

11

Chemical and enzymatic interesterification of lipids for use in food

X. Xu, Z. Guo, H. Zhang, A. F. Vikbjerg and M. L. Damstrup,
Technical University of Denmark, Denmark

11.1 Introduction

Interesterification, as defined in a lipid glossary (Gunstone and Herslöf, 1992), is a general term for alkali-catalyzed reaction of, for example, triacylglycerols producing a random distribution of ester groups within the molecules. This definition has been widely accepted as a term for chemical randomization, typically between liquid oils and solid fats, in the oil and fat modification industry, in particular for the manufacture of margarine or shortening fat products. A number of papers relating to this concept have recently been presented and its use in industry has expanded because it provides a better alternative to hydrogenation in the coming *trans*-free era (Rozendaal and Macrae, 1997; Rousseau and Marangoni, 2002).

The primary motivation for the modification of naturally occurring oils and fats by interesterification during the production of margarine and shortening fats stems from the increasing demand for lipids with specific melting behaviour and crystallization characteristics. This is because the supply of natural products capable of meeting these particular requirements is insufficient (Macrae, 1981; Gunstone, 1999; Baljit *et al.*, 2002). It is not surprising that the triacylglycerol structure of natural oils and fats is the result of either a regular distribution of fatty acids on the glycerol backbone or a limited statistical distribution, because these distributions are the intrinsic response of living organisms in adjusting their triacylglycerol structure to biological functions and to the environment (Ucciani and Debal, 1996). The functional properties of oils and fats are closely related to both their fatty acid profiles and the distribution of different fatty acid species on individual triacylglycerol molecules. Interesterification allows the rearranging of existing acyl groups or the incorporation of other fatty acids to create novel properties.

Intesterification in a chemical sense, as defined by Sonntag (1982), often refers to the reaction of triacylglycerols within or between the molecules or with other acyl donors or alcohol moieties, yielding new esters by an interchange of fatty acid groups. It can be classified into ester–ester interchange, acidolysis and alcoholysis, depending on the nature of the substrates. This definition covers more applications in terms of product development and process development, in particular with the boom in research on enzymes as catalysts. The subject is easily divided into chemical and enzymatic interesterification depending on the catalysts employed.

Therefore, in this chapter, the concept of interesterification for lipid modification will be expanded to include acidolysis and alcoholysis (glycerolysis) in addition to the ester–ester interchange, to which it usually refers. Several good papers have been presented on chemical interesterification, and since this technology is now mature the emphasis in this chapter will be on enzymatic interesterification, which is currently close to industrial development. With this wider definition in mind, the chapter will present a few typical and important applications in the area of lipid modification and discuss development of new products or technologies. The writing is primarily focused on our own research.

11.2 Interesterification in general practice: an introduction of potential reactions and applications

11.2.1 Chemical interesterification

Chemical interesterification has long been used to modify oils and fats into functional products (Marangoni and Rousseau, 1995). It modifies the physical properties of lipids by rearranging the distribution of fatty acids on the glycerol backbone without changing fatty acid profiles. During interesterification, fatty acids are exchanged within (intra) and among (inter) triacylglycerols until a thermodynamic equilibrium is reached. This equilibrium is based on the composition of the starting material and can be predicted from the laws of probability. The degree of difference depends upon the temperature, time and other conditions of the reaction (Marangoni and Rousseau, 1998; Rousseau *et al.*, 1998; Gunstone, 1999).

Early patents concerning chemical interesterification were filed by W. Normann between 1920 and 1930, and these contributed to the production of margarine with defined physical properties. The process was used in industrial applications from the 1940s. Typically, chemical interesterification can be carried out using sodium methoxide (0.2–0.3 %) or an alkali metal (0.1–9.2 %) as a catalyst (Anderson, 1996). Oil blends need to be dried under vacuum at a temperature higher than 90 °C for 0.5–1 hour. The reaction can be initiated by the addition of catalyst after the blend is cooled to 60–70 °C. At the beginning of the reaction, the reaction mixture becomes brown, which indicates the start of the reaction. Once such colour change occurs, the

reaction can be completed within 30 minutes. The reaction is stopped by the introduction of water, and subsequent processing usually includes washing, bleaching and deodorization to obtain the final randomized product.

Figure 11.1 illustrates the difference between chemical and specific lipase-catalyzed interesterification in two triacylglycerols. For the chemical randomization of ACA and BBB governed by thermodynamics and statistics, theoretically all 18 isomers (27 if stereoisomers are included) might be detected in the end reaction mixture. For comparison, with 1,3-specific lipase-catalyzed reaction governed mainly by enzyme specificity and dynamics, only four new isomers (six if stereoisomers are included) are expected, apart from the original ACA and BBB. The formation of isomers with acyl group C at the 1- or 3-positions and A at the 2-position of the glycerol backbone is theoretically impossible if acyl migration can be ruled out. The randomization of triacylglycerols can also be driven by non-specific lipases, yielding similar results to chemical interesterification. In practice, only a very few lipases are strictly non-specific. Most lipases are specific to a certain extent (Matori *et al.*, 1991). That is to say, the result of the reaction catalyzed even by a non-specific lipase will not be exactly the same as that with a chemical catalyst.

The widely used chemical catalysts are Na-K alloy or sodium alcoholate (e.g. CH_3ONa). However, they are believed not to be the real catalysts due to their ease of consumption during reaction. Glycerate is the real catalyst of interesterification. This mechanism explains the formation of both monoalcohol ester and diacylglycerol, the latter being formed by glycerate hydrolysis (Ucciani and Debal, 1996). Dijkstra *et al.* (2005) proposed a new mechanism in order to explain the experimental observations. This mechanism assumes that the reaction of a base (such as sodium methanolate) with the oil will eventually lead to the abstraction of an α -hydrogen from a fatty acid moiety and that the enolate anion thus formed acts as the catalytic intermediate. This enolate can re-abtract a proton from the hydroxyl group of a partial glyceride, whereupon the resulting alcoholate attacks the carbonyl group. This leads to a new ester and a glycerolate anion which then regenerates a new enolate anion.

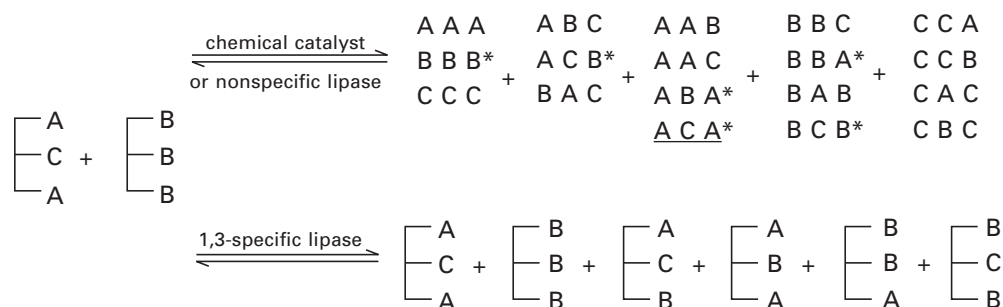


Fig. 11.1 Schematic illustration of the difference between randomization and 1,3-specific interesterification, exemplified by the reaction between ACA- and BBB-type triacylglycerols. Underlined molecules are starting materials and those with star are possible products for 1,3-specific lipase-catalyzed reaction.

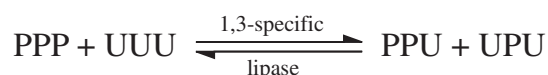
The reaction between oils and fats could yield the required plastic property and crystal forms (Rousseau *et al.*, 1998; Gunstone, 1999; Baljit *et al.*, 2002). For example, the shortening property of lard can be improved by randomization, which changes its crystalline form from β to β' by reducing the content of palmitic acid at the 2-position. *Trans*-fatty acid free margarine can be manufactured by interesterification from palm stearin and sunflower oil in appropriate ratios. Chemical interesterification can also take place between triacylglycerols and glycerol, and this method is currently used for the industrial production of partial acylglycerols (see Section 11.3.4) (Bornscheuer, 1995).

The reaction of triacylglycerols and methanol is now widely used for the preparation of methyl esters for gas chromatographic analysis of fatty acid composition. However, due to the predicted shortage of petroleum, this reaction has been utilized on a much larger scale to produce biodiesel (Korbitz, 1999; Ma and Hanna, 1999). Primary and secondary monohydric aliphatic alcohols having from one up to eight carbons can be used in the interesterification process. Among these, methanol and ethanol are used most frequently, especially methanol due to its low cost and its physical and chemical advantages. The reaction can be catalyzed by alkalis, acids or enzymes. Alkali-catalyzed interesterification is much faster than the acid-catalyzed reaction and is most often used commercially so long as the oil does not have a high acid value.

11.2.2 Enzymatic ester–ester exchange

Ester–ester exchange can occur between two triacylglycerols (TAGs), or between triacylglycerol and methyl or ethyl esters employing either a chemical or an enzymatic catalyst. Enzymatic TAG–TAG interchange, in principle, gives different triacylglycerol composition in the end product compared with the chemical process, depending on the regiospecificity of the lipase (Fig. 11.1). This reaction has been applied to margarine or shortening fat production and the manufacture of other structured lipids with specific functions (Negishi *et al.*, 2003; Zhang *et al.*, 2004a,b). Recently, an enzymatic scale-up formulation of butter incorporating polyunsaturated fatty acids (PUFA) in varied proportions has been studied. Nutritional concerns and sensory evaluation were also examined (Rønne *et al.*, 2005).

Triacylglycerols of UPU type (U = unsaturated fatty acids, P = palmitic acid) are believed to be readily absorbed by infants. Loders-Croklaan in the Netherlands manufactures such a product as a constituent of infant formula from tripalmitin and a vegetable oil with high oleic acid content confining the acyl exchange to the *sn*-1 and *sn*-3 positions by 1,3-specific lipases (Kavanagh, 1997).

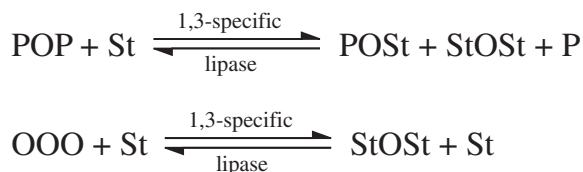


The reaction between triacylglycerols and fatty acid ethyl esters can be treated as an alternative to acidolysis of triacylglycerols for the production of, for example, fats or similar products with particular locations of specific fatty acids (see below). Therefore, the reaction will start from PPP and U-EE (ethyl esters of U) for the above product. After reaction, the remaining U-EE and released PEE have to be distilled to obtain the product (UPU + PPU).

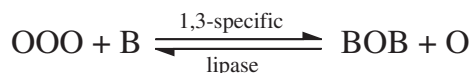
11.2.3 Enzymatic acidolysis

The acyl exchange reaction between triacylglycerols and fatty acids is called acidolysis. A particular interest in this area is that 1,3-specific lipases can selectively catalyze the acyl exchange at the *sn*-1 and *sn*-3 positions whilst leaving the *sn*-2 acyl group unchanged. This provides an opportunity to tailor some functional lipids with special requirements for the fatty acid types located in 1,3- or 2-positions. With this technology, a series of functional lipids, such as cocoa butter equivalents, human milkfat mimics or structured lipids containing medium- or short-chain fatty acids have been developed (Macrae, 1985; Balcao and Malcata, 1998; Gunstone, 1999; Xu, 2000c; Baljit *et al.*, 2002).

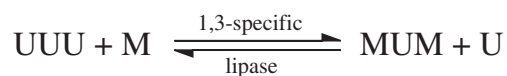
An important application of 1,3-specific lipase-catalyzed acidolysis is the enzymatic production of cocoa butter equivalents (Bloomer *et al.*, 1990; Undurraga *et al.*, 2001). For example, cocoa butter equivalents could be produced from palm mid-fraction (rich in POP) (O = oleic acid) or OOO-type oil by enzymatic incorporation of stearic acid (St) to the 1,3-positions:



An anti-blooming agent for chocolate, 1,3-dibehenoyl-2-oleoyl glycerol (BOB), has been prepared from triolein and behenic acid or its EE employing Lipozyme RM IM (Yoon *et al.*, 1996):



Structured lipids (MUM) with medium-chain fatty acids at 1,3-positions and unsaturated fatty acids at the 2-position are supposed to be an ideal energy and nutrition supplier, especially for patients suffering from malabsorption (Ikeda *et al.*, 1991; Ingle *et al.*, 1999). MUM are absorbed and digested more rapidly and the essential fatty acids at the *sn*-2 position meet the nutritional requirements. A substantial amount of work has been carried out in this area using oils with unsaturated fatty acids at the *sn*-2 position and medium-chain acyl donors (Xu, 2000c):



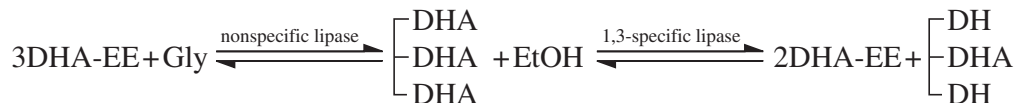
Lipase-catalyzed modification of vegetable oils by introducing certain polyunsaturated fatty acids, such as γ -linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), etc. from micro-organism oils, unusual plant oils and fish oils, in order to increase nutritional values constitutes another important aspect for lipase applications in lipid processing (Macrae, 1985; Huang and Akoh, 1994; Gunstone, 1999; Baljit *et al.*, 2002).

11.2.4 Enzymatic alcoholysis

Alcoholysis is the reaction between an ester and an alcohol, resulting in an alcohol exchange. The alcoholysis of triacylglycerols with glycerol is called glycerolysis and is widely used for production of mono- and diacylglycerols:



The reaction of triacylglycerols with monohydric alcohols generates the simple alcohol esters of fatty acids, which have found applications in the production of biodiesel, as well as alternative acyl donors of fatty acids, e.g. fatty acid ethyl esters. The intermediate product of 1,3-specific lipase-catalyzed alcoholysis, 2-monoglycerides, can also be used as starting materials for the synthesis of ABA-typed structured lipids:



More attention has been devoted to the enzymatic production of partial glycerol esters. Recently, we demonstrated a novel and efficient reaction system for enzymatic glycerolysis of oils and fats to produce a good yield of monoacylglycerols in a tetra-ammonium-based ionic liquid (Guo and Xu, 2005, 2006).

In general, many products and manifold process possibilities have been created with feasible reactions not only in theory but also in practice. Intensive demonstrations concerning important application areas are given in the following sections.

11.3 Typical interesterification in lipid modification

11.3.1 Enzymatic TAG–TAG interchange for plastic fat production

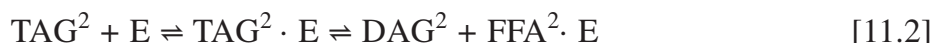
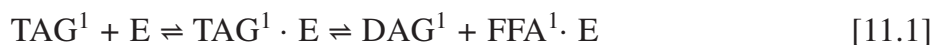
Since the 1990s, there has been a strong debate regarding *trans*-fatty acids (TFA) consumption and their relationship with high total blood cholesterol and low-density lipoprotein cholesterol levels, and the increase in death through coronary heart disease (Kris-Etherton and Nicolosi, 1995). The content of TFA in oils and fats has become a global issue. Legislation to limit the

TFA content of lipid and food products and to label the amount of TFA in fat-containing products has been issued in several countries in recent years. On July 11, 2003, the FDA announced final regulations for TFA labelling of foods in the USA from January 1, 2006. Demark issued such a requirement as early as 2004. To avoid *trans* fats produced by partial hydrogenation, interesterification of fully hydrogenated fats and liquid oils (List *et al.*, 1995) or some tropical oils (Lai *et al.*, 1998; Zhang *et al.*, 2004a, 2005) is frequently used for producing zero-*trans* plastic fats for industrial applications.

