: 23

Amount Standard: N/A Multiplier: 1.000000 Divisor: 1.000000

Baseline Offset: 21 microVolts

Noise (used): 190 microVolts - monitored before this run

Manual injection

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Appendix 2

Triglyceride composition method data

Triglyceride standards (Sigma) or a standardised reference milkfat were analysed with the samples. Relative response factors were calculated from comparing known concentrations to normalised concentrations of prepared standard.

Response factor calculation for each triglyceride carbon number group (peak):

$$\text{Response factor (peak)} = \frac{\text{Known value \% (peak)}}{\text{Area \% (peak)}}$$

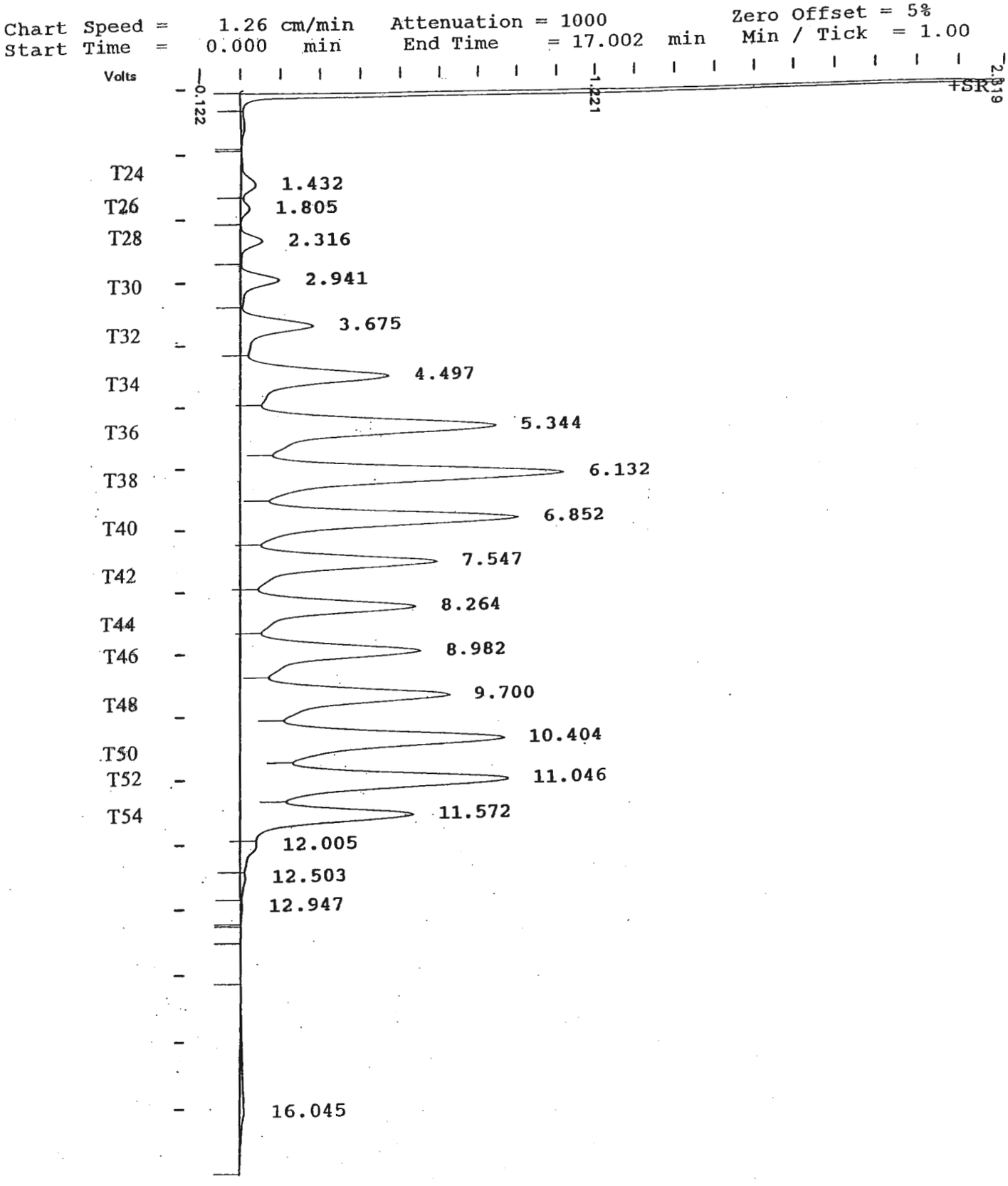
Sample triglyceride calculation:

Triglyceride sample (peak) % = Response factor (peak) x Area % (sample peak)

Table 2A Triglyceride composition data and typical response factors for standard reference milkfat.

Triglyceride group	Certified Value (%)	Peak Area (%)	Response factor
cholesterol +T24	0.37	0.45	0.82
T26	0.22	0.21	1.05
T28	0.57	0.52	1.10
T30	1.10	1.06	1.04
T32	2.43	2.40	1.01
T34	5.55	5.67	0.98
T36	10.42	10.65	0.98
T38	12.72	12.73	1.00
T40	10.28	10.19	1.01
T42	6.84	6.76	1.01
T44	6.31	6.14	1.03
T46	7.11	6.81	1.04
T48	8.94	8.42	1.06
T50	11.26	10.85	1.04
T52	10.42	10.22	1.02
T54	5.49	5.61	0.98

Figure 2.1A Chromatogram of standard reference milkfat



Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result (Area %)	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	T24	0.45	1.432	0.000	67129	BV	13.4	
2	T26	0.21	1.805	0.000	31009	VP	10.9	
3	T28	0.52	2.316	0.000	78534	PV	10.4	
4	T30	1.06	2.941	0.000	159360	VV	11.5	
5	T32	2.40	3.675	0.000	361604	VV	13.2	
6	T34	5.67	4.497	0.000	853354	VV	14.9	
7	T36	10.65	5.344	0.000	1601033	VV	16.7	
8	T38	12.73	6.132	0.000	1915014	VV	16.5	
9	T40	10.19	6.852	0.000	1532274	VV	15.3	
10	T42	6.76	7.547	0.000	1016895	VV	13.9	
11	T44	6.14	8.264	0.000	923106	VV	13.9	
12	T46	6.81	8.982	0.000	1023577	VV	14.8	
13	T48	8.42	9.700	0.000	1266158	VV	15.9	
14	T50	10.85	10.404	0.000	1632180	VV	16.6	
15	T52	10.22	11.046	0.000	1537419	VV	16.3	
16	T54	5.61	11.572	0.000	843309	VV	13.8	
17		0.52	12.005	0.000	78611	VV	34.2	
18		0.15	12.503	0.000	22031	VV	23.5	
19		0.03	12.947	0.000	5109	VB	11.5	
20		0.61	16.045	0.000	91083	BB	52.5	
	Totals:	100.00		0.000	15038789			

Total Unidentified Counts : 15038788 counts

Detected Peaks: 21 Rejected Peaks: 1 Identified Peaks: 0

Amount Standard: N/A Multiplier: 1.000000 Divisor: 1.000000

Baseline Offset: 839 microVolts

Noise (used): 140 microVolts - monitored before this run

Manual injection

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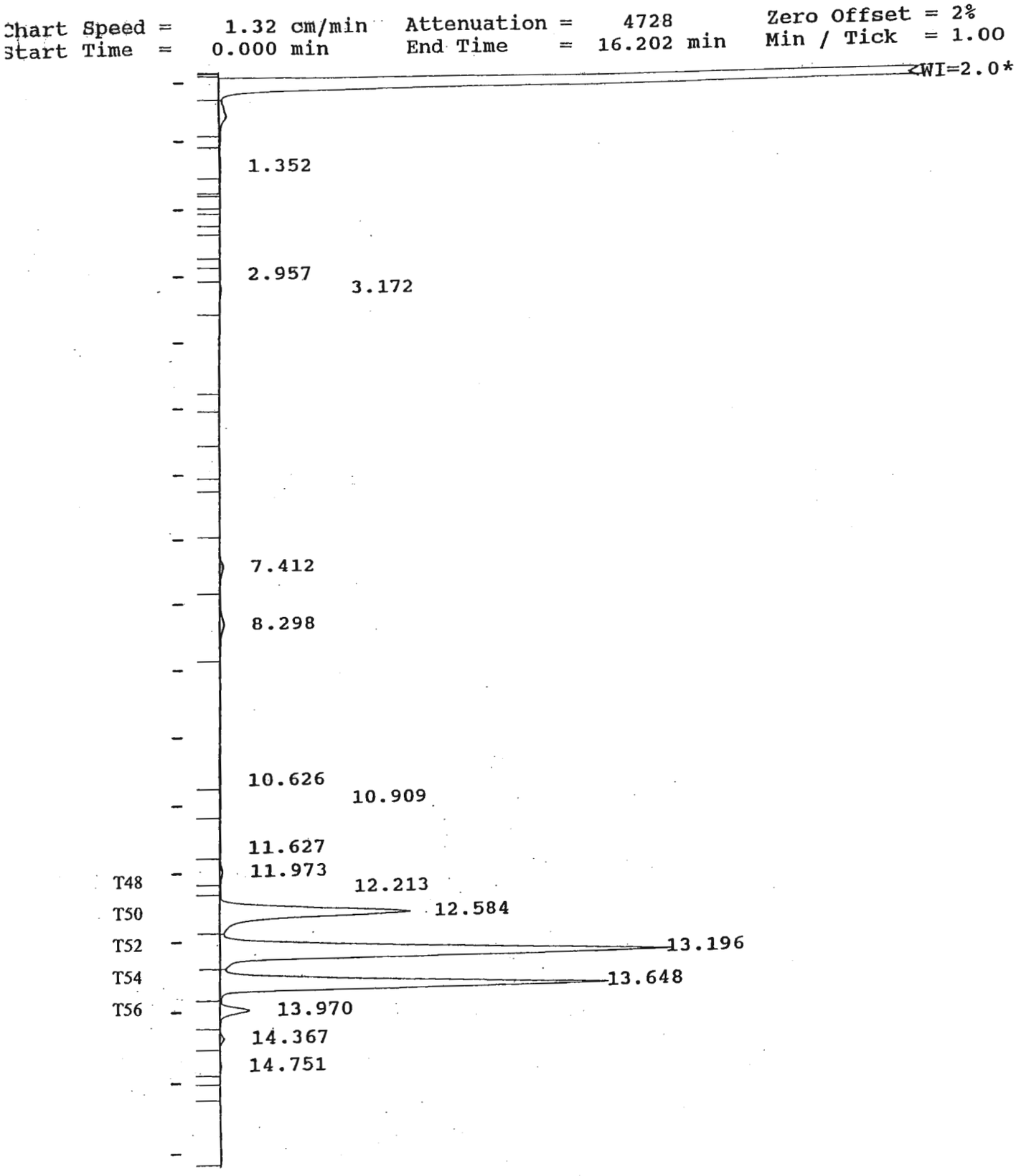
Figure 2.2A Chromatogram of cocoa butter



Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result (%)	Retention Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)
1		0.19	1.352		25140		0.0
2		0.06	2.957		8156		6.9
3		0.15	3.172		19433		7.8
4		0.54	7.412		72583		13.5
5		0.83	8.298		111079		17.6
6		0.42	10.626		55544		0.0
7		0.12	10.909		15986		0.0
8		0.17	11.627		22486		0.0
9	T48	0.35	11.973		46194		0.0
10		0.08	12.213		10259		0.0
11	T50	18.41	12.584		2461896		8.0
12	T52	44.75	13.196		5985829		9.0
13	T54	31.40	13.648		4199665		7.4
14	T56	1.99	13.970		265739		6.1
15		0.28	14.367		37487		8.4
16		0.10	14.751		13853		0.0
17		0.18	16.069		24726		0.0
	Totals:	100.00		0.000	13376054		

Total Unidentified Counts : 13376054 counts

Detected Peaks: 28 Rejected Peaks: 11 Identified Peaks: 0

Amount Standard: 1.000000 Multiplier: 1.000000 Divisor: 1.000000

Baseline Offset: -36 microVolts

Noise (used): 120 microVolts - monitored before this run

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Appendix 3

Lipid class and triglyceride composition method (1) data

Lipid class standards were analysed with the samples. Relative response factors were calculated for the four lipid class groups (FFA, MG, DG and TG) and the triglyceride groups by comparing known concentrations to normalised concentrations of prepared standard.