Enzymatic interesterification is now much studied for plastic fat production, although it is not a new technology. Unilever obtained a patent in 1977 on 'catalytic rearrangement of fatty acid groups in glycerol fats or oils – by contacting with an enzyme transesterification catalyst activated with water' (Unilever, 1977). However, the industrial breakthrough of lipase-catalyzed interesterification for plastic fat production has been delayed, mainly because the major commercially available immobilized lipases have hitherto been too expensive (Christensen *et al.*, 2001). Only high value-added oil and fat products have so far been processed in industry, such as cocoa butter substitutes (Chang *et al.*, 1990), human milk fat substitutes (Betapol™ from Loders Crokelaan), polyunsaturated fatty acid (PUFA)-enriched marine oils (Shimada *et al.*, 1997; Haraldsson and Kristinsson, 1998), functional 1,3-diglycerides and structured lipids (Soumanou *et al.*, 1998; Xu *et al.*, 1999a, 2000). However, in more recent years, the development of new cost-effective immobilized lipases is clearly providing more opportunities for the plastic fat industry to produce an economy-balanced product with better properties (Pedersen and Christensen, 2000). Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on silica granulates) is now successfully applied to bulk fat modifications (Novozymes A/S, 2005).

Reactions for enzymatic TAG–TAG interchange

Lipase-catalyzed interesterification involves water participation during the reactions and is accompanied by the formation of new triacylglycerol products as well as some diacylglycerol (DAG) and free fatty acid (FFA) by-products in the system (Wong, 1995). A simplified form, in which E stands for enzyme, instead of a detailed molecular structure is used to describe the process of lipase-catalyzed interesterification between triacylglycerols based on the formation of acyl enzyme complex (Zhang *et al.*, 2001):





The reaction will continue to reach equilibrium in the system involving production of new intermediates and formation of new TAG products (Eq. 11.1–6). If the water content increases in the system, the FFA content will increase (Eq. 11.7–8), whereas $\text{FFA}_1 \cdot \text{E}$ or $\text{FFA}_2 \cdot \text{E}$ decreases. As a consequence, the content of DAG will also increase (Eq. 11.3–4). The total yield of interesterified new TAG products will decrease. The free water is the main reason for the rise in the level of FFA in the system. One mole of free water in the reaction system generates one mole of FFA (Zhang *et al.*, 2000). Hence it is very important to control water content in the system to reduce the contents of FFA and DAG in the final products.

When an *sn*-1,3-specific lipase is used as a catalyst, the pathway for interesterification starts with the reaction of the TAG and an enzyme molecule (E), giving a 1,2(2,3)-DAG and $\text{FFA} \cdot \text{E}$ intermediate. A problem often encountered for a selective lipid modification is acyl migration, which leads to the non-specific distribution of fatty acids. Acyl migration is a non-enzymatic reaction catalyzed by acids, bases, ion exchange resin (carrier) and increased by heat and reaction time (Bloomer *et al.*, 1991; Xu *et al.*, 1998b). The mechanism of acyl migration has been studied (Fischer, 1920; Serdarevich, 1968) under the acidic condition. For enzymatic interesterification (ester-ester exchange), the acyl donors could be free fatty acids, which relate to the water content in the reaction system. Reaction temperature and reaction time are crucial for the control of acyl migration for enzymatic interesterification.

The suggested pathway of acyl migration is shown in Fig. 11.2. Acyl migration is initiated by the nucleophilic attack of a lone pair of electrons of the free hydroxyl oxygen on the ester carbonyl carbon (*sn*-2 position), resulting in a five-membered ring intermediate called an orthoester. The ring is unstable and opened by the cleavage of the original ester carbon single bond resulting in the formation of the 1,3-DAG. The initial driving force for the acyl migration is the efficiency of the nucleophilic attack and the formation of the orthoester intermediate. The primary hydroxyl oxygen is a better nucleophile than the secondary hydroxyl and, therefore, the acyl shift from the secondary hydroxyl to the primary is favoured (Kodali *et al.*, 1990). An equilibrium ratio between *sn*-1,3-DAG and *sn*-1,2(2,3)-DAG was reported to be between 3/2 and 2/1 (Jensen and Pitas, 1976; Zhang *et al.*, 2000).

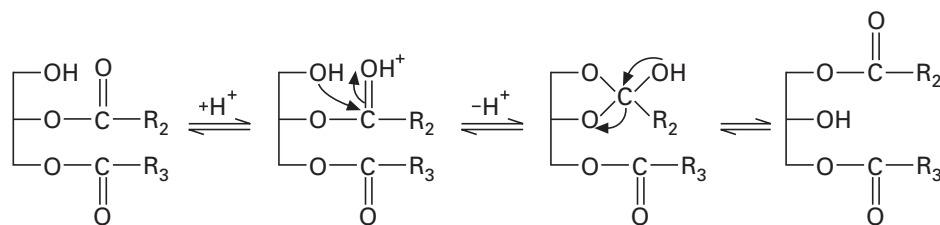


Fig. 11.2 Mechanism of acid-catalyzed acyl-migration (adapted from Serdarevich, 1968).

Bioreactors for enzymatic TAG–TAG interchange

Enzymatic interesterification can be carried out in a batch or a packed-bed reactor (PBR). Most reactions in the laboratory are carried out in a batch reactor. It requires less enzyme dosage and it is easy to apply vacuum or add molecule sieves to reduce the water content in the system. However, it also has some drawbacks. Longer reaction times are normally required due to the low enzyme dosage. It also gives rise to a high degree of acyl migration (Xu *et al.*, 1998c), which leads to non-specificity of the interesterified products. In a PBR, the ratio between enzyme and substrate is much higher and the reaction time much shorter than in a batch reactor. Therefore, the specificity of the enzyme can be better maintained since less acyl migration can occur. At the same time, it is suitable for continuous operation on an industrial scale.

For a batch reactor (Levenspiel, 1999), the relationship between enzyme load, flow rate, reaction rate and conversion degree is

$$\frac{w_b}{V_b}t = \int_0^{X_a} \frac{dX_a}{-r_a} \quad [11.9]$$

where w_b is the enzyme dosage, V_b is the amount of oil and t is the reaction time, X_a is the substance conversion degree and r_a is the reaction rate. For a PBR (Levenspiel, 1999), a similar relationship exists, which can be expressed as:

$$\frac{w_p}{V_p} = \int_0^{X_a} \frac{dX_a}{-r_a} \quad [11.10]$$

where w_p is the amount of enzyme in the PBR, F_p is the flow rate through the PBR.

Assuming the reaction rate and conversion degree are the same because the same enzyme is used, the right sides of Eqs 11.9 and 11.10 are the same. Therefore, the following equation is obtained:

$$\frac{w_p}{F_p} = \frac{w_b}{V_b}t \quad [11.11]$$

If no deactivation occurs in the PBR, Eq. 11.11 can be written as:

$$F_p = w_p \frac{V_b}{w_b \cdot t} \quad [11.12]$$

On the basis of experimental data in the batch reaction, Eq. 11.12 can be used to calculate the amount of enzyme and the flow rate in a PBR. Interesterified products with a desired conversion degree can be easily obtained by adjusting the flow rate for the PBR.

Monitoring of enzymatic TAG–TAG interchange process

During interesterification, the changes of chemical (*sn*-2 position, TAG composition) and physical properties [dropping point (DP), solid fat content

(SFC), differential scanning calorimeter (DSC) etc.] can be used to monitor the reaction. For industrial applications, SFC is mostly used for control of feedstock quality for plastic fat production. Therefore, SFC is chosen to monitor the interesterification process. For a batch reactor (Levenspiel, 1999), the mass balance for the reaction is shown as:

$$\text{input} = \text{output} + \text{accumulated} + \text{disappearance} \quad [11.13]$$

where input and output are zero. Therefore, based on the variation in SFC during the reaction, Eq. 11.13 can be written as:

$$0 = 0 + V \cdot dSFC - W \cdot r(SFC) \cdot dt \quad [11.14]$$

where r is the reaction rate, V is the volume of reactor, W is the amount of lipase and t is the reaction time. Eq. 11.14 then becomes:

$$\frac{dSFC}{dt} = \frac{W}{V} \cdot r(SFC) \quad [11.15]$$

Introducing a weight based reaction time, $\tau = (W/V) t$, gives the following equation:

$$d\tau = \frac{W}{V} \cdot dt \quad [11.16]$$

combining Eqs 11.15 and 11.16, gives the following relationship:

$$\frac{dSFC}{d\tau} = r(SFC) \quad [11.17]$$

Enzymatically catalyzed interesterification is assumed to be a first-order reversible reaction (Rozendaal and Macrae, 1997). Thus, Eq. 11.17 can be written as:

$$r(SFC) = -k \cdot SFC_r \quad [11.18]$$

where SFC_r is the reduced SFC content at reaction time τ and k is a constant. It is equal to:

$$SFC_r = SFC - SFC_\infty \quad [11.19]$$

where SFC_∞ is the SFC value when reaction reaches equilibrium. Combining Eqs 11.17–19 gives the following relationship:

$$\int_{SFC}^{SFC_\infty} \frac{dSFC}{SFC - SFC_\infty} = - \int_0^\tau k \cdot d\tau \quad [11.20]$$

After integration, Eq. 11.20 becomes:

$$\frac{SFC - SFC_\infty}{SFC_0 - SFC_\infty} = e^{-k\tau} \quad [11.21]$$

where SFC is the SFC value of the product at time τ and SFC_0 is the initial SFC value. ΔSFC is introduced to represent the maximum changes in SFC as follows:

$$\Delta SFC = SFC_0 - SFC_\infty \quad [11.22]$$

Therefore SFC can be written as:

$$SFC = SFC_0 - \Delta SFC(1 - e^{-k\tau}) \quad [11.23]$$

This model (Eq. 11.23) has three parameters which have physical and chemical meanings. The k value is related to the reaction rate of the enzyme on the given blend. SFC_0 and ΔSFC are related only to the types of blends and the blend ratios. The weight base reaction time (τ), adjusted for the volume of oil samples removed from the batch reactor, is expressed as follows:

$$\tau_n = t_{n-1} \cdot \frac{w_e}{V_{\text{Toil}}} \Big|_n + (t_n - t_{n-1}) \cdot \frac{w_e}{V_{\text{Toil}} - \sum_{i=1}^{n-1} S_i} \quad [11.24]$$

where t is the reaction time (min), n is the sampling times, w_e is the enzyme dosage in grams, V_{Toil} is the initial blend weight and S_i is the amount of sample withdrawn in grams at a given sampling time. The advantage of using weight-based reaction time is the ease with which these data can be applied in different reaction systems or enzyme dosages to obtain the same products. If the sampling amount is sufficiently small compared to the total volume during the reaction process, this means that $\tau = t$. Then the equation can be simplified:

$$SFC = SFC_0 - \Delta SFC (1 - e^{-kt}) \quad [11.25]$$

The model has been evaluated (Zhang *et al.*, 2004a) using different oil blends and different analytical methods (Fig. 11.3) for monitoring the enzymatic interesterification process.

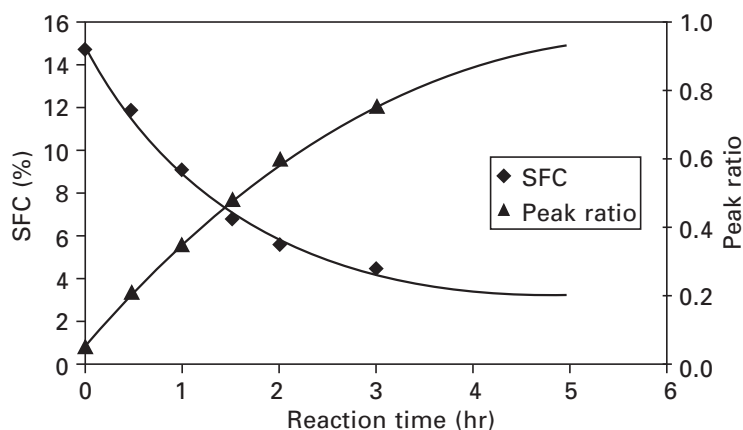


Fig. 11.3 Model fitting of experimental data (different signs) with exponential model (solid line) for an enzymatical interesterification process. (Reaction conditions: 70/30 blend of palm stearin and coconut oil; 4 wt % Lipozyme TL IM; 70 °C; three volumes of rapeseed oil were interesterified for 30 minutes at 70 °C to reduce the water content of Lipozyme TL IM, then Lipozyme TL IM was quickly washed by the blend. Finally, the blend was poured into the reactor and interesterified for three hours.) The peak ratio is the peak area between equivalent carbon number (ECN)44 and ECN48 analyzed by high-performance liquid chromatography (HPLC).

During enzymatic interesterification, degree of conversion can be controlled by different reaction times. The conversion degree (X) is defined as follows (left side); it can also be derived from Eq. 11.21 and redefined by the relationship between reaction time t and reaction rate k (right side):

$$X(\%) = \frac{SFC_0 - SFC}{SFC_0 - SFC_\infty} \times 100 = (1 - e^{-kt}) * 100 \quad [11.26]$$

where SFC_0 is the SFC at time 0, SFC is the SFC at reaction time t and SFC_∞ is the SFC at the equilibrium stage.

Recent progress for enzymatic TAG–TAG interchange

Current research work shows that Lipozyme TL IM-catalyzed interesterification presents no problem for the production of plastic fats. In general, the technology can easily be moved to the industrial sector for commercial exploitation. Both stirred tank reactors (Zhang *et al.*, 2001) and PBR (Rønne *et al.*, 2005) can be used for the production of plastic fats, and the system is generally straightforward. The control of water activity in the system presents no particular difficulty, as is often the case in other lipase-application systems, in which the lipase activity was not affected by the reduction of water content in the system (Zhang *et al.*, 2001). The process indeed shows tremendous advantage for plastic fat production compared to the chemical process with its additional washing and bleaching procedure or other lipase processes in which water content has to be adjusted during reuse (Zhang *et al.*, 2000). The exponential model (Eq. 11.23) is very useful for monitoring the enzymatic interesterification process. It has the potential to be implemented in routine industrial operations for practical control purposes.

The degree of conversion of enzymatic interesterification has significant effects on triacylglycerol composition (Zhang *et al.*, 2001, 2004a; Rønne *et al.*, 2005), crystallization behaviour (Zhang *et al.*, 2004b) and storage stability of the products (Zhang *et al.*, 2005, 2006). Based on the enzymatically-interesterified blend of palm stearin and coconut oil (70/30, w/w), it was observed that the increased conversion reduced the crystal size and, consequently, the crystal surface was changed from a coarse and condensed leaf-like crystal (β crystal) to a fine needle ball-like crystal (β' crystal, confirmed by X-ray analysis) (Fig. 11.4). At the same time, margarines made from enzymatically-interesterified fats became more physically stable with the increase in conversion degree. During a 12 week storage stability study at both 5 and 25 °C, the margarine made from enzymatically fully converted fat had the same physical properties as the margarine made from chemically interesterified fat. The margarines produced from enzymatically interesterified fats generally had much lower peroxide values compared to the margarine produced from chemically randomized fats stored at 25 °C (Zhang *et al.*, 2006). A similar observation was also apparent in a pure oil system (Tautorius and McCurdy, 1990). This confirms that enzymatic interesterification has advantages for the oxidative stability of the products.

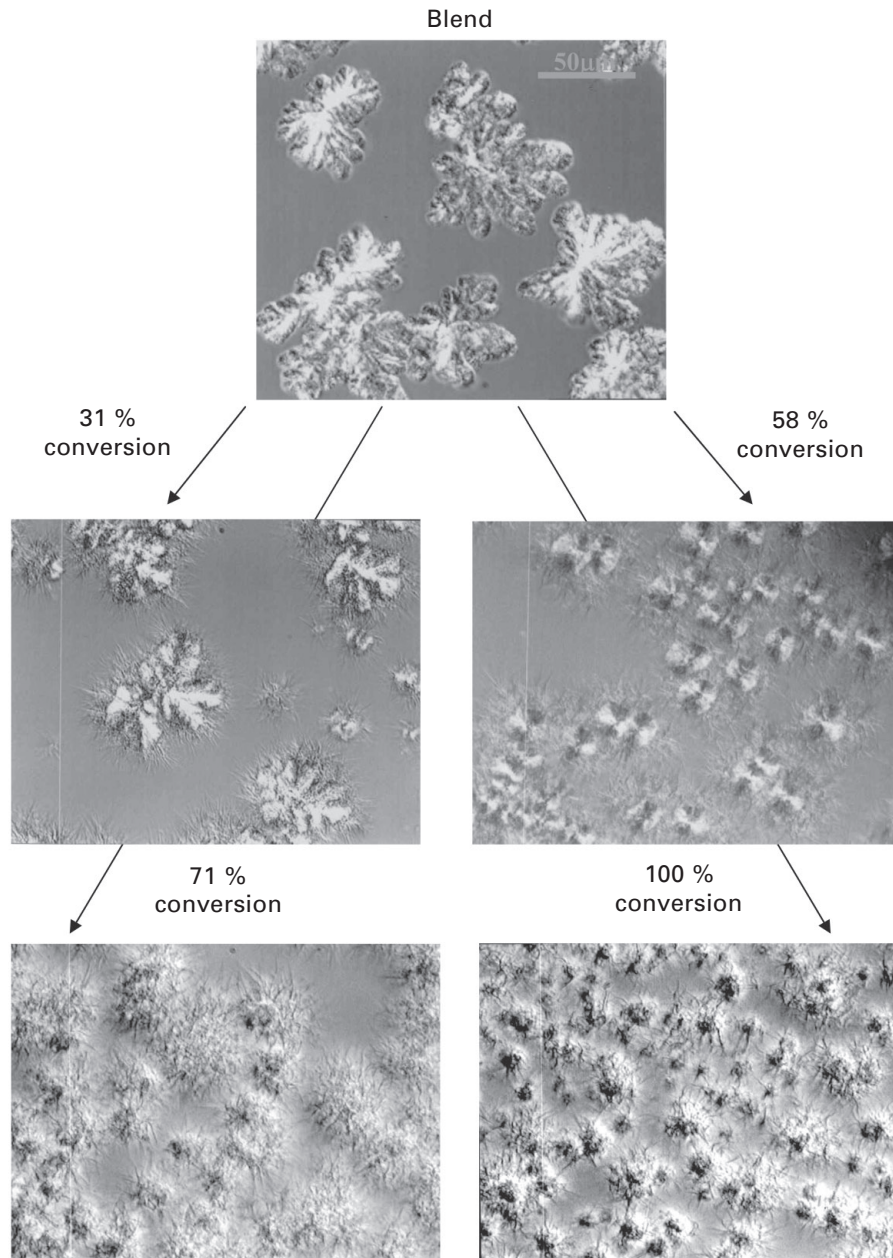


Fig. 11.4 Morphology of the blend (palm stearin/coconut oil, 70/30) and enzymatically interesterified products at different degrees of conversion (measured by HPLC). (Samples with 50 % added rapeseed oil were melted at 70 °C, and one drop was put on a slide. They were then cooled to room temperature. The morphology was observed after one hour at room temperature by polar light microscopy) (adapted from Zhang *et al.*, 2004b).

11.3.2 Enzymatic acidolysis for the production of structured lipids

Structured lipids (SL), in this text, means fats that are modified or restructured from natural oils and fats, or the fatty acids therefrom, with regio-positional preference of each fatty acid or each group of fatty acids in the glycerol backbone. Structured lipids are designed for a particular functionality or nutritional property for edible or pharmaceutical purposes. This definition

covers fats, particularly those produced by enzymatic methods using *sn*-1,3-specific lipases, including cocoa butter equivalents (SOS; S = saturated fatty acids, O = oleic acid), anti-bloom agent (BOB; B = behenic acid), breast milk fat substitutes (UPU; U = unsaturated fatty acids, P = palmitic acid), MLM-type oils containing long-chain (L, usually polyunsaturated or essential) and medium-chain (M) fatty acids. The chain length of fatty acids is normally defined as long-chain (more than 12), medium-chain (8–12) and short-chain (less than 8). The nutritional, physical or chemical properties of SL have been widely discussed in countless publications (Akoh, 1998; Mu and Porsgaard, 2005).

Acidolysis using enzyme technology has been more widely explored for the production of SL than other products. Chemical acidolysis has fewer practical applications due to its having no positional specificity. The reaction is between a triacylglycerol and a free fatty acid (FFA) with an *sn*-1,3-specific lipase. The acid will be exchanged with the acid in the ester. In fact, the production of SL can only be carried out by enzymatic interesterification, especially in large quantities. The reaction is normally conducted between a substrate oil and acyl donors (fatty acids or their ethyl esters) with *sn*-1,3-specific lipases as the biocatalysts, as demonstrated in industry for commercially available SL products (Quinlan and Moore, 1993).

Our research group has been studying structured lipids since 1996. A national framework program has been run for about seven years with the name LipoTech (www.biocentrum.dtu.dk/lipotech/). Process technology was widely studied in the early stages. Most of the progress made has been reviewed in a few early papers or chapters (Xu, 2000a,b,c; Høy and Xu, 2001). Concerning the enzymatic production of structured lipids in general, a few recent review publications from different groups have further addressed the issue (Kim and Yoon, 2003; Xu, 2003, 2004; Iwasaki and Yamane, 2004).

Acidolysis reaction with sn-1,3-specific lipases for production of structured lipids

Enzymatic acidolysis reaction is commonly considered as a two-step reaction: hydrolysis and esterification with diacylglycerols as reaction intermediates (Xu *et al.*, 1999b). Through these two steps, the new fatty acids are incorporated into triacylglycerols, and the reaction will finally reach equilibrium. This procedure for the reaction between a TAG (LLL) and a fatty acid (M) is depicted in Fig. 11.5.

Enzymatic acidolysis is a reversible reaction, and equilibrium will eventually be reached. The product yield under reaction equilibrium is decided by the ratio between fatty acids and esters (Xu *et al.*, 1998a). For the reaction between TAG and FFA, the incorporated fatty acids in the product can be calculated (Fig. 11.6). The higher the FFA to TAG substrate ratio, the higher the maximum incorporation of the FFA to TAG which can be expected. However, the time to reach equilibrium is related to many parameters, such as reaction temperature, enzyme dosage, water content and reaction system.

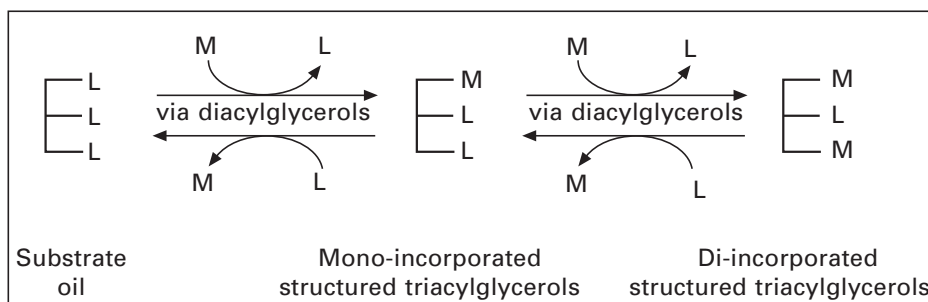


Fig. 11.5 Reaction principle of the lipase-catalyzed interesterification between LLL and M and the dynamic balance between LLL, *sn*-MLL/LLM and *sn*-MLM. L = long-chain fatty acid, M = medium-chain fatty acid.

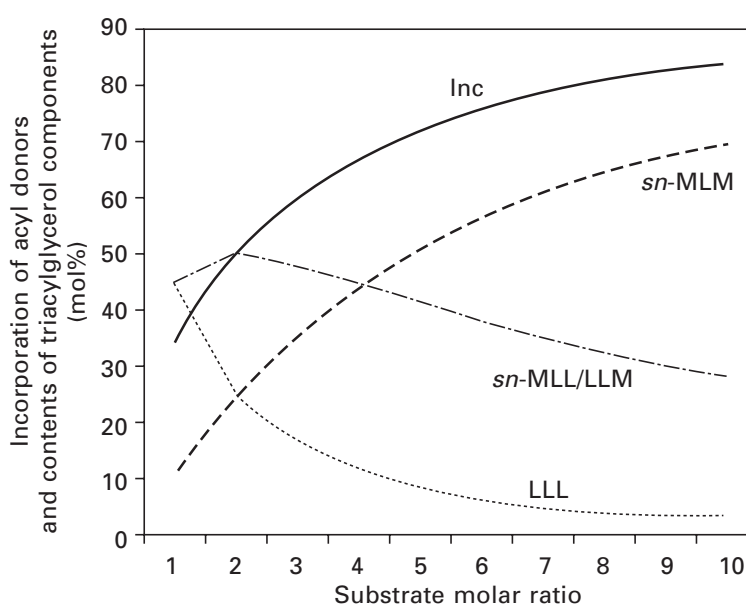


Fig. 11.6 The relationship between the substrate molar ratios (M/LLL) and the contents of substrate oil left (LLL), mono-incorporated SL (*sn*-MLL/LLM) and di-incorporated SL (*sn*-MLM), and the incorporation of the acyl donor (Inc) in the *sn*-1,3 regiospecific lipase-catalyzed interesterification (L = long-chain fatty acid, M = medium-chain fatty acid, SL = structured lipids).

In particular, when substrate molar ratios increase, the effect of substrate inhibition on the reaction activity will also increase and lead to prolongation of the equilibrating time of the reaction. This often reduces the productivity of the process. It is very important to choose a suitable substrate molar ratio in terms of reaction efficiency (incorporation level of acyl donors per unit time) and productivity (product quantity per unit time) with a specific reaction system. The choice of substrate molar ratio is also related to the downstream processing cost and associated difficulties of separating free fatty acids or acyl donors by evaporation and/or distillation. A high substrate molar ratio may reduce the number of reaction stages needed to obtain a suitable product, but the purification of the product may also be more difficult.

Depending on the products required, careful consideration should be given to the reaction system before embarking on the process design. There are many natural or synthetic oils and fats available for selection as substrate oils. In addition to the requirement of specific fatty acids at a specified position (usually at *sn*-2 position), purity and other characteristics should also be considered. Normally, free fatty acids are chosen because of their ready availability in large quantities, low price and high reactivity compared to the EE. Lipases differ in selectivity for various fatty acids (Halling, 1987). A screening of different lipases is necessary to find one appropriate for the specific reaction system. Commercially available lipases have been extensively documented in laboratory experiments (Xu, 2000c). A careful survey is necessary for the synthesis and production of the required products.

Recent progress in enzymatic production of structured lipids

Positional distribution in SL products is of particular concern, being one of the aspects in which enzyme process is important. However, during the reaction acyl migration occurs leading to non-specificity of the products. This issue has been widely discussed with respect to the effects of parameters and reactor designs (Xu *et al.*, 1998a,b,c). The mechanism of acyl migration has been illustrated in Fig. 11.1. The possible acyl migration steps during the enzyme acidolysis are illustrated in Fig. 11.7. In one recent study we have evaluated the possibility of using temperature programming for the suppression of acyl migration in batch reactions (Yang *et al.*, 2005a). Acyl migration is a thermodynamic process and one which is very difficult to fully stop in actual reactions. It seems likely that suppressing acyl migration by a programmed change of reaction temperature without loss of reaction yield is feasible. The model reactions were the acidolysis of tripalmitin (PPP) with conjugated linoleic acid (CLA) or with caprylic acid (CA, 8:0) intended for human milk fat substitutes. Acyl migration was considerably inhibited in the temperature-programmed acidolysis with only slight reduction of acyl incorporation. Temperature programming was more significant in solvent-

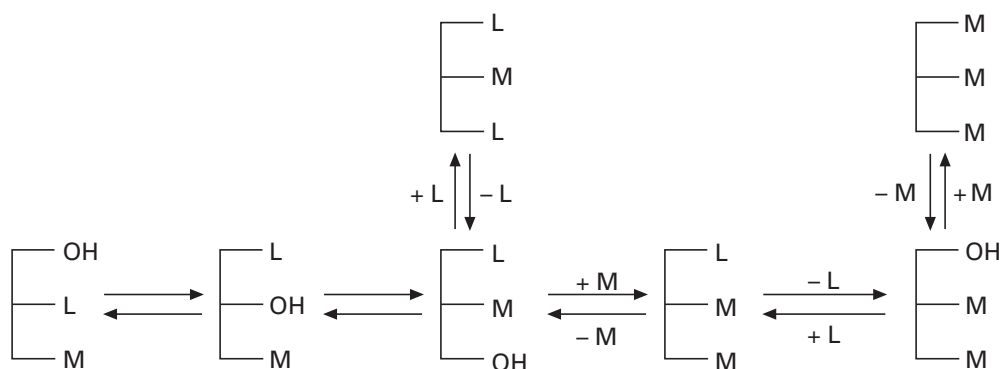


Fig. 11.7 Schematic diagram of acyl migration and formation mechanism of non-specific by-products during enzymatic production of specific structured lipids (L = long-chain fatty acids, M = medium-chain fatty acids).

free systems for the reduction of acyl migration. The study suggests that it is feasible to reduce acyl migration by means of programmed change of acidolysis temperature without significant loss of reaction yield.