Response factor calculation for each lipid class group (peak or total peaks in particluar group):

$$\text{Response factor (peak)} = \frac{\text{Known value weight \% (peak)}}{\text{Area \% (peak)}}$$

Sample lipid class calculation:

Lipid class sample (peak) weight % = Response factor (peak) x Area % (peak)

Table 3.1A Lipid class composition data and typical response factors for lipid class standards.

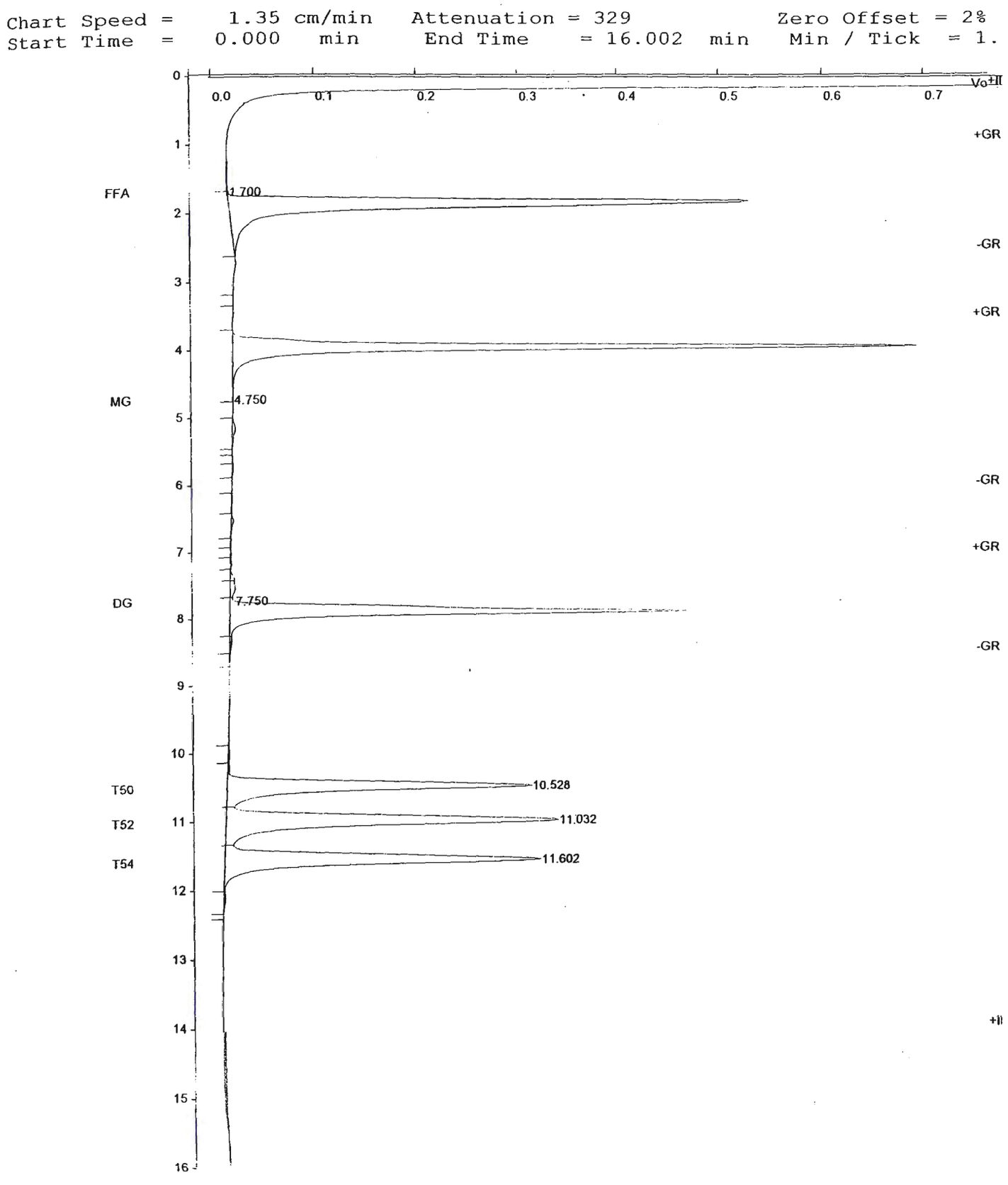
Lipid class	Relative weight % standards	Peak area (counts)	Relative Peak area %	Relative response factor
FFA	17.62	445482	21.03	0.84
MG	17.14	423581	20.00	0.72
DG	16.43	345783	16.33	0.85
TG (total)	48.82	903135	42.64	0.96

For the triglyceride composition, the response factors were calculated individually.

Table 3.2A Triglyceride composition data and typical response factors for triglyceride standards.

Triglyceride MW group	Relative weight (%)	Peak area (counts)	Relative peak area (%)	Response factor
T50 (PPO)	29.27	268537	29.73	0.98
T52 (POS)	34.14	329261	36.46	0.94
T54 (SSO)	36.59	305337	33.81	1.08

Figure 3.1A Chromatogram of lipid class and triglyceride standards.



Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Stat Code
1	FFA	21.0334	1.700	0.000	445482	GR	0.0	
2	MG	19.9993	4.750	0.000	423581	GR	0.0	
3	DG	16.3261	7.750	0.000	345783	GR	0.0	
4	T50	12.6789	10.528	-0.072	268537	VV	7.8	
5	T52	15.5460	11.032	-0.273	329261	VV	8.6	
6	T54	14.4164	11.602	-0.267	305337	VB	8.6	
	Totals:	100.0001		-0.612	2117981			

Total Unidentified Counts : 0 counts

Detected Peaks: 19 Rejected Peaks: 5 Identified Peaks: 6

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: 6 microVolts

Noise (used): 150 microVolts - monitored before this run

Manual injection

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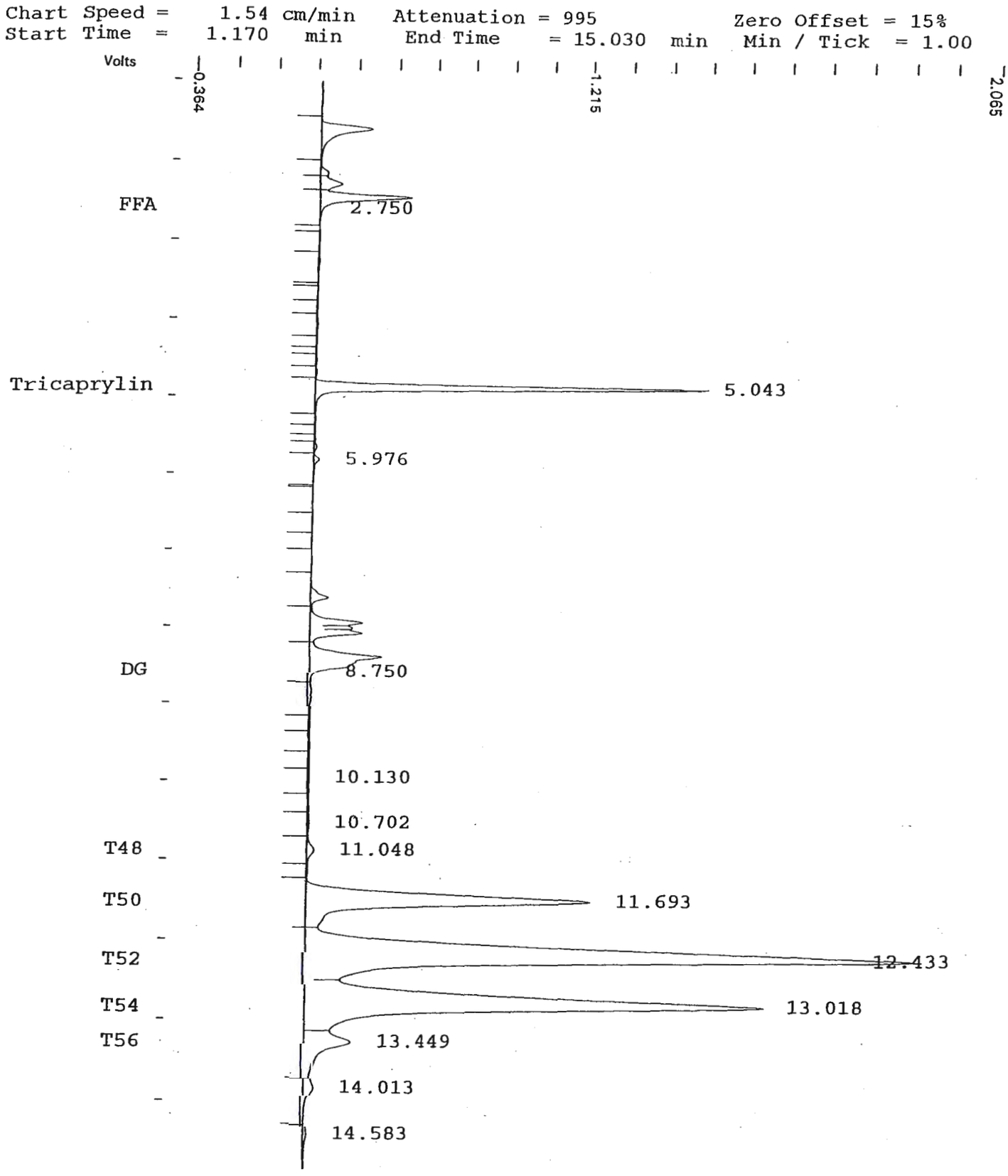
Figure 3.2A Chromatogram of cocoa butter



Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	FFA	4.91	2.750	0.000	327213	GR	0.0	
2	Tricaprylin	7.49	5.043	0.043	499030	VB	3.7	
3		0.14	5.976	0.000	9040	TF	0.0	
4	DG	7.52	8.750	0.000	501270	GR	0.0	
5		0.10	10.130	0.000	6937	VV	0.0	
6		0.12	10.702	0.000	7754	VV	0.0	
7	T48	0.32	11.048	-0.082	21339	VV	9.9	
8	T50	13.38	11.693	-0.007	891224	VV	8.6	
9	T52	35.21	12.433	0.133	2345640	VV	10.7	
10	T54	26.57	13.018	0.118	1770115	VV	10.3	
11	T56	3.21	13.449	-0.051	213922	VV	14.9	
12		0.79	14.013	0.000	52879	VV	27.8	
13		0.24	14.583	0.000	16190	VV	65.5	
	Totals:	100.00		0.154	6662553			

Total Unidentified Counts : 92800 counts

Detected Peaks: 43 Rejected Peaks: 16 Identified Peaks: 8

Amount Standard: N/A Multiplier: 1.000000 Divisor: 1.000000

Baseline Offset: -11 microVolts

Noise (used): 200 microVolts - monitored before this run

Manual injection

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Appendix 4

Lipid class and triglyceride composition method (2) data

Lipid class standards, including an internal standard, were analysed with the samples. Response factors were calculated for the four lipid class groups (FFA, MG, DG and TG) and the triglyceride groups using calculations that included an internal standard.

Response factor calculation for each lipid class group (peak or total peaks in particluar group) using an internal standard:

$$R_x = \frac{C_{IS}}{C_x} x \frac{A_x}{A_{IS}}$$

where R_x = response factor of reference substance x

C_{is} = concentration of internal standard, in mg/mL

C_x = concentration of reference substance x, in mg/mL

A_x = peak area of reference substance x

A_{is} = peak area of internal standard

Sample lipid class calculations:

$$m'_x = \frac{1}{R_x} x \frac{m'_{IS}}{m'_s} x \frac{A'_x}{A'_{IS}} x100\%$$

where m'_x = weight % of component x in sample

R_x = response factor of component x in sample

m'_{is} = mass of internal standard in sample, in mg

m'_s = mass of sample, in mg

A'_x = peak area of component x in sample

A'_{is} = peak area of internal standard in sample

Table 4.1A Lipid class composition data and typical response factors for lipid class standards using an internal standard.

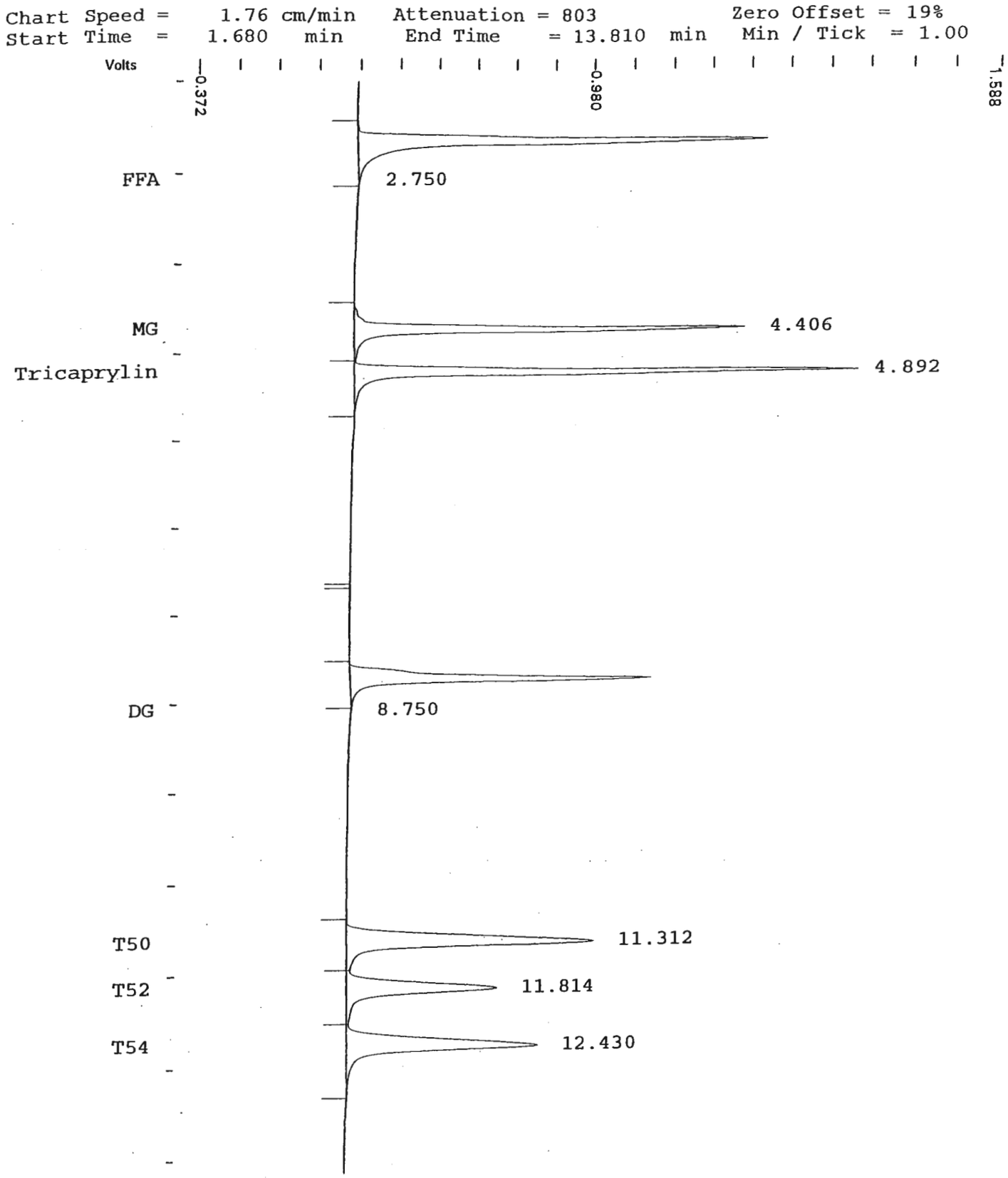
Lipid class	Concentration (mg/mL)	Peak area (counts)	Response factor
T24 (Tricaprylin)	0.408	572450	
Lipid class			
FFA	0.348	590830	1.21
MG	0.278	450064	1.15
DG	0.296	420044	1.01
TG (total)	0.964	1260921	0.93

For the triglyceride composition, the response factors were calculated individually:

Table 4.2A Triglyceride composition data and typical response factors for triglyceride standards using an internal standard.

Triglyceride group	Concentration (mg/mL)	Peak area (counts)	Response factor
T24 (Tricaprylin)	0.408	550528	
T50 (PPO)	0.386	494333	0.95
T52 (POS)	0.258	311236	0.89
T54 (SSO)	0.320	409768	0.95

Figure 4.1A Chromatogram of lipid class and triglyceride standards, including an internal standard (tricaprylin).



Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	FFA	18.06	2.750	0.000	575280	GR	0.0	
2	MG	13.95	4.406	-0.194	444419	BV	3.8	
3	Tricaprylin	17.28	4.892	-0.108	550528	VB	3.7	
4	DG	12.57	8.750	0.000	400329	GR	0.0	
5	T50	15.52	11.312	-0.188	494333	BV	7.0	
6	T52	9.77	11.814	-0.286	311236	VV	7.1	
7	T54	12.86	12.430	-0.270	409768	VB	7.4	
	Totals:	100.01		-1.046	3185893			

Total Unidentified Counts : 0 counts

Detected Peaks: 8 Rejected Peaks: 1 Identified Peaks: 7

Amount Standard: N/A Multiplier: 1.000000 Divisor: 1.000000

Baseline Offset: 7 microVolts

Noise (used): 300 microVolts - monitored before this run

Manual injection

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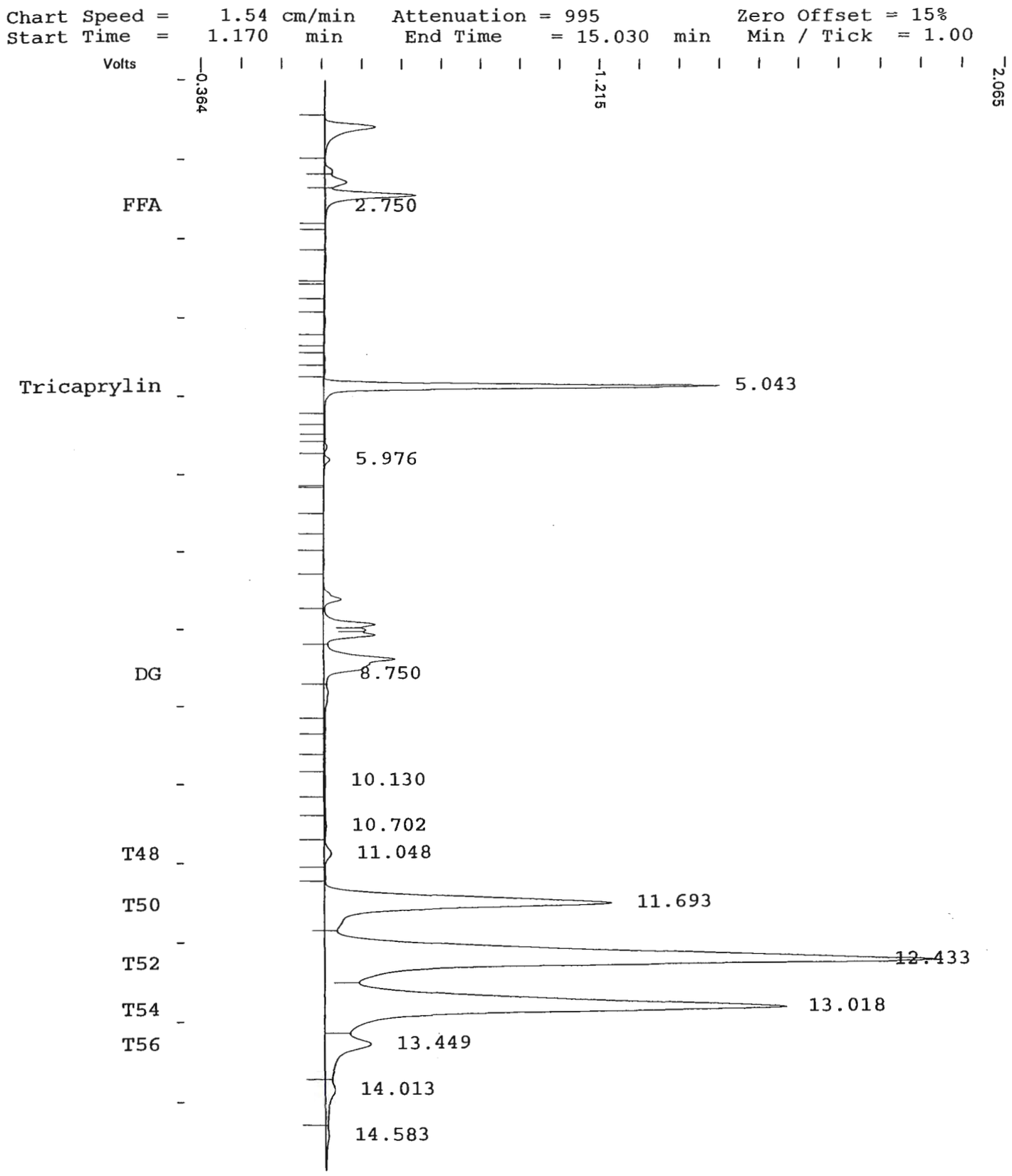
Figure 4.2A Chromatogram of cocoa butter, including an internal standard (tricaprylin).



Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1	FFA	4.91	2.750	0.000	327213	GR	0.0	
2	Tricaprylin	7.49	5.043	0.043	499030	VB	3.7	
3		0.14	5.976	0.000	9040	TF	0.0	
4	DG	7.52	8.750	0.000	501270	GR	0.0	
5		0.10	10.130	0.000	6937	VV	0.0	
6		0.12	10.702	0.000	7754	VV	0.0	
7	T48	0.32	11.048	-0.082	21339	VV	9.9	
8	T50	13.38	11.693	-0.007	891224	VV	8.6	
9	T52	35.21	12.433	0.133	2345640	VV	10.7	
10	T54	26.57	13.018	0.118	1770115	VV	10.3	
11	T56	3.21	13.449	-0.051	213922	VV	14.9	
12		0.79	14.013	0.000	52879	VV	27.8	
13		0.24	14.583	0.000	16190	VV	65.5	
	Totals:	100.00		0.154	6662553			

Total Unidentified Counts : 92800 counts

Detected Peaks: 43 Rejected Peaks: 16 Identified Peaks: 8

Amount Standard: N/A Multiplier: 1.000000 Divisor: 1.000000

Baseline Offset: -11 microVolts

Noise (used): 200 microVolts - monitored before this run

Manual injection

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Appendix 5

Calculation of theoretical fully interesterified triglyceride composition using Tricalc program

5.1A Interesterification function

Malaysian cocoa butter

OPTIONS SELECTED :

INPUT DATA AS % WEIGHT
INTERESTERIFIED

FATTY ACID		OVERALL % WT	OVERALL % MOL	MOL WT
M	14:0	0.07	0.08	228.38
P	16:0	24.40	26.31	256.43
S	18:0	37.32	36.27	284.48
A	20:0	1.23	1.09	312.54
T	16:1	0.21	0.23	254.41
O	18:1	33.80	33.09	282.47
I	18:2	2.57	2.53	280.45
N	18:3	0.17	0.17	278.44
U	17:0	0.22	0.22	270.46

PRINT LEVEL IS .5 % MOLE

TRIGLYCERIDE	% MOL	% WT	C.NO.
PPP	1.82	1.70	48
PPS	5.02	4.84	50
PPO	4.58	4.40	50
PSP	2.51	2.42	50
PSS	6.92	6.89	52
PSO	6.32	6.27	52
POP	2.29	2.20	50
POS	6.32	6.27	52
POO	5.76	5.71	52
SPS	3.46	3.45	52
SPO	6.32	6.27	52
SSS	4.77	4.90	54
SSO	8.71	8.93	54
SSI	0.67	0.68	54
SOS	4.35	4.46	54
SOO	7.94	8.12	54
SOI	0.61	0.62	54
SIO	0.61	0.62	54
OPO	2.88	2.85	52
OSO	3.97	4.06	54
OSI	0.61	0.62	54
OOO	3.62	3.70	54
OOI	0.55	0.56	54

CARBON NO.	% MOL	% WT	
12	0.00	0.00	I
14	0.00	0.00	I
16	0.00	0.00	I
18	0.00	0.00	I
20	0.00	0.00	I
22	0.00	0.00	I
24	0.00	0.00	I
26	0.00	0.00	I
28	0.00	0.00	I
30	0.00	0.00	I
32	0.00	0.00	I
34	0.00	0.00	I
36	0.00	0.00	I
38	0.00	0.00	I
40	0.00	0.00	I
42	0.00	0.00	I
44	0.00	0.00	I
46	0.02	0.02	I
48	2.01	1.88	I**
50	15.62	15.02	I***************
52	41.94	41.64	I**
54	38.68	39.61	I**************************************
56	1.70	1.80	I**
58	0.03	0.03	I
60	0.00	0.00	I
62	0.00	0.00	I
64	0.00	0.00	I
66	0.00	0.00	I
68	0.67	0.67	I*
70	0.00	0.00	I

N.B. IN THE ABOVE TABLE :
68 = TOTAL ODD CARBON NO. TRIGLYCERIDES
70 = TOTAL OTHER OR UNKNOWN C.NO. TRIGLYCERIDES

SUMMARY TABLE :

TRIGLYCERIDE	% MOL	% WT
SSS	26.19	25.90
SOS	13.64	13.63
SSO	27.28	27.26
SLS	1.11	1.10
SSL	2.21	2.21
SUU	16.60	16.76
USU	8.30	8.38
UUU	4.67	4.76

N.B. IN THE ABOVE TABLE ONLY :
S = TOTAL SATURATED ACIDS O = MONOUNSATURATED ACIDS
L = POLYUNSATURATED ACIDS U = TOTAL UNSATURATED ACIDS

TOTAL NUMBER OF TRIGLYCERIDES CALCULATED = 405
TOTAL NUMBER OF TRIGLYCERIDES PRINTED = 23

5.2A Lipolysis function

Malaysian cocoa butter

OPTIONS SELECTED :

INPUT DATA AS % WEIGHT
LIPOLYSIS

FATTY ACID		OVERALL % WT	OVERALL % MOL	2-POSITION % WT	2-POSITION % MOL	MOL WT
M	14:0	0.07	0.08	0.00	0.00	228.38
P	16:0	24.40	26.31	2.71	2.97	256.43
S	18:0	37.32	36.27	2.16	2.14	284.48
A	20:0	1.23	1.09	0.00	0.00	312.54
T	16:1	0.21	0.23	0.39	0.43	254.41
O	18:1	33.80	33.09	87.54	87.23	282.47
I	18:2	2.57	2.53	7.02	7.04	280.45
N	18:3	0.17	0.17	0.18	0.18	278.44
U	17:0	0.22	0.22	0.00	0.00	270.46

PRINT LEVEL IS .5 % MOLE

TRIGLYCERIDE	% MOL	% WT	C.NO.
PPS	1.21	1.16	50
PSS	0.87	0.86	52
POP	12.58	12.09	50
POS	35.34	35.10	52
POA	1.08	1.11	54
POO	3.99	3.95	52
PIP	1.02	0.97	50
PIS	2.85	2.83	52
SPS	0.85	0.84	52
SSS	0.61	0.62	54
SOS	24.82	25.45	54
SOA	1.52	1.61	56
SOO	5.60	5.73	54
SIS	2.00	2.05	54

CARBON NO.	% MOL	% WT	
12	0.00	0.00	I
14	0.00	0.00	I
16	0.00	0.00	I
18	0.00	0.00	I
20	0.00	0.00	I
22	0.00	0.00	I
24	0.00	0.00	I
26	0.00	0.00	I
28	0.00	0.00	I
30	0.00	0.00	I
32	0.00	0.00	I
34	0.00	0.00	I
36	0.00	0.00	I
38	0.00	0.00	I
40	0.00	0.00	I
42	0.00	0.00	I
44	0.00	0.00	I
46	0.00	0.00	I
48	0.60	0.56	I*
50	15.99	15.36	I****************
52	45.68	45.35	I**
54	35.82	36.70	I************************************
56	1.89	1.99	I**
58	0.03	0.03	I
60	0.00	0.00	I
62	0.00	0.00	I
64	0.00	0.00	I
66	0.00	0.00	I
68	0.67	0.67	I*
70	0.00	0.00	I

N.B. IN THE ABOVE TABLE :
68 = TOTAL ODD CARBON NO. TRIGLYCERIDES
70 = TOTAL OTHER OR UNKNOWN C.NO. TRIGLYCERIDES

SUMMARY TABLE :

TRIGLYCERIDE	% MOL	% WT
SSS	4.46	4.39
SOS	76.50	76.48
SSO	0.59	0.58
SLS	6.31	6.29
SSL	0.04	0.04
SUU	11.67	11.78
USU	0.02	0.02
UUU	0.41	0.42

N.B. IN THE ABOVE TABLE ONLY :
S = TOTAL SATURATED ACIDS O = MONOUNSATURATED ACIDS
L = POLYUNSATURATED ACIDS U = TOTAL UNSATURATED ACIDS

TOTAL NUMBER OF TRIGLYCERIDES CALCULATED = 405
TOTAL NUMBER OF TRIGLYCERIDES PRINTED = 14

Appendix 6

Genstat programs for equations describing lipid classes

6.1A Genstat program for linear equations describing lipid classes from results for the interesterification of Malaysian cocoa butter in Chapter 8.

```
"
.....  Cocoa Butter Experiment
         Ignoring blanks and the two unreplicated treatments
"
units [90]
open 'cocoa.prn'; ch=2; file=in
skip [ch=2] 1
read [ch=2; format=!(((0,-15,0.1,7,*)5,\
                     (*,(0,-15,0.1,7,*)4)10,5(*))2,10(*))]\
 %lipo,%water,time,FFA,MG,DG,TG
close ch=2; file=in

factor [lev=2] Week; values=!(45(1,2))
factor [lev=12] Flask; values=!((1,4(2...12))2)
factor [lev=4] Sample; values=!((1,(1...4)11)2)

factor [lev=2; lab=!t(cb,trt)] CB; values=!((1,44(2))2)
factor [lev=12; lab=!t(cb,e1,e2,e3,e4,e5,e6,e7,e8,e9,e10,e11)]\
   Trt; values=!((1,4(2...12))2)
factor [lev=!(0,2,8,24,48)] Time
factor [lev=4] Lipo; values=!((1,16(2,3),12(4))2)
factor [lev=8] Water; values=!((1,4(2,3,4,5),4(4,5,6,7),4(6,7,8))2)
calc Time = time

print Week,CB,Trt,Lipo,Water,Time,%lipo,%water,time,FFA,MG,DG,TG;\
 fieldw=5,4,5,5,6,5,6,7,5,4(7); deci=6(0),2,2,0,4(2)

variate [44] %Lipo,%Water,Hour,ffa,mg,dg,tg

units [44]
equate [oldf=!(-1,44,-45)] %lipo;  %Lipo
 &                         %water; %Water
 &                         time;   Hour
print %Lipo,%Water,Hour,ffa,mg,dg,tg; fieldw=7(10); deci=2,2,0,4(3)

"
.....  Regression Analyses Using Replicate Means
       and linear terms
"
for y=ffa,mg,dg,tg
 model y
 terms %Lipo + %Water + Hour
 fit [pr=mod,sum,est,acc; fprob=y; tprob=y] %Lipo + %Water + Hour
 rkeep res=r; fitted=f
 graph [nrow=20; ncol=60] r; f
  &                       r; %Water
  &                       r; Hour
 "rcheck [graph=high] ystat=res; xmeth=fitt,normal"
endfor
```

6.2A Averaged lipid class results for the treatments and replicates for Chapter 8.

```
48  print %Lipo,%Water,Hour,ffa,mg,dg,tg; fieldw=7(10); deci=2,2,0,4(3)
```