Fats for infant formula are one group of typical SL, where the second position is mostly occupied by palmitic acid. In two recent studies (Yang *et al.*, 2003b; Nielsen *et al.*, 2006) concerning the production of such fats, the source of the materials has been taken into consideration in a comparison with current commercial products, Betapol™ (Loders-Croklaan, Netherlands). Taking into account the high quantity of palmitic acid in lard (much of it already present in the *sn*-2 position), this fat has been evaluated for the production of infant fat using enzyme technology. Lard and soybean fatty acids were subject to acidolysis in a solvent-free system in a batch reaction (Yang *et al.*, 2003b). The reaction substrates for the production were specially chosen to mimic human milk fats. The characteristics of the product, produced in the scale-up acidolysis under selected conditions, were similar to the fat in mothers' milk. In another study, reactions were conducted in a PBR (Nielsen *et al.*, 2006). Residence time and production operational stability were investigated on the kg-scale with lard and soybean oil fatty acids as substrates. There was no significant effect of residence time and operation time on 18:2 and 18:3 incorporation or on acyl migration in the *sn*-2 position.

Adding antioxidants to the enzyme processes has been evaluated for the production of SL (Xu *et al.*, 2005) since, for various reasons, the products from enzymatic processes are generally less stable (Jacobsen *et al.*, 2003; Timm-Heinrich *et al.*, 2003a,b, 2004; Nielsen *et al.*, 2004). In the production process for structured lipids, the influence of addition of antioxidants before enzymatic acidolysis was investigated. Eight different antioxidants were screened: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) ascorbyl palmitate, citric acid, ethylene diamine tetraacetic acid (EDTA), a tocopherol blend and lecithin. As substrates, oils with different degree of unsaturation were used. Enzyme activity was not significantly influenced by the addition of antioxidants in either a batch process or a PBR operation. α -Tocopherol concentrations remained stable for those mixtures where tocopherols were added. Primary oxidation products were reduced after acidolysis in the PBR, possibly due to the adsorption in the enzyme bed. The study by Xu *et al.* (2005) shows that the addition of antioxidants before enzymatic reactions has no negative effects on the reaction process, but none of the antioxidants chosen had a significant positive effect on either the reaction process or the oxidative status of the structured lipid produced.

11.3.3 Enzymatic acidolysis for the modification of phospholipids

Phospholipids (PL) are used for different applications in food, pharmaceutical and cosmetic products, where they function as emulsifiers, stabilizers and antioxidants. Most applications of PL are based on natural products having

complex composition, such as lecithin, which is a by-product of vegetable oil refining. Chemical and enzymatic modifications of PL to alter emulsifying and dispersing properties are common practice in industry, as the range of application of PL is widely extended (Schneider, 1997). The aim of these modifications is to adapt PL for specific application requirements by providing technical or physiological properties that the natural substance does not possess. Chemical modification includes hydrolysis, hydroxylation, acylation and hydrogenation (Bueschelberger, 2004). Commercial use of enzymes for PL modification is significant only for partial hydrolysis to produce lyso-PL with improved emulsifying properties compared to natural PL (Schneider, 1997). However, since the 1980s interest in enzymatic acyl modification has increased continuously as enzymes are able to tailor PL with defined fatty acid composition at the *sn*-1 and *sn*-2 positions. Replacement of the existing fatty acids in original PL with desired fatty acids might also improve physical and chemical properties, or even nutritional, pharmaceutical and medical functions. Enzymatic synthesis of PL has some advantages compared to chemical synthesis, such as selectivity and specificity. Furthermore, the use of toxic and deleterious solvents is greatly reduced, which simplifies subsequent purification and reduces solvent residue in the final product.

For those interested in this area, published data concerning the use of lipases and PLA₂ for PL acyl modification with acidolysis reactions have recently been reviewed by Adlercreutz *et al.* (2003) and Guo *et al.* (2005).

Enzymes for acidolysis of phospholipids

Enzymes that utilize PL as their natural substrate are called phospholipases and are named according to hydrolytic action on the PL molecule. The general formula for glycerophospholipids, together with the enzymes which hydrolyze the different ester bonds in the molecules, is shown in Fig. 11.8. In principle, these enzymes can also catalyze the reverse reaction, i.e. the ester bond formation. Phospholipase A₁ and A₂ are acyl hydrolases acting on fatty acids at *sn*-1 and *sn*-2 positions, respectively. Phospholipase C and D are enzymes that cleave the phosphorus–oxygen bond between glycerol and

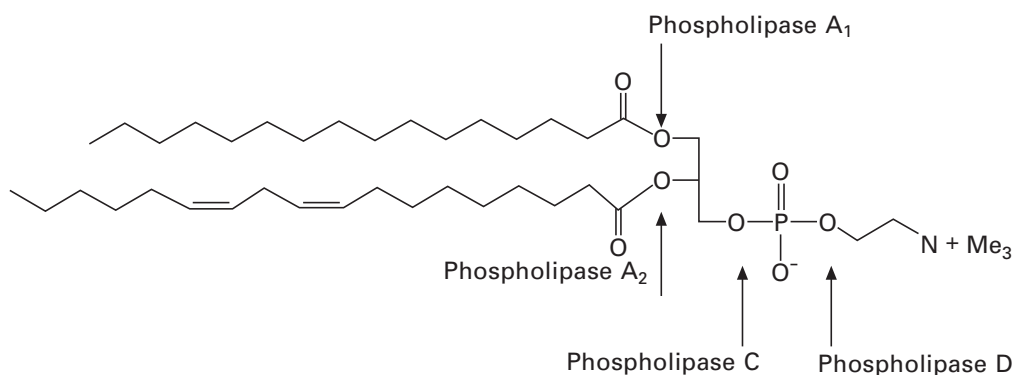


Fig. 11.8 1,2-Diacyl-*sn*-glycerophosphorylcholine molecule and phospholipases which can be used to hydrolyze or form different ester bonds.

phosphate, or phosphate and head group, respectively. Head group exchange of PLs will also change the chemical and physical properties; however, it is beyond the scope of this text to describe all reactions and products possible for these enzymes. The focus will be on the enzymatic acyl modification.

Besides phospholipases, there are many other enzymes that can be used for PL acyl modification, such as *sn*-1, 3-specific lipases, which work for the modification of acyl group in the *sn*-1 position of PL. Lipases are at a more mature stage of development than phospholipases in terms of theoretical and practical understanding, and have in practice been the most common enzymes used for PL acyl modification at the *sn*-1 position. Lipases are able to catalyze both esterification and interesterification reactions. Phospholipase A₂ (PLA₂) from porcine pancreas has been the most commonly used enzyme for the exchange of fatty acids on PL at the *sn*-2 position. Pancreatic PLA₂ functions reasonably well for esterification, but cannot catalyze interesterification since no acyl-enzyme intermediate is formed (Adlercreutz *et al.*, 2003). However, it is possible to use this enzyme to exchange the fatty acid in the *sn*-2 position in a one-step reaction. Hydrolysis and re-esterification then occur in parallel. For industrial applications, enzymes are preferred in an immobilized form because it is possible to reuse them. Only lipases can currently be obtained commercially in immobilized form for PL modification; phospholipases in immobilized forms are not yet available.

The majority of lipases sold commercially come from different microbial sources. Screening of different lipases showed that *Thermomyces lanuginosa* lipase (TLL) had higher activity compared to *Rhizomucor miehei* lipase (RML) and *Candida antarctica* lipases (CAL) during lipase-catalyzed acidolysis between soybean lecithin and caprylic acid (Peng *et al.*, 2002). CAL has in certain cases been claimed to be non-specific; however, in a recent study it has been shown that during PL modification it is specific for the *sn*-1 position (Lyberg *et al.*, 2005). Although lipases show good performance, the stability of the enzyme carrier is important. Commercial silica granulated TLL can easily be removed from the reaction medium in the presence of solvent during batch operation; however, in solvent-free systems, the immobilized lipase is not easily removed. During reaction, silica granulates are crushed and do not easily precipitate in the solvent-free system due to the highly viscous reaction medium. RML on resin carrier is more mechanically stable and can more easily be removed after solvent-free acidolysis reaction. However, during continuous operation in the PBR, the stability of silica granulated TLL can be maintained. Lipase from *Rhizopus oryzae* has also been reported to work well for PL modification at the *sn*-1 position (Adlercreutz *et al.*, 2002); however, this lipase cannot be obtained commercially in the immobilized form.

Reaction elucidation for enzymatic acidolysis of phospholipids

Product yield under reaction equilibrium during acidolysis of PLs is determined by the substrate ratio. Maximum incorporation of acyl donors to a phospholipid

($Inc_{\max}$) can be calculated at certain substrate ratios assuming no by-product formation. An equation is given below:

$$Inc_{\max} (\text{mol}\%) = \frac{50 \cdot S_r}{S_r + 1} \quad [11.27]$$

where S_r is the molar ratio between fatty acids and PL. Theoretical maximum of new fatty acids to be incorporated into PC is expected to reach 50 % for an *sn*-1,3-specific lipase or a PLA_2 . In practice, lipases are not equally specific towards different fatty acids and side reactions are difficult to avoid. A diagram of reaction and principle of the lipase-catalyzed interesterification and side reactions for the production of specific structured PL is given in Fig. 11.9. Lipase-catalyzed acidolysis of PLs is considered to be a two-step reaction involving hydrolysis and esterification. Lysophosphatidylcholine (LPC) produced in the first step is reactant in the second step. This intermediate is not thermodynamically stable, and intermolecular transfer of the fatty acid moiety from the *sn*-2 to the *sn*-1 position may occur. This non-enzymatic acyl migration is the reason for decreased yield and purity of the final product (Haraldsson and Thorarensen, 1999). LPC with fatty acids at the *sn*-1 position can be further hydrolyzed by the lipase to form glycerophosphorylcholine (GPC) which can be reacylated with novel fatty acids in the *sn*-1 position. If the acyl group migrates from the *sn*-1 position to the *sn*-2 position, the lipase has the potential to incorporate yet another new fatty acid into the *sn*-1 position, which would give rise to PC with novel fatty acid at both positions. Very little consideration has been given to the distribution of by-products formed during the reaction. The formation of GPC and LPC containing the novel fatty acids is a direct consequence of acyl migration and should be

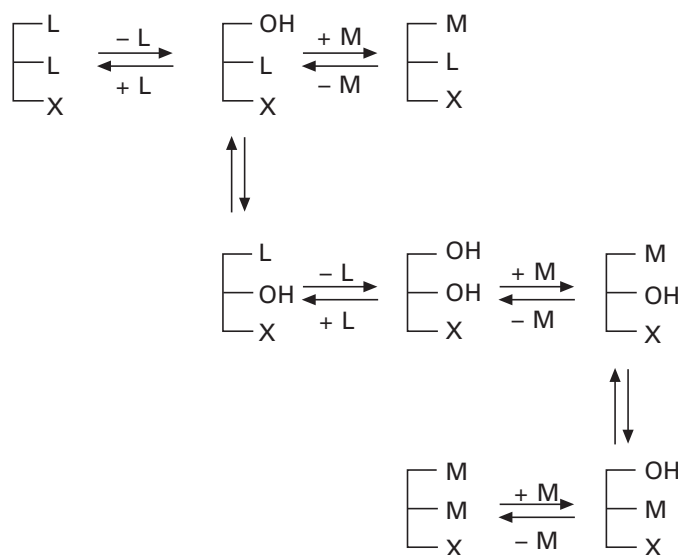


Fig. 11.9 Reaction process and mechanism for the production of specific structured phospholipids by enzymatic interesterification with *sn*-1,3 specific lipase (L = long-chain fatty acids, M = medium-chain fatty acids).

minimized in order to achieve a high product yield and high product purity. We have conducted reactions with different lipases in solvent and solvent-free systems. As is the case with the other enzymatic reactions mentioned in this chapter, the performance of enzymatic synthesis of structured PL depends on many factors involving type of reaction, enzyme, temperature, water content, composition of substrates, reaction time, mode of operation, etc. Incorporation and final yield are highly affected by the selected parameters.

Process technology for enzymatic acidolysis of phospholipids

The type of the acyl donors plays an important role in the reaction rate of lipase-catalyzed reactions. High incorporation can usually be obtained with saturated fatty acids; however, fatty acids with a high degree of unsaturation are usually more difficult to incorporate (Peng *et al.*, 2002; Hossen and Hernandez, 2005; Reddy *et al.*, 2005). Different lipases show discrimination of long-chain PUFAs. For example, RML, TLL and CAL give higher incorporation of EPA compared to DHA (Peng *et al.*, 2002). Reactivity also depends on the phospholipid head groups. During interesterification with EPA in hexane with RML the following order of reactivity was observed: PC > PI > PE > PS (PC is phosphatidylcholine, PI phosphatidylinositol, PE phosphatidylethanolamine, PS is phosphatidylserine) (Mutua and Akoh, 1993). The nature of PL also affects the incorporation rates of caprylic acid by TLL in hexane in the following order PC > PE > PA > PI (Peng *et al.*, 2002). PL can be obtained commercially at different purities. PC is the most abundant phospholipid in nature and has usually been the substrate selected for enzymatic modification. Incorporation of novel fatty acids has been made with both pure phospholipid compounds and de-oiled lecithin (Peng *et al.*, 2002; Vikbjerg *et al.*, 2005a). Purified compounds have considerably higher price compared to the de-oiled lecithin. Selection will depend on the purity requirements.

In most cases, lipase-catalyzed acidolysis reactions have been conducted with the assistance of organic solvents such as hexane or toluene (Mutua and Akoh, 1993; Adlercreutz *et al.*, 2002; Hossen and Hernandez, 2005). The use of solvents increases the capital investment when the process is scaled up. Furthermore, it has been reported that increasing the amount of solvent reduces the recovery of PL more strongly than it increases the incorporation during TLL-catalyzed acidolysis (Vikbjerg *et al.*, 2005a). If possible, it is recommended that the reaction should be conducted under solvent-free conditions. A clear elucidation of side reactions is important for practical operation in order to minimize by-products during reactions.

Recently we produced caprylic acid-containing PC in a batch reactor by RML-catalyzed acidolysis between PC and caprylic acid in a solvent-free system (Vikbjerg *et al.*, 2005b). A typical time course of the acidolysis reaction can be seen in Fig. 11.10. By-products were formed due to parallel hydrolysis reactions and acyl migration in the reaction system. Usually there was a tendency for a decrease in yields along with an increase in acyl incorporation. Response surface design was used to evaluate the influence of

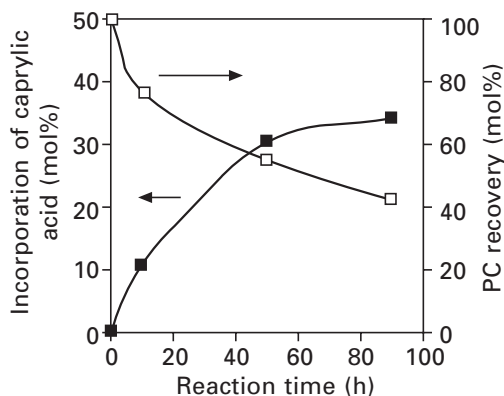


Fig. 11.10 Time course for immobilized *Rhizomucor miehei* lipase-catalyzed acidolysis reaction between phosphatidylcholine (PC) and caprylic acid. Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PC; enzyme dosage, 30 % (wt. % based on substrate); reaction temperature, 50 °C; water addition, 2 % (wt. % based on substrate).

major factors (enzyme dosage, reaction time, reaction temperature, substrate ratio and water addition) and their relationships on a number of responses reflecting the turnover of main reactions as well as side reactions. Several parameters important for the main reaction also affect by-product formation resulting in lower recoveries. All parameters besides water addition had an effect on the incorporation of caprylic acid into PC and LPC. Increased reaction time and enzyme dosage showed increased effect on incorporation into PC, while increased substrate ratio and reaction temperature showed opposite effects. The PC content decreased with increase of all parameters except for substrate ratio. In the solvent system using immobilized TLL, the incorporation of the desired acid was seen to increase with increase in temperature and substrate ratio (Vikbjerg *et al.*, 2005a). Clearly optimization must be individually performed in each case.