%Lipo	%Water	Hour	ffa	mg	dg	tg
0.65	0.08	2	1.335	0.000	4.700	93.965
0.65	0.08	8	1.560	0.000	5.465	92.975
0.65	0.08	24	1.790	0.000	5.320	92.890
0.65	0.08	48	1.880	0.070	5.535	92.510
0.65	0.11	2	1.945	0.000	5.825	92.230
0.65	0.11	8	1.910	0.000	5.825	92.265
0.65	0.11	24	1.785	0.020	5.990	92.200
0.65	0.11	48	2.060	0.000	5.810	92.125
0.65	0.13	2	2.110	0.010	5.810	92.070
0.65	0.13	8	1.675	0.035	5.110	93.180
0.65	0.13	24	2.250	0.000	6.355	91.395
0.65	0.13	48	2.600	0.000	6.680	90.720
0.65	0.20	2	2.695	0.040	7.115	90.145
0.65	0.20	8	2.910	0.015	7.845	89.225
0.65	0.20	24	2.865	0.085	8.055	88.995
0.65	0.20	48	3.155	0.045	7.720	89.080
2.00	0.13	2	2.030	0.000	5.550	92.420
2.00	0.13	8	2.175	0.000	5.895	91.930
2.00	0.13	24	2.145	0.000	6.100	91.755
2.00	0.13	48	2.305	0.000	6.290	91.410
2.00	0.20	2	3.855	0.025	9.205	86.920
2.00	0.20	8	4.180	0.010	9.810	86.000
2.00	0.20	24	3.985	0.010	9.695	86.305
2.00	0.20	48	4.290	0.015	10.180	85.520
2.00	0.27	2	4.460	0.120	9.645	85.775
2.00	0.27	8	4.760	0.085	9.960	85.200
2.00	0.27	24	4.795	0.180	10.190	84.830
2.00	0.27	48	5.265	0.125	10.650	83.960
2.00	0.48	2	5.765	0.160	11.690	82.390
2.00	0.48	8	6.785	0.340	12.775	80.095
2.00	0.48	24	7.350	0.265	13.795	78.590
2.00	0.48	48	7.040	0.300	13.655	79.010
5.48	0.27	2	4.030	0.035	9.000	86.940
5.48	0.27	8	4.220	0.000	9.495	86.290
5.48	0.27	24	4.305	0.040	8.840	86.820
5.48	0.27	48	4.735	0.030	9.880	85.360
5.48	0.48	2	7.060	0.395	13.830	78.715
5.48	0.48	8	7.680	0.230	14.515	77.575
5.48	0.48	24	8.055	0.335	14.775	76.835
5.48	0.48	48	8.140	0.195	15.260	76.400
5.48	0.66	2	8.435	0.405	13.660	77.500
5.48	0.66	8	10.135	0.420	15.130	74.315
5.48	0.66	24	10.075	0.395	15.260	74.270
5.48	0.66	48	9.565	0.710	15.125	74.595

6.3A Output data: Results from Genstat program for the linear equations to describe the lipid classes for Chapter 8.

Free fatty acids:

```
***** Regression Analysis *****

 Response variate: ffa
     Fitted terms: Constant + %Lipo + %Water + Hour


*** Summary of analysis ***

             d.f.          s.s.          m.s.      v.r.  F pr.
Regression      3       274.118       91.3728    409.74  <.001
Residual       40         8.920        0.2230
Total          43       283.039        6.5823

Change         -3      -274.118       91.3728    409.74  <.001

Percentage variance accounted for 96.6
Standard error of observations is estimated to be 0.472
* MESSAGE: The following units have large standardized residuals:
                29       -2.25
                41       -2.46


*** Estimates of regression coefficients ***

                            estimate          s.e.     t(40)  t pr.
Constant                       0.287         0.155      1.86  0.071
%Lipo                         0.1225        0.0548      2.23  0.031
%Water                        12.910         0.595     21.70  <.001
Hour                         0.01203       0.00400      3.01  0.005


*** Accumulated analysis of variance ***

Change                         d.f.          s.s.          m.s.      v.r.  F pr.
+ %Lipo                           1      167.0525      167.0525    749.10  <.001
+ %Water                          1      105.0489      105.0489    471.06  <.001
+ Hour                            1        2.0171        2.0171      9.05  0.005
Residual                         40        8.9201        0.2230

Total                            43      283.0386        6.5823
```

Diglycerides:

```
***** Regression Analysis *****

 Response variate: dg
     Fitted terms: Constant + %Lipo + %Water + Hour


*** Summary of analysis ***

             d.f.          s.s.          m.s.      v.r.  F pr.
Regression      3       467.91       155.970    141.48  <.001
Residual       40        44.10         1.102
```

```
Total            43       512.01       11.907

Change           -3      -467.91      155.970    141.48  <.001

Percentage variance accounted for 90.7
Standard error of observations is estimated to be 1.05
* MESSAGE: The following units have large standardized residuals:
                41       -2.37
* MESSAGE: The residuals do not appear to be random;
           for example, fitted values in the range 5.41 to 7.42
           are consistently larger  than observed values
           and fitted values in the range 12.46 to 13.76
           are consistently smaller than observed values
* MESSAGE: The error variance does not appear to be constant:
           large responses are more variable than small responses


*** Estimates of regression coefficients ***

                              estimate         s.e.     t(40)  t pr.
Constant                         3.921        0.344     11.41  <.001
%Lipo                            0.167        0.122      1.37  0.177
%Water                           16.78         1.32     12.69  <.001
Hour                           0.01812      0.00889      2.04  0.048


*** Accumulated analysis of variance ***

Change                          d.f.          s.s.          m.s.       v.r.  F pr.
+ %Lipo                            1       285.828       285.828    259.27  <.001
+ %Water                           1       177.505       177.505    161.01  <.001
+ Hour                             1         4.576         4.576      4.15  0.048
Residual                          40        44.097         1.102

Total                             43       512.006        11.907
```

Triglycerides:

```
***** Regression Analysis *****

 Response variate: tg
     Fitted terms: Constant + %Lipo + %Water + Hour


*** Summary of analysis ***

             d.f.          s.s.          m.s.       v.r.  F pr.
Regression      3       1533.46       511.154    247.14  <.001
Residual       40         82.73         2.068
Total          43       1616.19        37.586

Change         -3      -1533.46       511.154    247.14  <.001

Percentage variance accounted for 94.5
Standard error of observations is estimated to be 1.44
* MESSAGE: The following units have large standardized residuals:
                41        2.55
* MESSAGE: The residuals do not appear to be random;
           for example, fitted values in the range 89.54 to 92.12
```

```
          are consistently smaller than observed values
          and fitted values in the range 78.22 to 80.41
          are consistently larger  than observed values


*** Estimates of regression coefficients ***

                            estimate         s.e.     t(40)  t pr.
Constant                      95.912        0.471    203.78  <.001
%Lipo                         -0.275        0.167     -1.65  0.107
%Water                        -30.64         1.81    -16.91  <.001
Hour                         -0.0308       0.0122     -2.53  0.015


*** Accumulated analysis of variance ***

Change                        d.f.          s.s.          m.s.       v.r.  F pr.
+ %Lipo                          1       928.579       928.579    448.97  <.001
+ %Water                         1       591.625       591.625    286.05  <.001
+ Hour                           1        13.257        13.257      6.41  0.015
Residual                        40        82.730         2.068

Total                           43      1616.191        37.586
```

6.4A Genstat program for linear equations describing lipid classes from results for the interesterification of Brazilian cocoa butter in Chapter 9.

```
"
.....  Cocoa Butter Experiment

"
units [84]
open 'lipid4.prn'; ch=2; file=in
skip [ch=2] 1
read [ch=2; format=!(((8,*)7)12)] \
expt,%lipo,%water,time,FFA,MG,DG,TG
close ch=2; file=in

factor [lev=12] Flask; values=!(7(1...12))
factor [lev=7] Sample; values=!((1...7)12)


factor [lev=1; lab=!t(trt)] CB; values=!(84(1))
factor [lev=12; lab=!t(e1,e2,e3,e4,e5,e6,e7,e8,e9,e10,e11,e12)]\
    Trt; values=!(7(1...12))
factor [lev=!(1,2,4,6,8,24,48)] Time
factor [lev=3] Lipo; values=!((28(1,2,3)))
factor [lev=7] Water; values=!(14(1),7(2,3,4),14(5),7(6),14(7,6))
calc Time = time

print expt,CB,Trt,Lipo,Water,Time,%lipo,%water,time,FFA,MG,DG,TG;\
 fieldw=5,4,5,5,6,5,6,7,5,4(7); deci=6(0),2,2,0,4(2)


variate [84] %Lipo,%Water,Hour,ffa,mg,dg,tg

"for Y=FFA,MG,DG,TG; Z=ffa,mg,dg,tg
 endfor
"
```

```
blocks Flask/Sample
treatments CB/(Trt*Time)

units [84]
equate [oldf=!(84)] %lipo;  %Lipo
 &                          %water; %Water
 &                          time;   Hour
print %Lipo,%Water,Hour,FFA,MG,DG,TG; fieldw=7(10); deci=2,2,0,4(3)

"
.....  Regression Analyses Using linear terms
"
for y=FFA,MG,DG,TG
 model y
 terms %Lipo + %Water + Hour
 fit [pr=mod,sum,est,acc; fprob=y; tprob=y] %Lipo + %Water + Hour
 rkeep res=r; fitted=f
 graph [nrow=20; ncol=60] r; f
  &                       r; %Water
  &                       r; Hour
 "rcheck [graph=high] ystat=res; xmeth=fitt,normal"
endfor
```

6.5A Input data for the development of linear equations describing the lipid classes for Chapter 9.

%Lipo	%Water	Hour	FFA	MG	DG	TG
1.25	0.12	1	2.490	0.000	4.830	92.680
1.25	0.12	2	2.610	0.000	5.130	92.260
1.25	0.12	4	2.580	0.000	4.780	92.640
1.25	0.12	6	2.700	0.000	5.640	91.660
1.25	0.12	8	2.790	0.000	5.430	91.770
1.25	0.12	24	2.700	0.000	5.360	91.940
1.25	0.12	48	2.710	0.000	5.490	91.810
1.25	0.20	1	3.360	0.000	5.840	90.790
1.25	0.20	2	3.590	0.000	6.830	89.580
1.25	0.20	4	3.770	0.000	7.370	88.860
1.25	0.20	6	3.850	0.000	6.800	89.360
1.25	0.20	8	4.010	0.000	7.700	88.290
1.25	0.20	24	4.060	0.000	7.270	88.670
1.25	0.20	48	4.150	0.000	8.210	87.640
1.25	0.20	1	2.880	0.000	5.740	91.380
1.25	0.20	2	3.060	0.000	6.520	90.420
1.25	0.20	4	3.030	0.000	6.520	90.440
1.25	0.20	6	3.340	0.000	7.080	89.580
1.25	0.20	8	3.270	0.000	6.800	89.940
1.25	0.20	24	3.320	0.000	7.300	89.380
1.25	0.20	48	3.360	0.000	7.310	89.330
1.25	0.40	1	6.780	0.310	10.670	82.240
1.25	0.40	2	7.560	0.240	13.100	79.100
1.25	0.40	4	7.750	0.170	13.860	78.220
1.25	0.40	6	8.010	0.120	14.190	77.680
1.25	0.40	8	7.970	0.240	14.440	77.340
1.25	0.40	24	8.350	0.110	15.150	76.390
1.25	0.40	48	8.270	0.130	15.160	76.440
2.50	0.17	1	3.770	0.000	7.890	88.340
2.50	0.17	2	4.610	0.000	8.670	86.720
2.50	0.17	4	4.170	0.000	9.720	86.100
2.50	0.17	6	4.010	0.000	8.840	87.150