The exchange of fatty acids in PL has mainly been performed batch-wise in small screw cap vessels or glass bottles with either orbital or magnetic stirring. For larger scale production, it would be more convenient to operate in PBR as this allows continuous operation. Furthermore, due to the high enzyme dosages usually applied for lipase-catalyzed PL modification (> 40 % based on substrate), the final product is not easily removed from the reaction mixture after batch operation. To obtain the same conversion degree with the same enzyme load in the PBR, a very long residence time is required. Trials have been made for the continuous operation of the acidolysis reaction (Vikbjerg *et al.*, 2005c). If no water was added to the substrate during reaction in the solvent-free system, very low incorporation of novel fatty acids was observed. Operative stability was tested for several days. Incorporation was highest at the beginning and decreased in the first 30 hours, where it stabilized afterwards. Incorporation of novel fatty acids was slightly higher when the water content was increased. In the presence of hexane, incorporations into PL were considerably higher and increased continuously in the first two

days, and thereafter were stable with only a slight decline in incorporation over several days. Using PBR with solvent-free systems, it seems that small amounts of water are beneficial for the incorporation of desired fatty acids. Due to the high PL concentration, the substrates probably remove bound water from the enzymes, thus reducing the catalytic activity. Increasing water content will at the same time decrease the yield. Therefore, some kind of compromise needs to be made.

11.3.4 Glycerolysis for partial glyceride production

Partial glycerides, more commonly known as mono- and diglycerides, have been produced commercially for many years. Today, they are widely used in the food, cosmetic and pharmaceutical industries as well as in the textile, fibre and plastic industries (Bornscheuer, 1995; Coteron *et al.*, 1998; Elfman-Borjesson and Harrod, 1999; Bellot *et al.*, 2001; Ferreira-Dias *et al.*, 2001; Kaewthong and H-Kittikun, 2004). Mono- and diacylglycerols of edible fatty acids are approved by the EU as food grade additives. They have been given 'Generally Recognized As Safe' (GRAS) status by the US Food and Drug Administration (FDA), and can be used *quantis satis* (no permitted maximum level is specified) according to the European Parliament and Council Directive (European Parliament Council, 2004). According to World Health Organization (WHO) and the EU directive, mono- and diglycerides of fatty acids are required to contain at least 70 wt % MAG + DAG (mono- + diacylglycerols), at least 30 wt % MAG and maximum 7 wt % glycerol (European Food Emulsifier Manufacturers' Association, 2004; European Parliament Council, 2004).

Pure monoacylglycerols have excellent emulsifying properties, superior to diacylglycerols and mixtures of partial glycerol esters (Bornscheuer, 1995; Peng *et al.*, 2000; Kaewthong and H-Kittikun, 2004). This is due to the MAG molecular structure with a favourable distribution between one hydrophobic fatty acid moiety esterified to a hydrophilic glycerol moiety. This combination, as well as the dietary safety profile, makes monoacylglycerols and mono/diacylglycerol mixtures very popular additives for facilitating uniform quality of food products among other applications (Krog, 1997). Accordingly, mixtures of mono- and diacylglycerols and distilled monoacylglycerols contribute to a market estimated at around 75 % of the worldwide emulsifier production. This corresponds to approximately 200 000–250 000 tonnes produced per year (Moonen and Bas, 2004).

The main applications of mono-diacylglycerols in foods are typically in fat-based products such as margarine, spreads, bakery products, cake mixtures, confectionery and the like (Krog, 1997; Kaewthong and H-Kittikun, 2004). Mono/diacylglycerols are often added to industrial food formulations in combination with other more hydrophilic emulsifiers, for instance in combination with hydrocolloids in dairy emulsions such as ice cream (Krog, 1997).

Recent studies on DAG nutritional properties and dietary effects suggest that oils with high DAG content play an important role in reducing levels of serum TAG (Flickinger and Matsou, 2003; Watanabe *et al.*, 2005). Thus, to fight obesity and other lifestyle-related diseases, substitution of TAG oils with DAG oils has attracted much attention recently. The first DAG cooking oil entered the Japanese market after its introduction in February 1999. With more than 70 million bottles sold (year 2003) and launched in the USA in 2005, the interest in a global DAG-market has indeed begun (Flickinger and Matsou, 2003; Kristensen *et al.*, 2005).

Chemical glycerolysis

Today, commercial mono-diacylglycerol mixtures are widely manufactured using a glycerolysis reaction between glycerol and fats or oils, as illustrated in Fig. 11.11. The currently used glycerolysis reaction is performed at high temperatures (220–260 °C) with inorganic alkaline catalysts, such as NaOH or $\text{Ca}(\text{OH})_2$. Approximately 30 to 60 minutes of processing leads to an equilibrium mixture with partial glycerol esters and excess glycerol. To remove impurities and achieve high-purity products, subsequent removal of excess glycerol by distillation followed by molecular/short path distillation (SPD) processing is often performed (Bornscheuer, 1995; Krog, 1997; Rosu *et al.*, 1997; Elfman-Borjesson and Harrod, 1999; Xu *et al.*, 2000, 2002; Xu, 2000b; Bellot *et al.*, 2001; Kaewthong and H-Kittikun, 2004; Lee *et al.*, 2004).

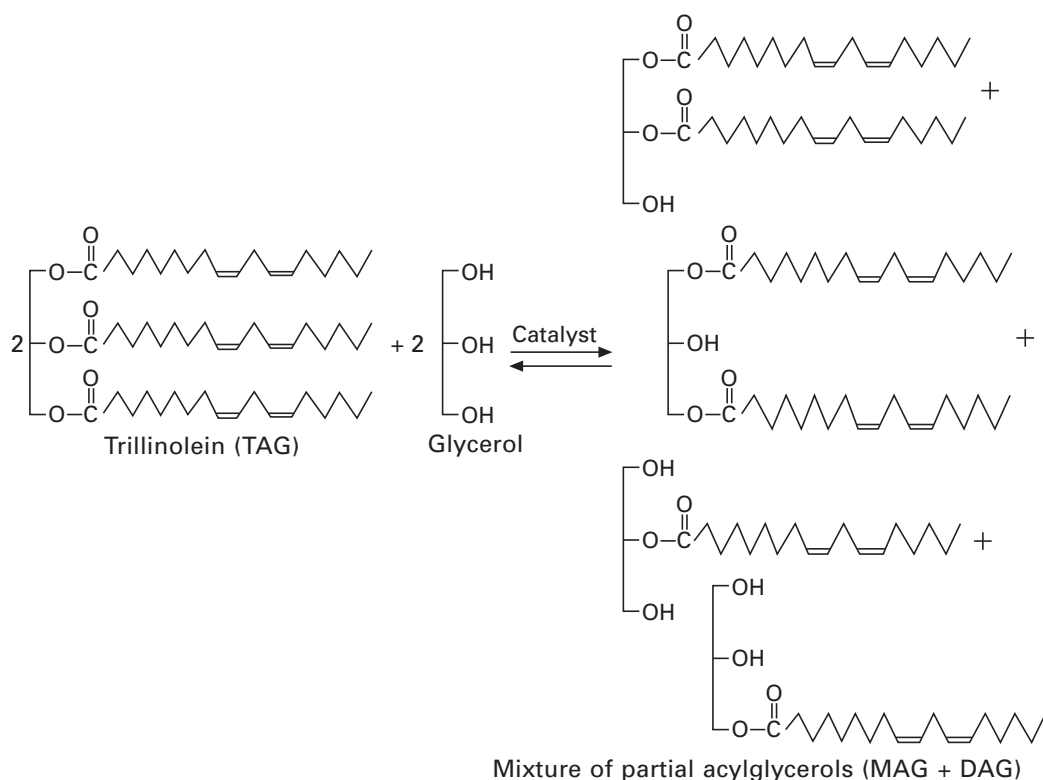


Fig. 11.11 Reaction scheme for production of mono-diacylglycerols by glycerolysis.

The reaction can, to a certain extent, favour the formation of certain glycerol esters depending on the reaction conditions such as glycerol to oil ratio, reaction time and pressure (Peng *et al.*, 2000). The effect on the product distribution of MAG, DAG, TAG and glycerol at equilibrium conditions after glycerolysis of different blended glycerol to oil ratios is illustrated in Fig. 11.12. Current chemical glycerolysis processing is usually conducted with a relatively low molar ratio of glycerol to oil of about two (Rendon *et al.*, 2001). This provides an amount of non-reacted glycerol of about 8 wt% (Fig. 11.12b) and a distribution between MAG, DAG and TAG of 45–55 %, 38–45 % and 8–12 %, respectively (Krog, 1997).

Enzymatic glycerolysis

Today, it is well known that the Western diet is characterized by a general low intake of essential n-3 polyunsaturated fatty acids (PUFAs) and an

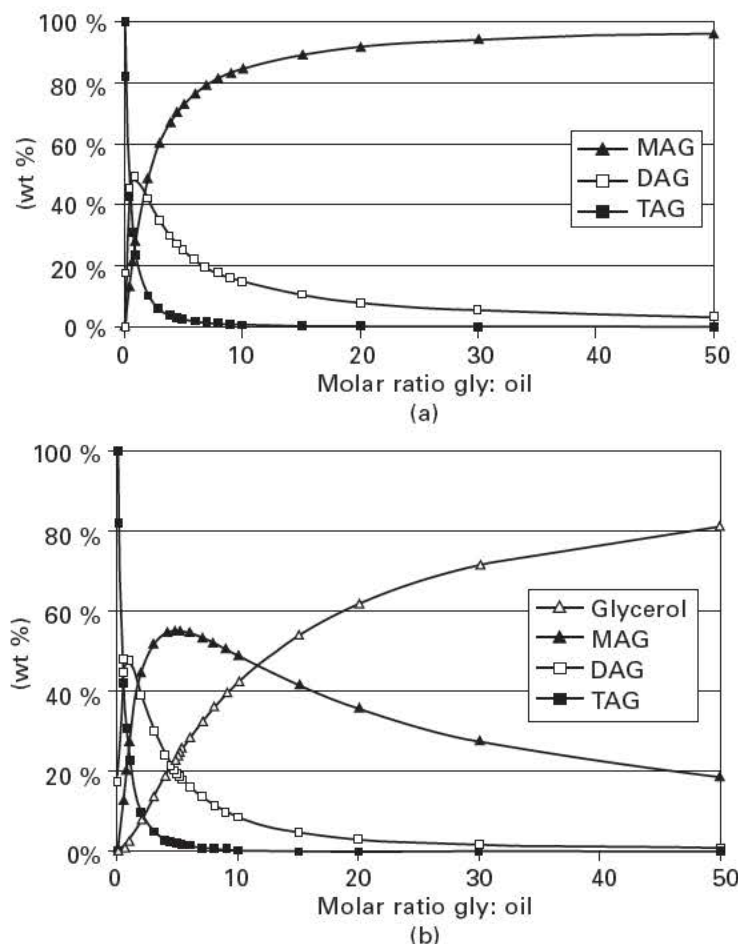


Fig. 11.12 Calculated product distribution at equilibrium conditions after glycerolysis reaction with different molar ratios of glycerol to oil. (a): Distribution between MAG, DAG and TAG (fat phase). (b): Distribution in the complete reaction mixture (fat phase and glycerol). Calculations are based on binomial random distribution with probability calculations of ester formation between mole fatty acids (FA) and mole hydroxyl groups (OH) and expressed as weight percentages (adapted from Damstrup *et al.*, 2006).

overabundance of n-6 fatty acids compared to international recommendations (Volker and Garg, 2001). In Australia and the USA, the current intakes of n-3 PUFAs are for instance less than 100–200 mg/day (Peters and Nurmikko, 2002). The intake is far from the recommendations of 1–2 g/day from organizations such as WHO, FDA and American Health Association (AHA) (Anselmino, 2004). This has led to a great deal of commercial interest in producing health-promoting and functional lipids with specific fatty acid profiles to overcome the gap between actual and recommended n-3 PUFA intake.

Vegetable oils are considered as easily accessible, relatively cheap and neutral flavoured triacylglycerol raw materials for carrying nutritionally important PUFAs. However, the current chemical glycerolysis process makes the use of raw materials based on PUFA-vegetable oils difficult due to their sensitivity to oxidation at high temperatures (Peng *et al.*, 2000). In contrast, enzymatic glycerolysis requires only a low temperature, below approximately 80 °C, which makes the use of heat-sensitive MAG and DAG with PUFA-profile feasible. Furthermore, the gentle enzyme technology reduces some of the heat-accelerated problems with development of bad flavour, dark colour and unwanted side reactions (Bornscheuer, 1995; Elfman-Borjesson and Harrod, 1999; Bellot *et al.*, 2001; Kaewthong and H-Kittikun, 2004). Accordingly, lipase-catalyzed glycerolysis of vegetable oils is believed to be a potential alternative/supplementary industrial method for the production of nutritional high-valued monoacylglycerols with important n-3 PUFAs. This opportunity for product and process development has led to a great interest in the enzymatic glycerolysis process, not only from academia but also from industry since the mid-1980s.

Glycerolysis under enzymatic catalysis has similar reaction routes to chemical glycerolysis, as shown in Fig. 11.11. Reaction equilibrium is still a central determinant for the evaluation of the product yield as shown in Fig. 11.12. However, because of its use of low temperature and its possible use of solvents, reaction equilibrium is not a target, which is imperative to achieve through the enzymatic glycerolysis process. This is because the operating conditions make it possible for a product ‘fishing’ strategy. All possible effort is being made to speed up the procedure of reaching reaction equilibrium on the other hand. This is illustrated in Fig. 11.13. Possible methods for such ‘fishing’ have been tested using crystallization, chromatographic separation, extraction, etc. as a step to break the reaction equilibrium and force the reaction to form more product (Peng *et al.*, 2000). Recent progress has been made with the idea of using solvent partitioning to push the reactions beyond reaction equilibrium (Guo and Xu, 2005; Yang *et al.*, 2005b, Damstrup *et al.*, 2005, 2006). In such systems with the selected media, the reaction efficiency and effectiveness have been dramatically improved, and higher yields of monoacylglycerols and diacylglycerols have been obtained. Figure 11.14 compares the reaction behaviour of glycerolysis of sunflower oil using ionic liquid and tert-butanol as system media with a solvent-free system. The first

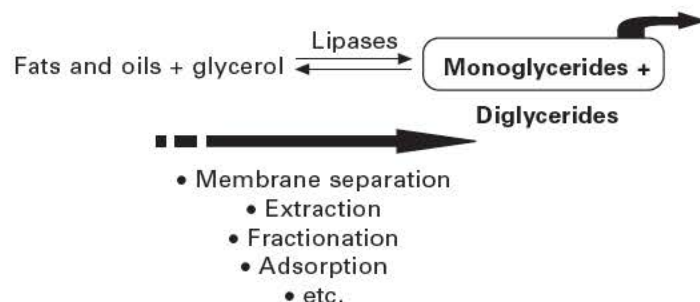


Fig. 11.13 Illustration of product ‘fishing’ for the enzymatic production of partial glycerol ester glycerides.

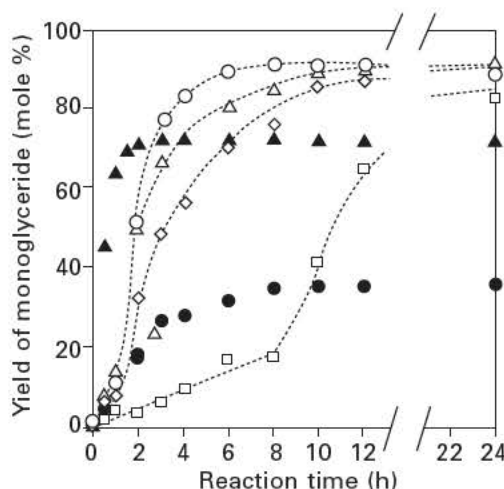


Fig. 11.14 Different reaction behaviours of glycerolysis of sunflower oil in ionic liquid, tert-butanol and the solvent-free system (0.5 mmol sunflower oil and 5×0.5 mmol glycerol for reaction at □, 40 °C; ◇, 50 °C; △, 60 °C; ○, 70 °C in 2.2 g ionic liquid; ▲, 50 °C in 2.5 g tert-butanol; ●, 2 mmol sunflower oil and 5×2 mmol glycerol for reaction at 70 °C in solvent-free system) (adapted from Guo and Xu, 2005).

two systems have been shown to have good potential for industrial implementation.

Recent progress in enzymatic glycerolysis

In general, the major challenge in using enzymes in low-temperature glycerolysis reactions is that the system comprises of three heterogeneous phases: a hydrophobic oil phase, a hydrophilic glycerol phase and a solid enzyme phase, as illustrated in Fig. 11.15. Since enzymes in their native forms have hydrophilic characteristics, glycerol often binds to the enzyme particles and makes the access of the hydrophobic oil molecules to the enzyme difficult (Kristensen, *et al.*, 2005; Yang *et al.*, 2005b). Poor miscibility of the glycerol and oil at low temperatures in combination with high reactant viscosity results in a reaction system with high mass transfer limitations. As a result, long reaction times and/or low conversion of reactants generally make the reaction inefficient.

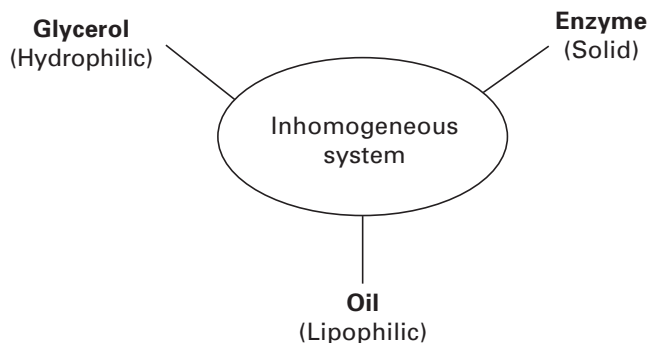


Fig. 11.15 Illustration of the heterogeneous reactant mixture used for enzymatic glycerolysis.

However the sustainability of an effective system has, to a certain extent, relationships with the content of glycerol in the system. In the case of DAG production, the theoretical stoichiometric amount of glycerol in the reaction system is low. Although the reaction time remains long, it is possible to have a sustainable system for DAG production without the usage of any medium assistance (Kristensen *et al.*, 2005). With a sufficiently low glycerol amount, a conversion of TAG up to 60–70 % can be achieved (Fig. 11.16). However, for MAG production, assistance of media or involvement of alternative techniques is normally required to improve the formation of MAG in reasonable time and amounts. Solid phase crystallization (McNeill *et al.*, 1990; Bornscheuer *et al.*, 1994; Rosu *et al.*, 1997), glycerol adsorbed to silica gel (Aha *et al.*, 1998; Elfman-Borjesson and Harrod, 1999; Rendon *et al.*, 2001), usage of protected glycerol (Akoh, 1993) and reactions performed in different media such as supercritical CO₂ (Jackson and King, 1997) or organic solvents (Bornscheuer *et al.*, 1994; Kwon *et al.*, 1995; Rendon *et al.*, 2001) are among the many interesting approaches to improve reaction efficiency and/or product quality. Many of the approaches work successfully on a laboratory scale, and a great deal of knowledge, revealed from 20 years of progressing research, has been accessible.

However, due to practical difficulties, the big industrial breakthrough for MAG production has still not taken place. For instance, solid phase crystallization and glycerol adsorption are difficult to handle continuously, and the reaction time required is still lengthy from an industrial point of view. At present, the high cost of the enzyme also makes the use of lipases in industrial applications uneconomic (Yang *et al.*, 2003a; Kaewthong *et al.*, 2005). To overcome some of the problems with high enzyme costs, a widely used strategy is to employ the lipase in an immobilized form. Immobilization of the enzyme onto a solid support material allows easy separation and reutilization of the enzyme. Furthermore, the use of immobilized enzymes on porous supports eliminates some of the problems arising with the use of suspended enzyme powders, such as the tendency to aggregation and attachment to the wall of the reactor, as the enzyme spreads on a large surface area (Barros *et al.*, 1998).

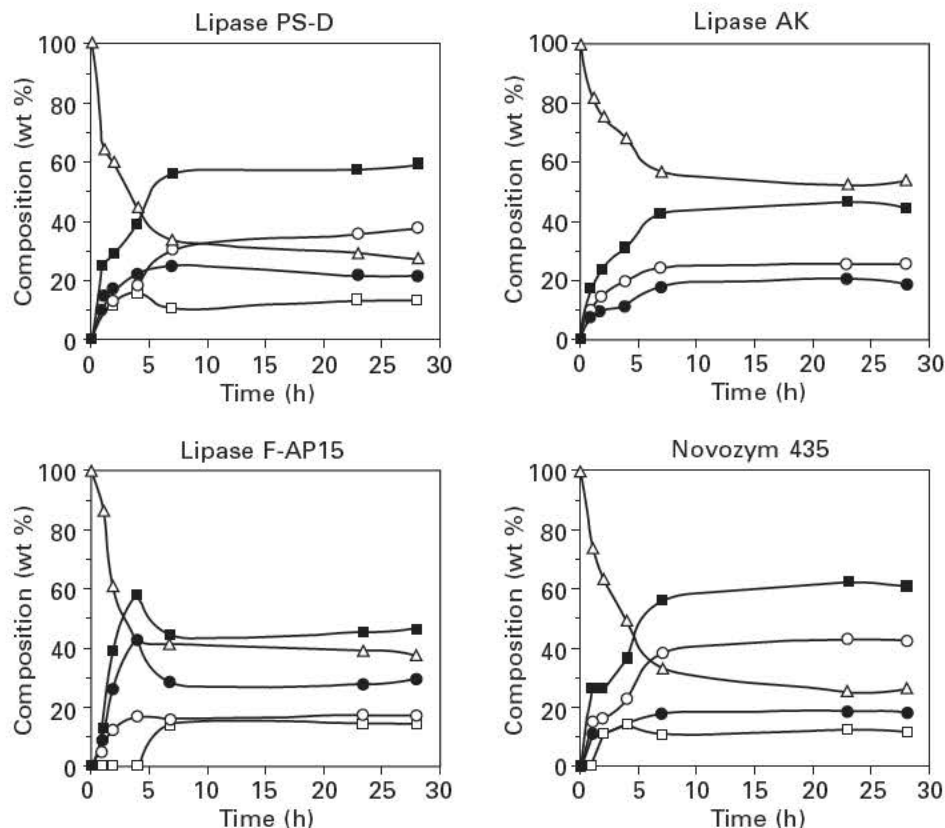


Fig. 11.16 Time courses for enzymatic glycerolysis with different lipases in batch reactors (Δ , TAG; $\circ$, 1,3-DAG; $\bullet$, 1,2-DAG; $\square$, MAG; $\blacksquare$, total DAG (adapted from Kristensen *et al.*, 2005).

Other benefits from immobilized enzymes compared to non-immobilized enzymes are improved activity and long-time stability in various organic media (Goto *et al.*, 2005; Nakaoki *et al.*, 2005). For instance, it has been shown that when lipases are immobilized onto a hydrophobic matrix, their activity increases 51-fold and exhibits superior heat resistance compared to the native lipase in esterification reactions of lauric acid in isooctane system (Goto *et al.*, 2005). Since Zaks and Klivanov discovered that lipase enzymes act well in organic solvents (Zaks and Klivanov, 1984, 1985), many investigations of lipase-catalyzed interesterification reactions in solvents confirm the benefits of using non-conventional media. Among the useful solvents in interesterification reactions are dioxan, *n*-hexane, *n*-heptane, acetonitrile, acetone, isooctane and *tert*-butanol and *tert*-pentanol (Goto *et al.*, 1995; Hess *et al.*, 1995; Elfman-Borjesson and Harrod, 1999; Bellot *et al.*, 2001; Rendon *et al.*, 2001; Kaewthong and H-Kittikun, 2004; Yang *et al.*, 2005b).

In our experience, the usage of selected media is believed to provide potential approaches for glycerolysis with industrial applications (Guo and Xu, 2005; Damstrup *et al.*, 2005). In such systems, commercially available immobilized CALB is shown to be one of the most stable enzymes. In spite

of lowered productivity due to the presence of solvent, glycerolysis reaction in the tertiary alcohols, *tert*-pentanol (2-methyl-2-butanol) and *tert*-butanol (2-methyl-2-propanol), is found to have great potential for efficient glycerolysis (Damstrup, *et al.*, 2005, 2006). These solvents offer benefits beyond the drawbacks related to lowered productivity. Amongst these advantages are high MAG yield/purity in the product mixture and very short reaction time together with a well-preserved PUFA profile of the MAGs (Damstrup *et al.*, 2005, 2006; Yang *et al.*, 2005b).

In addition, fluid solvent systems make continuous operation in PBR feasible. The obvious benefits of using continuous PBR are: ease of separation between reactant mixture and catalyst; reuse of the enzyme without the need for a prior separation; a cost-effective reactor design; and long-term production due to high density loading of the immobilized enzyme into the reactor (Kaewthong *et al.*, 2005; González Moreno *et al.*, 2005). These advantages indeed promote the arguments for using solvents in enzymatic glycerolysis. Finally, a process design including reusability of the solvent minimizes the drawbacks from lowered space-time yield.

A good review of lipase-catalyzed production of high-quantity monoacylglycerols by Peng *et al.* (2000) is recommended for further reading. In addition, a recently published and excellent patent review on lipid technology including patent literature involving monoglycerides by Lai and Lo (2005) is also recommended.

11.4 Remarks and future trends

The concept of interesterification, as defined in this chapter, with catalysis either by enzymes or chemicals, can be widely explored for unlimited product or process development. The subject is becoming more interesting and attracting more attention.

Chemical interesterification, to a large extent, is a mature technology that offers the prospect of many new applications in industry. Chemical randomization of oils and fats for plastic fat production, although in practice for about a century, is gaining new momentum with the coming ‘*trans*-free’ ruling for the future market. The method has been widely used but the ‘share’ of industrial capacity using this method for plastic fat production is expanding. Chemical glycerolysis is a common method for monoacylglycerol production in the present food industry, and it is expected to dominate monoacylglycerol production in the foreseeable future. Another case of chemical interesterification, which is receiving more and more importance, is chemical methanolysis for the production of biodiesel. Energy crises in the past year have led to the expansion of biodiesel production capacity from oils and fats.

Enzymes as catalysts for the interesterification of oils and fats, although far from mature and a long way from achieving widespread implementation

in industry, have been a focus for extensive studies for decades. Even though nobody expects a fast increase in the applications of enzymatic interesterification in industry, interest in using enzyme processes and realization of their benefits have increased in the industrial sector. More and more industrial think-tanks are including this area in their development strategy.

As an alternative to chemical randomization for plastic fat production, enzyme processes, even with such a low-priced product, have made an entry in the oils and fats industry in very recent years. Fats produced from the enzyme process in general can be used for margarine production without significant change of properties, though there are some unusual aspects compared to either blended or chemically randomized fats (Zhang, 2005). From the point of view of oxidation during storage, the enzymatically-produced fats have advantages over the chemically randomized product; the latter develops high peroxide values during storage. With regard to physical properties, the former can form the required crystal type and textures along with suitable solid fat profiles which are comparable to those of the chemically randomized fats.

However, margarines produced from the enzymatically-interesterified fats become more physically stable with a higher conversion degree of the interesterification. The control of the conversion degree, therefore, becomes highly critical to achieving good quality products. Furthermore, the optimization of margarine formulation, such as the use of diacylglycerols to postpone crystal transformation and the optimization of process conditions for margarine production, needs more studies to cater for the new requirement of using the partially interesterified fats from the enzymatic processes.

For the enzymatic production of structured lipids with structural specificity concerning the locations of fatty acids, enzyme processes are the only potential methods for large-scale production. Products of high value intended for functional foods and pharmaceutical applications provide only a small market. So far, a few products, such as fats for infant formula, fats for confectionery products, etc. have been available in the market. The initial criteria are the functional values of the products and their market demands. The economical balance of the process is the crucial point for industrial consideration. So far, technical issues for enzymatic interesterification concerning the production of structured lipids are not, in general crucial. The benefits of such products in human nutrition remain to be explored. Process optimization for a defined product needs a lot more work to reduce the cost of the process at engineering and management levels. Concept development for process engineering is a high priority for future research. A package of knowledge for building up a new plant is still far from available.

For enzymatic modification of PL, an understanding of the reaction systems is just the beginning. Before these types of reactions can be implemented industrially, a lot more work will need to be done to increase efficiency. For these reactions to be applicable, their operative stability needs to be better understood and a longer lifetime of the immobilized enzyme is desired.

Although solvent-free systems may seem to work well during batch operation, there is no information as to whether the lipase can actually be re-used. In terms of stability during continuous operation, it may be beneficial to use non-polar solvents as higher incorporation can be obtained for a prolonged time compared to solvent-free systems. Even though major by-product formation occurs during lipase-catalyzed acidolysis, it should be kept in mind that these by-products (LPC and GPC) are themselves valuable products having wide applications in the same area as the original material. These compounds can be purchased in purified form from different companies, and are usually sold at considerably higher price compared to natural PL.