2.50	0.17	8	4.740	0.000	8.810	86.440
2.50	0.17	24	4.620	0.000	9.350	86.030
2.50	0.17	48	4.850	0.000	11.270	83.880
2.50	0.30	1	4.450	0.000	8.110	87.430
2.50	0.30	2	4.610	0.000	8.750	86.640
2.50	0.30	4	4.870	0.070	9.160	85.900
2.50	0.30	6	4.740	0.000	8.900	86.360
2.50	0.30	8	4.870	0.080	9.560	85.490
2.50	0.30	24	4.950	0.070	9.510	85.480
2.50	0.30	48	5.130	0.070	9.830	84.960
2.50	0.30	1	4.590	0.080	8.140	87.200
2.50	0.30	2	4.710	0.000	8.490	86.800
2.50	0.30	4	4.910	0.090	9.220	85.780
2.50	0.30	6	4.820	0.090	9.240	85.840
2.50	0.30	8	5.110	0.080	9.590	85.220
2.50	0.30	24	4.940	0.080	9.120	85.850
2.50	0.30	48	5.250	0.090	10.130	84.530
2.50	0.48	1	6.490	0.140	11.270	82.090
2.50	0.48	2	7.100	0.140	12.150	80.600
2.50	0.48	4	7.260	0.170	12.590	79.980
2.50	0.48	6	7.320	0.170	13.320	79.180
2.50	0.48	8	7.760	0.180	13.660	78.400
2.50	0.48	24	7.570	0.160	13.520	78.740
2.50	0.48	48	7.830	0.160	13.790	78.220
4.00	0.24	1	3.450	0.000	6.820	89.730
4.00	0.24	2	3.580	0.000	7.100	89.320
4.00	0.24	4	3.660	0.000	7.120	89.230
4.00	0.24	6	3.340	0.030	6.800	89.830
4.00	0.24	8	3.880	0.000	7.650	88.470
4.00	0.24	24	3.950	0.050	8.070	87.930
4.00	0.24	48	4.020	0.090	8.070	87.810
4.00	0.24	1	3.630	0.000	6.870	89.500
4.00	0.24	2	3.670	0.000	7.000	89.330
4.00	0.24	4	3.920	0.000	7.380	88.700
4.00	0.24	6	3.830	0.000	7.410	88.760
4.00	0.24	8	4.080	0.000	7.660	88.260
4.00	0.24	24	4.140	0.030	7.820	88.010
4.00	0.24	48	4.290	0.030	7.990	87.690
4.00	0.48	1	7.450	0.070	11.580	80.890
4.00	0.48	2	6.790	0.070	11.370	81.770
4.00	0.48	4	7.420	0.140	11.950	80.490
4.00	0.48	6	7.260	0.150	12.270	80.320
4.00	0.48	8	7.490	0.150	12.890	79.470
4.00	0.48	24	8.230	0.070	13.350	78.350
4.00	0.48	48	7.850	0.080	13.620	78.450
4.00	0.48	1	7.450	0.070	11.580	80.890
4.00	0.48	2	6.790	0.070	11.370	81.770
4.00	0.48	4	7.420	0.140	11.950	80.490
4.00	0.48	6	7.260	0.150	12.270	80.320
4.00	0.48	8	7.490	0.150	12.890	79.470
4.00	0.48	24	8.230	0.070	13.350	78.350
4.00	0.48	48	7.850	0.080	13.620	78.450

6.6A Output data: Results from Genstat program for the linear equations to describe the lipid classes for Chapter 9.

Free fatty acids:

```
***** Regression Analysis *****

 Response variate: FFA
     Fitted terms: Constant + %Lipo + %Water + Hour

*** Summary of analysis ***

             d.f.         s.s.         m.s.      v.r.  F pr.
Regression      3       254.17      84.7223    243.10  <.001
Residual       80        27.88       0.3485
Total          83       282.05       3.3982

Change         -3      -254.17      84.7223    243.10  <.001

Percentage variance accounted for 89.7
Standard error of observations is estimated to be 0.590
* MESSAGE: The following units have large standardized residuals:
                30        2.64
                33        2.73
* MESSAGE: The residuals do not appear to be random;
           for example, fitted values in the range 4.087 to 5.067
           are consistently larger  than observed values
           and fitted values in the range 6.734 to 7.284
           are consistently smaller than observed values
* MESSAGE: The error variance does not appear to be constant:
           intermediate responses are less variable than small or large
responses

*** Estimates of regression coefficients ***

                            estimate         s.e.     t(80)  t pr.
Constant                       1.120        0.199      5.62  <.001
%Lipo                        -0.2282       0.0633     -3.60  <.001
%Water                        14.716        0.575     25.59  <.001
Hour                         0.01287      0.00406      3.17  0.002


*** Accumulated analysis of variance ***

Change                      d.f.         s.s.         m.s.      v.r.  F pr.
+ %Lipo                        1      22.4595      22.4595     64.44  <.001
+ %Water                       1     228.1966     228.1966    654.78  <.001
+ Hour                         1       3.5107       3.5107     10.07  0.002
Residual                      80      27.8807       0.3485

Total                         83     282.0475       3.3982
```

Diglycerides:

```
***** Regression Analysis *****

 Response variate: DG
     Fitted terms: Constant + %Lipo + %Water + Hour
```

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	549.4	183.141	130.61	<.001
Residual	80	112.2	1.402		
Total	83	661.6	7.971		
Change	-3	-549.4	183.141	130.61	<.001

Percentage variance accounted for 82.4
Standard error of observations is estimated to be 1.18
* MESSAGE: The following units have large standardized residuals:
31 2.91
35 3.08
* MESSAGE: The residuals do not appear to be random;
for example, fitted values in the range 8.15 to 9.06
are consistently larger than observed values
and fitted values in the range 13.04 to 13.26
are consistently smaller than observed values
* MESSAGE: The error variance does not appear to be constant:
intermediate responses are less variable than small or large responses

*** Estimates of regression coefficients ***

	estimate	s.e.	t(80)	t pr.
Constant	3.573	0.400	8.94	<.001
%Lipo	-0.417	0.127	-3.29	0.002
%Water	21.55	1.15	18.68	<.001
Hour	0.03301	0.00814	4.06	<.001

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ %Lipo	1	36.992	36.992	26.38	<.001
+ %Water	1	489.341	489.341	349.00	<.001
+ Hour	1	23.088	23.088	16.47	<.001
Residual	80	112.171	1.402		
Total	83	661.593	7.971		

Triglycerides:

***** Regression Analysis *****

Response variate: TG
Fitted terms: Constant + %Lipo + %Water + Hour

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	1589.9	529.977	174.07	<.001
Residual	80	243.6	3.045		
Total	83	1833.5	22.090		
Change	-3	-1589.9	529.977	174.07	<.001

```
Percentage variance accounted for 86.2
Standard error of observations is estimated to be 1.74
* MESSAGE: The following units have large standardized residuals:
                31       -2.61
                35       -2.80
* MESSAGE: The residuals do not appear to be random;
           for example, fitted values in the range 85.89 to 87.73
           are consistently smaller than observed values
           and fitted values in the range 80.37 to 81.38
           are consistently larger  than observed values


*** Estimates of regression coefficients ***

                              estimate         s.e.     t(80)  t pr.
Constant                        95.356        0.589    161.96  <.001
%Lipo                            0.666        0.187      3.56  <.001
%Water                         -36.80         1.70     -21.65  <.001
Hour                           -0.0459       0.0120     -3.83  <.001


*** Accumulated analysis of variance ***

Change                          d.f.           s.s.          m.s.       v.r.  F pr.
+ %Lipo                            1        118.115       118.115      38.79  <.001
+ %Water                           1       1427.153      1427.153     468.74  <.001
+ Hour                             1         44.661        44.661      14.67  <.001
Residual                          80        243.573         3.045

Total                             83       1833.502        22.090
```

Appendix 7

Genstat Fitcurve programs for individual equations describing random and 1,3-specific triglyceride reaction profiles

7.1A Genstat programs.

Random interesterification LDL fitcurve program:

```
"
.....  Brazilian Cocoa Butter
"
units [96]
open 'rxndata.prn'; ch=2; file=in; width=120
skip [2] 1
read [ch=2; format=!((7,*)96)] \
 expt,%Lipo,%Water,hr,Orig,Rand,Spec13
close ch=2; file=in
scalar Ex,%L,%W
variate [12] Expt,%LIPO,%WATER,a,b,r,g,RSS,RDF,S2
for ex=1...12
 restrict expt,hr,%Lipo,%Water,Rand; expt.eq.ex
 calc Ex = mean(expt)
 calc %L = mean(%Lipo)
 calc %W = mean(%Water)
 print [iprint=*] '...  Curve Fit for
Expt',Ex,'%Lipo',%L,'%Water',%W;\
  fieldw=24,4,7,6,8,6; deci=0,0,0,2,0,2
 model Rand
 fitcurve [curve=ldl] hr
 rkeep estim=bhat; dev=rss; df=rdf
 calc b$[ex] = bhat$[2]
 calc r$[ex] = bhat$[1]
 calc g$[ex] = bhat$[3]
 calc Expt$[ex] = Ex
 calc %LIPO$[ex] = %L
 calc %WATER$[ex] = %W
 calc RSS$[ex] = rss
 calc RDF$[ex] = rdf
 rgraph [graph=hi]
  "rgraph"
 restrict expt,hr,%Lipo,%Water,Rand
endfor

calc S2 = RSS/RDF

print Expt,%LIPO,%WATER,b,r,g,RSS,RDF,S2;\
 fieldw=5,6,7,3(10),8,4,7; deci=0,2,2,3(4),3,0,3
```

Random interesterification QDL fitcurve program:

```
"
.....  Brazilian Cocoa Butter
"
units [96]
open 'rxndata.prn'; ch=2; file=in; width=120
skip [2] 1
read [ch=2; format=!((7,*)96)] \
 expt,%Lipo,%Water,hr,Orig,Rand,Spec13
close ch=2; file=in
scalar Ex,%L,%W
variate [12] Expt,%LIPO,%WATER,a,b,r,g,RSS,RDF,S2
for ex=1...12
 restrict expt,hr,%Lipo,%Water,Rand; expt.eq.ex
 calc Ex = mean(expt)
 calc %L = mean(%Lipo)
 calc %W = mean(%Water)
 print [iprint=*] '...  Curve Fit for
Expt',Ex,'%Lipo',%L,'%Water',%W;\
  fieldw=24,4,7,6,8,6; deci=0,0,0,2,0,2
 model Rand
 fitcurve [curve=qdl] hr
 rkeep estim=bhat; dev=rss; df=rdf
 calc a$[ex] = bhat$[4]
 calc b$[ex] = bhat$[2]
 calc r$[ex] = bhat$[1]
 calc g$[ex] = bhat$[3]
 calc Expt$[ex] = Ex
 calc %LIPO$[ex] = %L
 calc %WATER$[ex] = %W
 calc RSS$[ex] = rss
 calc RDF$[ex] = rdf
rgraph [graph=hi]
"  rgraph"
 restrict expt,hr,%Lipo,%Water,Rand
endfor

calc S2 = RSS/RDF

print Expt,%LIPO,%WATER,a,b,r,g,RSS,RDF,S2;\
 fieldw=5,6,7,4(10),8,4,7; deci=0,2,2,4(4),3,0,3
```