Concerning the production of partial glycerol esters, it is well documented that enzymatic glycerolysis has become a powerful method in terms of MAG/DAG quality and functionality, which makes it possible to expand their fields of applications into functional foods, pharmaceuticals, etc. Of particular interest is the possibility of producing 'new' MAGs with PUFA profiles that differ significantly from MAGs produced by traditional chemical glycerolysis methods. With the introduction of efficient long-lasting commercially available immobilized lipases and the benefits of large-scale PBR, the industrial breakthrough for enzyme-catalyzed glycerolysis has moved closer. Accordingly, in the future, it is most likely that the fat and oil industry will, to a certain extent, adapt the enzyme-catalyzed glycerolysis into industrial plants, even although the chemical process will still occupy the main position for many years to come.

As discussed in the above section, a medium, usually an organic solvent, has been an advantage in achieving a high degree of efficiency and effectiveness in the process and good product yields. The use of solvents, however, is contrary to the aim of an environmentally friendly and energy-reduced enzymatic process due to the extra processing required for solvent removal. From this point of view a solvent-free medium or the application of non-evaporative solvents such as ionic liquids could be a better solution. This certainly opens a new window for future visions.

11.5 Sources of further information and advice

Enzymatic interesterification has been attracting significant interest since the 1980s and chemical interesterification for even longer. A large amount of information has been collected and accumulated. A large number of recent review papers and chapters concerning each topic have been cited in the relevant sections.

A number of company homepages are worth visiting, including technology providers (e.g. Desmet Ballestra, Alfa Laval), enzyme providers (e.g. Novozymes, Amano) and product providers (e.g. ADM, Unilever, Danisco, Degussa, Karlshmsns, Aarhus United, Kao, Nisshin Olillio, Loders-Croklaan). These companies provide a real picture of interesterification in industry.

11.6 Acknowledgements

Financial support for our group research activity is acknowledged from the Research Council for Technology and Production, the Danish Dairy Foundation, Danisco, and the Centre for Advanced Food Studies as well as other assistance from industry and collaboration partners.

11.7 References

- ADLERCREUTZ D, BUDDE H and WEHTJE E (2002), Synthesis of phosphatidylcholine with defined fatty acid in the *sn*-1 position by lipase-catalyzed esterification and transesterification reaction, *Biotech Bioeng*, **78**(4), 403–411.
- ADLERCREUTZ P, LYBERG A-M and ADLERCREUTZ D (2003), Enzymatic fatty acid exchange in glycerophospholipids, *Eur J Lipid Sci Technol*, **105**(10), 638–645.
- AHA B, BERGER M, HERMANN J, KEIL O, WALDINGER C and SCHNEIDER M (1998), Food emulsifiers: enzyme assisted synthesis of isomerically pure mono- and diglycerides, *Food Sci Technol Today*, **12**, 30–34.
- AKOH C C (1993), Lipase-catalyzed synthesis of partial glyceride, *Biotechnol Lett*, **15**(9), 949–954.
- AKOH C C (1998), Structured lipids, in Akoh C C and Min D B, *Food Lipids: Chemistry, Nutrition, and Biotechnology*, New York, Marcel Dekker, Inc., 699–727.
- ANDERSON D (1996), A primer on oils processing technology, in Hui Y H, *Bailey's Industrial Oil & Fat Products*, 5th edn, New York, Wiley Interscience, 40–41.
- ANSELMINO C (TRANSLATED by HORNSTRA G) (2004), *Omega-3 Long Chain Polyunsaturated Fatty Acids and Health Benefits*, Maastricht, Netherlands, NutriScience.
- BALCAO V M and MALCATA F X (1998), Lipase catalyzed modification of milkfat, *Biotechnol Adv*, **16**(2), 309–341.
- BALJIT S G, DYAL S D and NARINE S S (2002), Lipid shortenings: a review, *Food Res Int*, **35**(10), 1015–1048.
- BARROS R J, WEHTJE E and ADLERCREUTZ P (1998), Mass transfer studies on immobilized α -chymotrypsin biocatalysts prepared by deposition for use in organic medium, *Biotechnol Bioeng*, **59**(3), 364–373.
- BELLOT J C, CHOISNARD L, CASTILLO E and MARTY A (2001), Combining solvent engineering and thermodynamic modelling to enhance selectivity during monoglyceride synthesis by lipase-catalyzed esterification, *Enzyme Microb Technol*, **28**(4–5), 362–369.
- BLOOMER S, ADLERCREUTZ P and MATTIASSEN B (1990), Triglyceride interesterification by lipases 1. Cocoa butter equivalents from a fraction of palm oil, *J Am Oil Chem Soc*, **67**(8), 519–524.
- BLOOMER S, ADLERCREUTZ P and MATTIASSEN B (1991), Triglyceride interesterification by lipases 2. Reaction parameters for the reduction of trisaturated impurities and diglycerides in batch reactions, *Biocatalysis*, **5**, 145–162.
- BORNSCHEUER U T (1995), Lipase-catalyzed syntheses of monoacylglycerols, *Enzyme Microb Technol*, **17**(7), 578–586.
- BORNSCHEUER U T, STAMATIS H, XENAKIS A, YAMANE T and KOLISIS F N A (1994), Comparison of different strategies for lipase-catalyzed synthesis of partial glycerides, *Biotechnol Lett*, **16**(7), 697–702.
- BUESCHELBERGER H-G (2004), Lecithins, in Whitehurst R J, *Emulsifiers in Food Technology*, Oxford, Blackwell Publishing, 1–39.
- CHANG M K, ABRAHAM G and JOHN V T (1990), Production of cocoa butter-like fat from interesterification of vegetable oils, *J Am Oil Chem Soc*, **67**(4), 832–834.

- CHRISTENSEN M W, ANDERSEN L, KIRK O and HOLM H C (2001), Enzymatic interesterification of commodity oils and fats: approaching the tonnes scale, *Lipid Technol*, **4**, 33–37.
- COTERON A, MARTINEZ M and ARACIL J (1998), Reactions of olive oil and glycerol over immobilized lipases, *J Am Oil Chem Soc*, **75**(5), 657–660.
- DAMSTRUP M L, JENSEN T, SPARSØ F V, KIIL S Z, JENSEN A D and XU X (2005), Solvent optimization for efficient enzymatic monoacylglycerol production based on a glycerolysis reaction, *J Am Oil Chem Soc*, **82**(8), 559–564.
- DAMSTRUP M L, JENSEN T, SPARSØ F V, KIIL S Z, JENSEN A D and XU X (2006), Production of heat sensitive monoacylglycerols by enzymatic glycerolysis in *tert*-pentanol: process optimization by response surface methodology, *J Am Oil Chem Soc*, **83**, 27–33.
- DIJKSTRA A L, TÖKE E R, KOLONITS P, RECSEG K, KÖVÁRI K and POPPE L (2005), The base-catalyzed, low-temperature interesterification mechanism revisited, *Eur J Lipid Sci Technol*, **107**(12), 912–921.
- ELFMAN-BORJESSON I and HARROD M (1999), Synthesis of monoglycerides by glycerolysis of rapeseed oil using immobilized lipase, *J Am Oil Chem Soc*, **76**(6), 701–707.
- EUROPEAN FOOD EMULSIFIER MANUFACTURERS' ASSOCIATION (2004), *Index of Food Emulsifiers*, 4th edn, Brussels, EFEMA, 51–54.
- EUROPEAN PARLIAMENT COUNCIL (2004), *European parliament council directive No. 95/2/EC on food additives other than colours and sweeteners*, CONSLEG: 1995L0002-29/01/2004, Office for Official Publications of the European Communities.
- FERREIRA-DIAS S, CORREIA A C, BAPTISTA F O, DA FONSECA M M R (2001), Contribution of response surface design to the development of glycerolysis systems catalyzed by commercial immobilized lipases, *J Mol Catal B-Enz*, **11**(4–6) 699–711.
- FISCHER E (1920), Wanderung von acyl bei den glyceriden, *Ber Deut Chem Ges*, **53**, 1621–1633.
- FLICKINGER B D and MATSOU N (2003), Nutritional characteristics of DAG oil, *Lipids*, **38**(2), 129–132.
- GONZALEZ MORENO P A, ROBLES MEDINA A, CAMACHO RUBIO F, CAMACHO PAEZ B, ESTEBAN CERDAN L and MOLINA GRIMA E (2005), Production of structured triacylglycerols in an immobilised lipase packed-bed reactor: batch mode operation, *J Chem Technol Biotechnol*, **80** (1), 35–43.
- GOTO M, KAMIYA N and NAKASHIO F (1995), Enzymatic interesterification of triglyceride with surfactant-coated lipase in organic media, *Biotechnol Bioeng*, **45**(1), 27–32.
- GOTO M, HATANAKA C and GOTO M (2005), Immobilization of surfactant-lipase complexes and their high heat resistance in organic media, *Biochem Eng J*, **24**(1), 91–94.
- GUNSTONE F D (1999), Enzymes as biocatalysts in the modification of natural lipids *J Sci Food Agric*, **79**, 1535–1549.
- GUNSTONE F D and HERSLÖF B G (1992), *A Lipid Glossary*, Ayr, Oily Press.
- GUO Z and XU X (2005), New opportunity for enzymatic modification of fats and oils with industrial potentials, *Org Biomol Chem*, **3**, 2615–2619.
- GUO Z and XU X (2006), Lipase-catalyzed glycerolysis of fats and oils in ionic liquids: a further study on the reaction system, *Green Chem*, **8**, 54–62.
- GUO Z, VIKBJERG A F and XU X (2005), Enzymatic modification of phospholipids for functional applications and human nutrition, *Biotechnol Advances*, **23**(3), 203–259.
- HALLING P J (1987), Rates of enzymatic reactions in predominately organic low water systems, *Biocatalysis*, **1**, 109–115.
- HARALDSSON G G and KRISTINSSON B (1998), Separation of eicosapentaenoic acid and docosahexaenoic acid in fish oil by kinetic resolution using lipase, *J Am Oil Chem Soc*, **75**(11), 1551–1556.
- HARALDSSON G G and THORARENSEN A (1999), Preparation of phospholipids highly enriched with n-3 polyunsaturated fatty acids by lipase, *J Am Oil Chem Soc*, **76**(10), 1143–1149.
- HESS R, BORNSCHEUER U, CAPEWELL A and SCHEPER T (1995), Lipase-catalyzed synthesis of monostearoylglycerol in organic-solvents from 1,2-o-isopropylidene glycerol, *Enzyme Microb Technol*, **17**(8), 725–728.

- HOSSEN M and HERNANDEZ E (2005), Enzyme-catalyzed synthesis of structured phospholipids with conjugated linoleic acid, *Eur J Lipid Sci Technol*, **107**(10), 730–736.
- HØY C-E and XU X (2001), Structured triacylglycerols, in Gunstone F D, *Structured and Modified Lipids*, New York, Marcel Dekker, Inc., 209–240.
- HUANG K and AKOH C C (1994), Lipase-catalyzed incorporation of n-3 polyunsaturated fatty acids into vegetable oils, *J Am Oil Chem Soc*, **71**(11), 1277–1280.
- IKEDA I, TOMARI Y, SUGANO M, WATANABE B N and NAGATA J (1991), Lymphatic absorption of structured glycerolipids containing medium-chain fatty acids and linoleic acid, and their effect on cholesterol absorption in rats, *Lipids*, **26**(5), 369–373.
- INGLE D L, DRIEDGER A, TRAU L K A and NAKHASI D K (1999), Concise reviews in food service – dietary energy value of medium-chain triglycerides, *J Food Sci*, **64**(6), 960–963.
- IWASAKI Y and YAMANE T (2004), Enzymatic synthesis of structured lipids, *Adv Biochem Eng Biot*, **90**, 151–171.
- JACKSON M A and KING J W (1997), Lipase-catalyzed glycerolysis of soybean oil in supercritical carbon dioxide, *J Am Oil Chem Soc*, **74**(2), 103–106.
- JACOBSEN C, XU X, NIELSEN N S and TIMM-HEINRICH M (2003), Oxidative stability of mayonnaise containing structured lipids produced from sunflower oil and caprylic acid, *Eur J Lipid Sci Technol*, **105**(8), 449–458.
- JENSEN R G and PITAS R E (1976), Synthesis of some acylglycerols and phosphoglycerides, *Adv Lipid Res*, **12**, 213–247.
- KAETHONG W and H-KITTIKUN A (2004), Glycerolysis of palm olein by immobilized lipase PS in organic solvents, *Enzyme Microb Technol*, **35**(2–3), 218–222.
- KAETHONG W, SIRISANSANEYAKUL S, PRASERTSAN P and H-KITTIKUN A (2005), Continuous production of monoacylglycerols by glycerolysis of palm oil with immobilized lipase, *Process Biochem*, **40**(5), 1525–1530.
- KAVANAGH A R (1997), A breakthrough in infant formula fats, *Oleagineux Corps Gras Lipides*, **4**, 165–168.
- KIM E J and YOON S H (2003), Recent progress in enzymatic production of structured lipids, *Food Sci Biotechnol*, **12**, 721–726.
- KODALI D R, TERCYAK A, FAHEY D A and SMALL D M (1990), Acyl migration in 1,2-diapamitoyl-sn-glycerol, *Chem Phys Lipids*, **52**(3–4), 163–170.
- KORBITZ W (1999), Biodiesel production in Europe and North America, an encouraging prospect, *Renewable Energy*, **16**(1–4), 1078–1083.
- KRIS-ETHERTON P M and NICOLSI R J (1995), *Trans Fatty Acids and Coronary Heart Disease Risk*, International Life Sciences Institute, Washington, ILSI Press.
- KRISTENSEN J B, XU X, MU H (2005), Diacylglycerol synthesis by enzymatic glycerolysis: Screening of commercially available lipases, *J Am Oil Chem Soc*, **82**(5), 329–334.
- KROG N (1997), Food emulsifiers, in Gunstone F D, *Lipid Technologies and Applications*, New York, Marcel Dekker, Inc., 521–534.
- KWON S J, HAN J J and RHEE J S (1995), Production and *in-situ* separation of monoacylglycerol or diacylglycerol catalyzed by lipases in n-hexane, *Enzyme Microb Technol*, **17**(8), 700–704.
- LAI O M and LO S K (2005), Patent review on lipid technology, in Akoh C C and Lai O M, *Healthful Lipids*, Champaign, Ill, AOCS Press, 433–507.
- LAI O M, GHAZALI H M and CHONG C L (1998), Effect of enzymatic transesterification on the fluid of palm stearin–palm kernel mixtures, *Food Chem*, **63**(2), 155–159.
- LEE G-C, WANG D-L, HO Y-F and SHAW J-F (2004), Lipase-catalyzed alcoholysis of triglycerides for short-chain monoglyceride production, *J Am Oil Chem Soc*, **81**(6), 533–536.
- LEVENSPIEL O (1999), *Chemical Reaction Engineering*, 3rd edn, New York, John Wiley & Sons, Inc., 38–60 and 427–446.
- LIST G R, MOUNTS T L, ORTHOEFER F and NEFF W E (1995), Margarine and shortening oils by interesterification of liquid and trisaturated triglycerides, *J Am Oil Chem Soc*, **72**(3), 379–382.
- LYBERG A M, ADLERCREUTZ D and ADLERCREUTZ P (2005), Enzymatic and chemical synthesis