1,3-specific interesterification QDL fitcurve program:

```
"
.....  Brazilian Cocoa Butter
"
units [96]
open 'rxndata.prn'; ch=2; file=in; width=120
skip [2] 1
read [ch=2; format=!((7,*)96)] \
 expt,%Lipo,%Water,hr,Orig,Rand,Spec13
close ch=2; file=in
scalar Ex,%L,%W
variate [12] Expt,%LIPO,%WATER,a,b,r,g,h,RSS,RDF,S2
for ex=1...12
 restrict expt,hr,%Lipo,%Water,Spec13; expt.eq.ex
 calc Ex = mean(expt)
 calc %L = mean(%Lipo)
```

```
 calc %W = mean(%Water)
 print [iprint=*] '...  Curve Fit for
Expt',Ex,'%Lipo',%L,'%Water',%W;\
  fieldw=24,4,7,6,8,6; deci=0,0,0,2,0,2
 model Spec13
 fitcurve [curve=qdl] hr
 rkeep estim=bhat; dev=rss; df=rdf
 calc a$[ex] = bhat$[4]
 calc b$[ex] = bhat$[2]
 calc r$[ex] = bhat$[1]
 calc g$[ex] = bhat$[3]
 "calc h$[ex] = bhat$[5]"
 calc Expt$[ex] = Ex
 calc %LIPO$[ex] = %L
 calc %WATER$[ex] = %W
 calc RSS$[ex] = rss
 calc RDF$[ex] = rdf
 rgraph [graph=hi]
 " rgraph"
 restrict expt,hr,%Lipo,%Water,Spec13
endfor

calc S2 = RSS/RDF

print Expt,%LIPO,%WATER,a,b,r,g,RSS,RDF,S2;\
 fieldw=5,6,7,4(10),8,4,7; deci=0,2,2,4(4),3,0,3
```

1,3-specific interesterification QDQ fitcurve program:

```
"
.....  Brazilian Cocoa Butter
"
units [96]
open 'rxndata.prn'; ch=2; file=in; width=120
skip [2] 1
read [ch=2; format=!((7,*)96)] \
 expt,%Lipo,%Water,hr,Orig,Rand,Spec13
close ch=2; file=in
scalar Ex,%L,%W
variate [12] Expt,%LIPO,%WATER,a,b,r,g,h,RSS,RDF,S2
for ex=1...12
 restrict expt,hr,%Lipo,%Water,Spec13; expt.eq.ex
 calc Ex = mean(expt)
 calc %L = mean(%Lipo)
 calc %W = mean(%Water)
 print [iprint=*] '...  Curve Fit for
Expt',Ex,'%Lipo',%L,'%Water',%W;\
  fieldw=24,4,7,6,8,6; deci=0,0,0,2,0,2
 model Spec13
 fitcurve [curve=qdq] hr
 rkeep estim=bhat; dev=rss; df=rdf
 calc a$[ex] = bhat$[4]
 calc b$[ex] = bhat$[2]
 calc r$[ex] = bhat$[1]
 calc g$[ex] = bhat$[3]
 calc h$[ex] = bhat$[5]
 calc Expt$[ex] = Ex
 calc %LIPO$[ex] = %L
 calc %WATER$[ex] = %W
```

```
 calc RSS$[ex] = rss
 calc RDF$[ex] = rdf
 rgraph [graph=hi]
 " rgraph"
 restrict expt,hr,%Lipo,%Water,Spec13
endfor

calc S2 = RSS/RDF

print Expt,%LIPO,%WATER,a,b,r,g,h,RSS,RDF,S2;\
 fieldw=5,6,7,5(10),8,4,7; deci=0,2,2,5(4),3,0,3
```

7.2A Input data for Fitcurve function Genstat programs: Rxndata.prn

Name	Lipo	Water	Hour	Orig	Rand	Spec13
1	1.25	0.12	0	0	36.62	7.3
1	1.25	0.12	1	0.4	31.64	4.44
1	1.25	0.12	2	0.5	30.71	4.14
1	1.25	0.12	4	1.05	28.88	3
1	1.25	0.12	6	1.35	28.3	2.52
1	1.25	0.12	8	2.18	26.99	1.71
1	1.25	0.12	24	5	21.01	0.48
1	1.25	0.12	48	8.57	12.92	1.33
2	1.25	0.2	0	0	45.05	9.84
2	1.25	0.2	1	0.28	39.66	6.81
2	1.25	0.2	2	0.91	36.99	4.95
2	1.25	0.2	4	1.75	34.68	3.49
2	1.25	0.2	6	2.56	32.4	2.51
2	1.25	0.2	8	3.39	30.96	1.86
2	1.25	0.2	24	7.87	20.39	0.23
2	1.25	0.2	48	14.32	10.62	1.85
3	1.25	0.2	0	0	30.22	6.24
3	1.25	0.2	1	0.46	26.09	3.54
3	1.25	0.2	2	0.66	25.68	3.03
3	1.25	0.2	4	1.75	21.84	1.9
3	1.25	0.2	6	2.36	21.17	1.43
3	1.25	0.2	8	3.78	19.36	0.96
3	1.25	0.2	24	7.22	13.16	1.45
3	1.25	0.2	48	13.66	5.11	6.06
4	1.25	0.4	0	0	36.33	7.07
4	1.25	0.4	1	1.03	26.81	3.37
4	1.25	0.4	2	2.86	22.2	1.77
4	1.25	0.4	4	7.34	16.58	1.2
4	1.25	0.4	6	12.13	12.63	2.39
4	1.25	0.4	8	15.65	10.61	3.84
4	1.25	0.4	24	34.79	2.13	16.83
4	1.25	0.4	48	46.44	1.02	28.65
5	2.5	0.17	0	0	49.64	12.4
5	2.5	0.17	1	0.72	41.37	7.26
5	2.5	0.17	2	1.1	41.7	6.51
5	2.5	0.17	4	2.42	36.85	4.11

5	2.5	0.17	6	3.96	33.7	2.64
5	2.5	0.17	8	4.58	31.75	2.06
5	2.5	0.17	24	10.04	17.98	0.71
5	2.5	0.17	48	17.19	8.7	4.03
6	2.5	0.3	0	0	43.8	9.14
6	2.5	0.3	1	0.7	36.92	4.95
6	2.5	0.3	2	2.22	32.14	2.57
6	2.5	0.3	4	4.52	28.07	1.03
6	2.5	0.3	6	6.81	23.93	0.37
6	2.5	0.3	8	8.64	20.75	0.2
6	2.5	0.3	24	18.76	7.13	3.73
6	2.5	0.3	48	31.5	1.07	12.76
7	2.5	0.3	0	0	32.08	6.12
7	2.5	0.3	1	0.76	25.65	2.92
7	2.5	0.3	2	2.03	22.44	1.63
7	2.5	0.3	4	5.03	18.4	0.87
7	2.5	0.3	6	7.21	15.25	1.24
7	2.5	0.3	8	8.73	13.59	1.64
7	2.5	0.3	24	20.65	3.19	9.73
7	2.5	0.3	48	33.45	0.27	22.19
8	2.5	0.48	0	0	45.66	9.67
8	2.5	0.48	1	1.29	34.79	4.02
8	2.5	0.48	2	3.9	29.06	1.57
8	2.5	0.48	4	9.17	21.72	0.32
8	2.5	0.48	6	13.85	16.76	0.82
8	2.5	0.48	8	15.97	14.07	1.34
8	2.5	0.48	24	31.88	2.05	10.4
8	2.5	0.48	48	42.54	0.11	19.9
9	4	0.24	0	0	41.6	9.04
9	4	0.24	1	1.31	36.78	4.18
9	4	0.24	2	1.7	35.09	3.43
9	4	0.24	4	3.5	29.56	1.47
9	4	0.24	6	6.09	23.34	0.4
9	4	0.24	8	6.56	23.37	0.27
9	4	0.24	24	15.23	8.31	2.94
9	4	0.24	48	24.95	2.76	13.21
10	4	0.24	0	0	41.05	8.54
10	4	0.24	1	1.15	32.85	3.58
10	4	0.24	2	2.66	29.04	1.83
10	4	0.24	4	5.12	24.89	0.62
10	4	0.24	6	7.32	20.36	0.21
10	4	0.24	8	8.46	18.09	0.3
10	4	0.24	24	19.52	4.84	5.73
10	4	0.24	48	32.46	0.51	15.83
11	4	0.48	0	0	42.47	8.69
11	4	0.48	1	1.98	31.01	2.58
11	4	0.48	2	4.21	26.48	1.02
11	4	0.48	4	10.73	18.73	0.53
11	4	0.48	6	14.26	14.44	1.32
11	4	0.48	8	17.81	11.04	2.64

11	4	0.48	24	33.72	0.97	13.76
11	4	0.48	48	43.15	0.21	23.02
12	4	0.48	0	0	47.56	10.94
12	4	0.48	1	1.76	35.99	4.09
12	4	0.48	2	5.52	28.72	1.21
12	4	0.48	4	9.55	23.06	0.25
12	4	0.48	6	14.94	16.26	0.68
12	4	0.48	8	17.41	13.32	1.29
12	4	0.48	24	30.38	2.05	10.15
12	4	0.48	48	40.41	0.7	19.13

7.3A Summary tables of output results from Genstat programs.

Random interesterification LDL equation:

Expt	%LIPO	%WATER	b	r	g	RSS	RDF	S2
1	1.25	0.12	32.0807	0.0353	1.9006	14.969	5	2.994
2	1.25	0.20	44.5587	0.0480	-1.9206	14.366	5	2.873
3	1.25	0.20	31.5150	0.0514	-2.8951	10.481	5	2.096
4	1.25	0.40	37.7733	0.2721	-1.9554	2.880	5	0.576
5	2.50	0.17	51.4734	0.0575	-4.4106	17.127	5	3.425
6	2.50	0.30	49.2618	0.1051	-6.8173	6.649	5	1.330
7	2.50	0.30	35.4230	0.1386	-4.3938	4.920	5	0.984
8	2.50	0.48	49.4144	0.2280	-4.4182	4.394	5	0.879
9	4.00	0.24	48.5079	0.0843	-7.1296	7.234	5	1.447
10	4.00	0.24	45.4940	0.1239	-6.0223	9.294	5	1.859
11	4.00	0.48	45.8471	0.2629	-3.9238	5.251	5	1.050
12	4.00	0.48	50.8063	0.2545	-3.7914	7.227	5	1.445

Random interesterification QDL equation:

Expt	%LIPO	%WATER	a	b	r	g	RSS	RDF	S2
1	1.25	0.12	29.3880	7.2162	1.5716	-0.3472	0.310	4	0.077
2	1.25	0.12	-40.0715	84.8330	0.0232	0.2221	86.472	4	21.618
3	1.25	0.20	19.6844	10.4190	0.4115	-0.3139	1.748	4	0.437
4	1.25	0.40	-1.6189	37.4672	0.2772	-0.0068	2.871	4	0.718
5	2.50	0.17	21.4830	26.6206	0.1525	-0.3400	13.235	4	3.309
6	2.50	0.30	1.7266	41.1336	0.1403	-0.1303	5.565	4	1.391
7	2.50	0.30	-0.8688	32.1176	0.1651	-0.0580	4.647	4	1.162
8	2.50	0.48	-4.3729	49.3725	0.2284	-0.0009	4.394	4	1.099
9	4.00	0.24	-12.6545	53.8995	0.0737	0.0727	7.019	4	1.755
10	4.00	0.24	-0.5333	40.3193	0.1523	-0.0872	8.767	4	2.192
11	4.00	0.48	-6.5065	48.2180	0.2358	0.0511	4.789	4	1.197
12	4.00	0.48	-5.4910	52.3663	0.2384	0.0335	7.031	4	1.758

1,3-specific interesterification QDL equation:

Expt	%LIPO	%WATER	a	b	r	g	RSS	RDF	S2
1	1.25	0.12	-0.3472	7.3990	0.3600	0.0226	1.010	4	0.252
2	1.25	0.20	-2.5805	12.2790	0.2963	0.0723	0.495	4	0.124
3	1.25	0.20	-4.5552	10.4505	0.2231	0.1994	0.893	4	0.223
4	1.25	0.40	-1.7247	8.8885	1.4284	0.6529	10.456	4	2.614
5	2.50	0.17	-3.8015	15.7925	0.3138	0.1377	2.168	4	0.542
6	2.50	0.30	-6.8507	15.9425	0.3955	0.3897	0.224	4	0.056
7	2.50	0.30	-4.4547	10.6073	0.5742	0.5495	0.147	4	0.037
8	2.50	0.48	-3.7145	13.4855	1.0327	0.4999	3.971	4	0.993
9	4.00	0.24	-9.3492	17.9115	0.2631	0.4376	2.209	4	0.552
10	4.00	0.24	-5.5288	14.0262	0.5861	0.4340	0.102	4	0.025
11	4.00	0.48	-1.9446	10.6751	2.1362	0.5401	9.244	4	2.311
12	4.00	0.48	-3.5635	14.5902	1.2524	0.4822	4.418	4	1.104

1,3-specific interesterification QDQ equation:

Expt	%LIPO	%WATER	a	b	r	g	h
1	1.25	0.12	-0.2998	-0.0177	0.4856	6.2581	0.9027
2	1.25	0.20	-0.3762	-0.0112	0.4199	9.7809	0.0652
3	1.25	0.20	1.1652	-0.0091	0.5742	8.5931	-2.4064
4	1.25	0.40	-22.7852	0.0091	0.3349	-51.6869	58.7633
5	2.50	0.17	-0.3453	-0.0117	0.5188	12.1627	0.1055
6	2.50	0.30	34.4200	-0.0019	0.4746	86.3328	-77.1733
7	2.50	0.30	-117.5309	0.0012	0.4734	-237.7984	243.9115
8	2.50	0.48	-30.5629	0.0097	0.5153	-41.6230	51.3001
9	4.00	0.24	8.4843	-0.0059	0.5433	27.7335	-18.7993
10	4.00	0.24	179.3669	-0.0009	0.6289	298.1205	-289.6031
11	4.00	0.48	-32.7876	0.0169	0.5976	-37.9186	46.5732
12	4.00	0.48	-35.6580	0.0128	0.6441	-36.6207	47.5768

RSS	RDF	S2
0.518	3	0.173
0.044	3	0.015
0.213	3	0.071
0.017	3	0.006
1.048	3	0.349
0.058	3	0.019
0.027	3	0.009
0.064	3	0.021
0.799	3	0.266
0.073	3	0.024
0.143	3	0.048
0.115	3	0.038

Appendix 8

Genstat programs for the development of overall equations describing random and 1,3-specific triglyceride reaction profiles

8.1A Input data from individual equations, Chapter 9, the coefficients of LDL equations for random interesterification and the results for the regression of coefficient D with Lipozyme and water content.

Input data:

Trt!	%Lipozyme	%Water	A	B	D	R2	se	S2
1	1.25	0.12	1.9	32.08	0.0353	94.3	1.73	2.99
2	1.25	0.2	-1.92	44.56	0.0480	97.6	1.7	2.87
3	1.25	0.2	-2.9	31.52	0.0514	96.7	1.45	2.1
4	1.25	0.4	-1.96	37.77	0.2721	99.6	0.76	0.58
5	2.5	0.17	-4.41	51.47	0.0575	98.1	1.85	3.43
6	2.5	0.3	-6.82	49.26	0.1051	99.4	1.15	1.33
7	2.5	0.3	-4.32	35.42	0.1386	99.2	0.99	0.98
8	2.5	0.48	-4.42	49.41	0.2280	99.6	0.94	0.88
9	4	0.24	-7.13	48.51	0.0843	99.2	1.2	1.45
10	4	0.24	-6.02	45.49	0.1239	99	1.36	1.86
11	4	0.48	-3.92	45.85	0.2629	99.5	1.02	1.05
12	4	0.48	-3.79	50.81	0.2545	99.5	1.2	1.45

Output data:

```
   Identifier      Values   Missing    Levels
          Trt          12         0        12

    Identifier   Minimum      Mean   Maximum    Values   Missing
     %Lipozym     1.250     2.583     4.000        12         0
       %Water    0.1200    0.3008    0.4800        12         0
            A    -7.130    -3.809     1.900        12         0
            B     31.52     43.51     51.47        12         0
            D    0.0400    0.1383    0.2700        12         0
          R2     94.30     98.47     99.60        12         0
          se     0.760     1.279     1.850        12         0
          S2     0.580     1.747     3.430        12         0


***** Regression Analysis *****

 Response variate: D
     Fitted terms: Constant, %Lipozym, %Water
```

```
 *** Summary of analysis ***

             d.f.          s.s.          m.s.       v.r.  F pr.
Regression      2      0.081037     0.0405186      45.99  <.001
Residual        9      0.007929     0.0008811
Total          11      0.088967     0.0080879

Change         -2     -0.081037     0.0405186      45.99  <.001

Percentage variance accounted for 89.1
Standard error of observations is estimated to be 0.0297
* MESSAGE: The following units have large standardized residuals:
                   4        2.48


*** Estimates of regression coefficients ***

                estimate          s.e.       t(9)  t pr.
Constant         -0.0547        0.0255      -2.15  0.060
%Lipozym        -0.00452       0.00842      -0.54  0.605
%Water           0.6806        0.0765       8.90  <.001
```

8.2A Input data from individual equations, Chapter 9, the coefficients of QDL equations for random interesterification and the results for the regression of coefficients C and D with Lipozyme and water content.

Input data:

Trt!	%Lipozyme	%Water	A	B	D	C	R2	se	S2
1	1.25	0.12	-0.3500	7.4000	0.360	0.0226	94.6	0.5	0.25
2	1.25	0.2	-2.5800	12.2800	0.296	0.0723	98.7	0.35	0.12
3	1.25	0.2	-4.5600	10.4500	0.223	0.1994	94.8	0.47	0.22
4	1.25	0.4	-1.7200	8.8900	1.428	0.6529	97.2	1.62	2.61
5	2.5	0.17	-3.8000	15.7900	0.314	0.1377	96.1	0.74	0.54
6	2.5	0.3	-6.8500	15.9400	0.396	0.3897	99.7	0.24	0.06
7	2.5	0.3	-4.4500	10.6100	0.574	0.5495	99.9	0.19	0.04
8	2.5	0.48	-3.7100	13.4900	1.032	0.4999	97.9	1	0.99
9	4	0.24	-9.3500	17.9200	0.263	0.4376	97.3	0.74	0.55
10	4	0.24	-5.5300	14.0300	0.586	0.4340	99.9	0.16	0.03
11	4	0.48	-1.9400	10.6800	2.136	0.5401	96.4	1.52	2.31
12	4	0.48	-3.5600	14.5900	1.252	0.4822	97.6	1.05	1.1

Output data:

```
  Identifier     Values    Missing     Levels
         Trt         12          0         12
```

Identifier	Minimum	Mean	Maximum	Values	Missing
%Lipozym	1.250	2.583	4.000	12	0
%Water	0.1200	0.3008	0.4800	12	0
A	-9.350	-4.033	-0.350	12	0
B	7.40	12.67	17.92	12	0
D	0.2200	0.7375	2.1400	12	0
C	0.0200	0.3675	0.6500	12	0
R2	94.60	97.51	99.90	12	0
se	0.1600	0.7150	1.6200	12	0
S2	0.0300	0.7350	2.6100	12	0

***** Regression Analysis *****

Response variate: C
Fitted terms: Constant, %Lipozym, %Water

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	2	0.3101	0.15505	8.63	0.008
Residual	9	0.1617	0.01797		
Total	11	0.4718	0.04289		
Change	-2	-0.3101	0.15505	8.63	0.008

Percentage variance accounted for 58.1
Standard error of observations is estimated to be 0.134
* MESSAGE: The residuals do not appear to be random;
for example, fitted values in the range 0.342 to 0.440
are consistently smaller than observed values
and fitted values in the range 0.117 to 0.214
are consistently larger than observed values

*** Estimates of regression coefficients ***

	estimate	s.e.	t(9)	t pr.
Constant	-0.060	0.115	-0.52	0.614
%Lipozym	0.0313	0.0380	0.82	0.431
%Water	1.153	0.346	3.34	0.009

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.....

***** Regression Analysis *****

Response variate: D
Fitted terms: Constant, %Lipozym, %Water

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.

Regression	2	2.847	1.4235	11.33	0.003
Residual	9	1.131	0.1257		
Total	11	3.978	0.3616		
Change	-2	-2.847	1.4235	11.33	0.003

Percentage variance accounted for 65.3
Standard error of observations is estimated to be 0.354
* MESSAGE: The following units have large standardized residuals:
11 2.36

*** Estimates of regression coefficients ***

	estimate	s.e.	t(9)	t pr.
Constant	-0.440	0.305	-1.45	0.182
%Lipozym	-0.005	0.101	-0.05	0.965
%Water	3.954	0.914	4.33	0.002

List of publications

1. *Ainsworth, S.*, Versteeg, C., Palmer, M. and Millikan, M. B. 'Enzymatic Interesterification' (oral presentation) *Milkfat Update Conference*, February 1996, Werribee, Victoria.
   Paper published in *Aust. J. Dairy Technol.*, **51**, 105-107, 1996.

2. *Ainsworth, S.*, Versteeg, C., Palmer, M. and Millikan, M. B. 'A Model for Solvent-free Enzymatic Interesterification of Fats and Oils' (poster presentation) *AIFST '96*, May 1996, Gold Coast, QLD.
   Research note published in *Food Australia*, **49 (6)**, 281-282, 1997.

3. *Ainsworth, S.*, Versteeg, C., Palmer, M. and Millikan, M. B. 'Solvent-free Enzymatic Interesterification – Product and process development' (oral presentation) *AOCS - Australasian Workshop*, February 1997, Canberra, ACT.
   Full paper published in proceedings.

4. i) *Ainsworth, S.*, Versteeg, C., Palmer, M. and Millikan, M. B. 'Characterisation and optimisation of the solvent-free enzymatic interesterification of fats using cocoa butter as a model' (oral presentation)

   ii) Ainsworth, S., Versteeg, C., Palmer, M. and *Millikan, M. B.* 'Characterisation of solvent-free enzymatic interesterification of fats and oils' (poster presentation) *22nd World Congress and Exhibition of the International Society for Fat Research (ISF),* September 1997, Kuala Lumpur, Malaysia.

5. *Ainsworth, S.*, Versteeg, C., Palmer, M. and Millikan, M. B. 'Effect of water content on the solvent-free enzymatic interesterification of fats and oils' (poster presentation) *AIFST '98*, April 1998, Melbourne, Victoria

6. Ainsworth, S., Versteeg, C., Palmer, M. and *Millikan, M. B.* 'Potential for better fat through biotechnology' (poster presentation) *European Research towards Safer and Better Food – A Review and Transfer Congress*, October 1998, Karlsruhe, Germany.

7. Ainsworth, S., Versteeg, C., Palmer, M. and *Millikan, M. B.* 'Enzymatic interesterification of fats: a predictive model' *Institute of Food Technologists Annual Meeting (IFT)*, June 2000, Dallas, Texas, USA.