- of phosphatidylcholine regioisomers containing eicosapentaenoic acid or docosahexaenoic acid, *Eur J Lipid Sci Technol*, **107**(5), 279–290.
- MA F and HANNA M A (1999), Biodiesel production: a review, *Bioresource Technol*, **70**(1), 1–15.
- MACRAE A R (1985), Enzyme-catalysed modification of oils and fats, *Phil Trans R Soc Lond B, Biol Sci*, **311**(1144), 227–233.
- MARANGONI A G and ROUSSEAU D (1995), Engineering triacylglycerols: the role of interesterification, *Trends Food Sci Technol*, **6**, 329–335.
- MARANGONI A G and ROUSSEAU D (1998), The influence of chemical interesterification on physicochemical properties of complex fat systems 1. Melting and crystallization, *J Am Oil Chem Soc*, **75**(10), 1265–1272.
- MATORI M, ASAHARA T and OTA Y (1991), Positional specificity of microbial lipases, *J Ferment Bioeng*, **72**(5), 397–398.
- MCNEILL G P, SHIMIZU S and YAMANE T (1990), Solid-phase enzymatic glycerolysis of beef tallow resulting in a high-yield of monoglyceride, *J Am Oil Chem Soc*, **67**(11), 779–783.
- MOONEN H and BAS H (2004), Mono- and diglycerides, in Whitehurst R J, *Emulsifiers in Food Technology*, Oxford, Blackwell Publishing, 40–58.
- MU H and PORSGAARD T (2005), The metabolism of structured triacylglycerols, *Progress Lipid Res*, **44**(6), 430–448.
- MUTUA L N and AKOH C C (1993), Lipase-catalyzed modification of phospholipids: Incorporation of n-3 fatty acids into biosurfactants, *J Am Oil Chem Soc*, **70**(2), 125–128.
- NAKAOKI, T, MEI Y, MILLER L M, KUMAR A, KALRA B, MILLER M E, KIRK O, CHRISTENSEN M, NEGISHI S, SHIRASAWA S, ARAI Y, SUZUKI J and MUKATAKA S (2003), Activation of powdered lipase by cluster water and the use of lipase powers for commercial esterification of food oils, *Enzyme Microb Technol*, **32**(1), 66–70.
- NIELSEN N S, XU X, TIMM-HEINRICH M and JACOBSEN C (2004), Oxidative stability during storage of structured lipids produced from fish oil and caprylic acid, *J Am Oil Chem Soc*, **81**(4), 375–384.
- NIELSEN N S, YANG T, XU X and JACOBSEN C (2006), Production and oxidative stability of a human milk fat substitute produced from lard by enzyme technology in a pilot packed-bed reactor, *Food Chem*, **94**(1), 53–60.
- NOVOZYMES A/S (2005) Award-winning technology gives healthier fats, *BioTimes*, Sept.
- PEDERSEN S and CHRISTENSEN M W (2000), Immobilized biocatalyst, in Straathof A and Adlercreutz P, *Applied Biocatalysis*, 2nd edn, New York, Harwood Academic, 213–228.
- PENG L, XU X and TAN T (2000), Lipase-catalyzed production of high quantity monoglycerides, in Mohan R M, *Research Advances in Oil Chemistry*, Kerala, GRN, 1, 53–78.
- PENG L, XU X, MU H, HØY C-E and ADLER-NISSEN J (2002), Production of structured phospholipids by lipase-catalyzed acidolysis: optimization using response surface methodology, *Enzyme Microb Technol*, **31**(4), 523–532.
- PETERS G and NURMIKKO T J (2002), Peripheral and gasserian ganglion-level procedures for the treatment of trigeminal neuralgia, *Clin J Pain*, **18**(1), 28–34.
- QUINLAN P and MOORE S (1993), Modification of triglycerides by lipases: Process technology and its application to the production of nutritionally improved fats, *INFORM*, **4**, 580–585.
- REDDY J R C, VIJEETA T, KARUNA M S L, RAO B V S K and PRASAD R B N (2005), Lipase-catalyzed preparation of palmitic and stearic acid-rich phosphatidylcholine, *J Am Oil Chem Soc*, **82**(10), 727–730.
- RENDON X, LOPEZ-MUNGUÍA A and CASTILLO E (2001), Solvent engineering applied to lipase-catalyzed glycerolysis of triolein, *J Am Oil Chem Soc*, **78**(10), 1061–1066.
- RØNNE T H, YANG T, MU H, JACOBSEN C and XU X (2005), Enzymatic interesterification of butterfat with rapeseed oil in a continuous packed bed reactor, *J Agric Food Chem*, **53**(14), 5617–5624.

- ROSU R, UOZAKI Y, IWASAKI Y and YAMANE T (1997), Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil, *J Am Oil Chem Soc*, **74**(4), 445–450.
- ROUSSEAU D and MARANGONI A G (2002), Chemical interesterification of food lipids: theory and practice, in Akoh C C and Min D B, *Food Lipids: Chemistry, Nutrition, and Biotechnology*, New York, Marcel Dekker, Inc., 301–334.
- ROUSSEAU D, MARANGONI A G and JEFFREY K R (1998), Influence of chemical interesterification on the physicochemical properties of complex fat systems 2. Morphology and polymorphism, *J Am Oil Chem Soc*, **75**(12), 1833–1839.
- ROZENDAAL A and MACRAE A R (1997), Interesterification of fats and oils, in Gunstone F D and Padley F B, *Lipid Technologies and Applications*, New York, Marcel Dekker, Inc., 223–263.
- SCHNEIDER M (1997), Phospholipids, in Gunstone F D and Padley F B, *Lipid Technologies and Applications*, New York, Marcel Dekker, Inc., 51–78.
- SERDAREVICH B (1968), Glyceride isomerizations in lipid chemistry, *J Am Oil Chem Soc*, **44**(7), 381–393.
- SHIMADA Y, SUGIHARA A, KURAMOTO H, NAGAO T, GEMBA M and TOMINAGA Y (1997), Purification of docosahexaenoic acid by selective esterification of fatty acids from tuna oil with *Rhizopus delemar* lipase, *J Am Oil Chem Soc*, **74**(11), 97–101.
- SONNTAG N O V (1982), Fat splitting, esterification, and interesterification, in Swern D, *Bailey's Industrial Oil and Fat Products*, 4th edn, New York, Wiley Interscience, 97–174.
- SOUMANOU M M, BORNSCHEUER U T, SCHMID R D (1998), Synthesis of structured triglycerides by lipase catalysis, *Fett/Lipid*, **100**(4–5), 156–160.
- TAUTORUS C L and MCCURDY A R (1990), Effect randomization on oxidative stability of vegetable oils at two different temperatures, *J Am Oil Chem Soc*, **67**(8), 525–530.
- TIMM-HEINRICH M, XU X, NIELSEN N S and JACOBSEN C (2003a) Oxidative stability of milk drinks containing structured lipids produced from sunflower oil and caprylic acid, *Eur J Lipid Sci Technol*, **105**(8), 459–470.
- TIMM-HEINRICH M, XU X, NIELSEN N S and JACOBSEN C (2003b), Oxidative stability of structured lipids produced from sunflower oil and caprylic acid, *Eur J Lipid Sci Technol*, **105**(6), 436–448.
- TIMM-HEINRICH M, XU X, NIELSEN N S and JACOBSEN C (2004), Oxidative stability of mayonnaise and milk drink produced with structured lipids based on fish oil and caprylic acid, *Eur Food Res Technol*, **219**(1), 32–41.
- UCCIANI E and DEBAL A (1996), Chemical properties of fats, in Karleskind A and Wolff J-P, *Oils and Fats Manual*, Paris, Lavoisier Publishing, 1, 325–332.
- UNDURRAGA D, MARKOVITS A and ERAZO S (2001), Cocoa butter equivalent through enzymatic interesterification of palm oil midfraction, *Proc Biochem*, **36**(10), 933–939.
- UNILEVER N V (1977), Catalytic rearrangement of fatty acid groups in glyceride fats or oils – by contacting with an enzyme transesterification catalyst activated with water, Patent, GB765376.
- VIKBJERG A F, MU H and XU X (2005a), Lipase-catalyzed acyl exchange of soybean phosphatidylcholine in n-hexane: a critical evaluation of both acyl incorporation and product recovery, *Biotechnol Prog*, **21**(2), 397–404.
- VIKBJERG A F, MU H and XU X (2005b), Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system, *J Mol Cat B*, **36**(1–6), 14–21.
- VIKBJERG A F, PENG L, MU H and XU X (2005c), Continuous production of structured phospholipids in a packed bed reactor with lipase from *Thermomyces lanuginosa*, *J Amer Oil Chem Soc*, **82**(4), 237–242.
- VOLKER D H and GARG M L (2001), Omega-3 polyunsaturated fatty acids and rheumatoid arthritis, in Handmen R E, *Handbook of Nutraceuticals and Functional Foods*, Boca Raton, FL, CRC Press, 353–376.

- WATANABE T, SUGIURA M, SATO M, YAMADA N and NAKANISHI K (2005), Diacylglycerol production in a packed bed bioreactor, *Process Biochem*, **40**(2), 637–643.
- WONG D W S (1995), *Food Enzymes: Structure and Mechanism*, New York, Chapman & Hall, 37–84.
- XU X (2000a), Enzymatic production of structured lipids: process reactions and acyl migration, *INFORM*, **11**, 1121–1131.
- XU X (2000b), Modification of oils and fats by lipase-catalyzed interesterification: aspects of process engineering, in Bornscheuer U T, *Enzymes in Lipid Modification*, Weinheim, Wiley-VCH, 190–215.
- XU X (2000c), Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review, *Eur J Lipid Sci Technol*, **102**(4), 287–303.
- XU X (2003), Engineering of enzymatic reactions and reactors for lipid modification, *Eur J Lipid Sci Technol*, **105**(6), 289–304.
- XU X (2004), Biocatalysis for lipid modification, in Dunford N T and Dunford H B, *Nutritionally Enhanced Edible Oil Processing*, Champaign IL, AOCS Press, 162–196.
- XU X, BALCHEN S, HØY C E and ADLER-NISSEN J (1998a), Production of specific-structured lipids by enzymatic interesterification in a pilot continuous enzyme bed reactor, *J Am Oil Chem Soc*, **75**(11), 1573–1579.
- XU X, BALCHEN S, HØY C-E and ADLER-NISSEN J (1998b), Pilot batch production of specific-structure lipids by lipase-catalyzed interesterification: preliminary study on incorporation and acyl migration, *J Am Oil Chem Soc*, **75**(11), 301–308.
- XU X, MU H, HØY C-E and ADLER-NISSEN J (1999a), Production of specific structured lipids by enzymatic interesterification in a pilot enzyme bed reactor: process optimisation by response surface methodology, *Fett/Lipid*, **101**(6), 203–214.
- XU X, MU H, SKANDS A, HØY C-E and ADLER-NISSEN J (1999b), Parameters affecting diacylglycerol formation during the production of specific-structured lipids by lipase-catalyzed interesterification, *J Am Oil Chem Soc*, **76**(2), 175–181.
- XU X, SKANDS A R H, HØY C-E, MU H, BALCHEN S and ADLER-NISSEN J (1999c), Production of specific-structured lipids by enzymatic interesterification: elucidation of acyl migration by response surface design, *J Am Oil Chem Soc*, **75**(9), 1179–1186.
- XU X, SKANDS A, JONSSON G and ADLER-NISSEN J (2000), Production of structured lipids by lipase-catalysed interesterification in an ultrafiltration membrane reactor, *Biotechnol Lett*, **22**(21), 1667–1671.
- XU X, JACOBSEN C, NIELSEN N S, HEINRICH M T and ZHOU D Q (2002), Purification and deodorization of structured lipids by short path distillation, *Eur J Lipid Sci Technol*, **104**(11), 745–755.
- XU X, TIMM-HEINRICH M, NIELSEN N S, PORSGAARD T and JACOBSEN C (2005), Effects of antioxidants on the lipase-catalyzed acidolysis during production of structured lipids, *Eur J Lipid Sci Technol*, **107**(7), 464–468.
- YANG T K, FRUEKILDE M B and XU X (2003a), Applications of immobilized *Thermomyces lanuginosa* lipase in interesterification, *J Am Oil Chem Soc*, **80**(9), 881–887.
- YANG T K, XU X, HE C and LI L T (2003b), Lipase-catalyzed modification of lard to produce human milk fat substitutes, *Food Chem*, **80**(4), 473–481.
- YANG T K, FRUEKILDE M B and XU X (2005a), Suppression of acyl migration in enzymatic production of structured lipids through temperature programming, *Food Chem*, **92**(1), 101–107.
- YANG T, REBSDORF M, ENGELRUD U and XU X (2005b), Enzymatic production of monoacylglycerols containing polyunsaturated fatty acids through an efficient glycerolysis system, *J Agric Food Chem*, **53**(5), 1475–1481.
- YOON S H, MIYAWAKI O, PARK K H and NAKAMURA K (1996), Transesterification between triolein and ethylbehenate by immobilized lipase in supercritical carbon dioxide, *J Ferment Bioeng*, **82**(4), 334–340.
- ZAKS A and KLIBANOV A M (1984), Enzymatic catalysis in organic media at 100 °C, *Science*, **224**(4654), 1249–1251.

- ZAKS A and KLIBANOV A M (1985), Enzyme-catalyzed processes in organic solvents, *Proc Natl Acad Sci*, **82**(10), 3192–3196.
- ZHANG H (2005), Lipase-Catalysed Interesterification for Margarine Fat Production, PhD Thesis, Technical University of Denmark, Lyngby.
- ZHANG H, XU X, MU H, NILSSON J, ADLER-NISSEN J and HØY C-E (2000), Lipozyme IM-catalyzed interesterification for the production of margarine fats in a 1 kg scale stirred tank reactor, *Eur J Lipid Sci Technol*, **102**(6) 411–418.
- ZHANG H, JACOBSEN C, PEDERSEN L S, CHRISTENSEN M W and ADLER-NISSEN J (2006), Storage stability study for margarines produced by enzymatically interesterified fats compared to the margarines by the conventional methods – chemical properties, *Eur J Lipid Sci Technol*, **108**(4) 227–238.
- ZHANG H, XU X, NILSSON J, MU H, ADLER-NISSEN J and HØY C-E (2001), Production of margarine fats by enzymatic interesterification with silica-granulated *Thermomyces lanuginosus* lipase in a large-scale study, *J Am Oil Chem Soc*, **78**(1), 57–64.
- ZHANG H, PEDERSEN L S, KRISTENSEN D, ADLER-NISSEN J and HOLM H C (2004a), Modification of margarine fats by enzymatic interesterification: evaluation of a solid-fat-content-based exponential model with two groups of oil blends, *J Am Oil Chem Soc*, **81**(7), 653–658.
- ZHANG H, SMITH P and ADLER-NISSEN J (2004b), Effects of degree of enzymatic interesterification on the physical properties of margarine fats – solid fat content, crystallization behavior, crystal morphology and crystal network, *J Agr Food Chem*, **52**(14), 4423–4431.
- ZHANG H, JACOBSEN C and ADLER-NISSEN J (2005), Storage stability study for margarines produced by enzymatically interesterified fats compared to the margarines by the conventional methods I. Physical properties, *Eur J Lipid Sci Technol*, **107**(7–8), 530–539.