# Enzymatic synthesis of polyol esters in aqueous-organic two-phase systems

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# Enzymatic synthesis of polyol esters in aqueous-organic two-phase systems

Proefschrift
ter verkrijging van de graad van
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op gezag van de rector magnificus,
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# STELLINGEN

1. Tsai and Chiang voorspellen het concentratieverloop als functie van de tijd van de hydrolyse van olijfolie met behulp van een model waarbij oliezuur optreedt als competitieve remmer. Zij verwaarlozen hierbij echter onterecht de teruggaande reactie.

Tsai, S.W., Chiang, C.L. 1991. Kinetics, mechanism, and time course analysis of lipase-catalyzed hydrolysis of high concentration olive oil in AOT-isooctane reversed micelles. Biotechnol. Bioeng. 38: 206-211.

2. De lage productie van ethylbutyraat in oplosmiddelen met een lage log P waarde, is niet te wijten aan verstoring van de essentiële waterlaag rond het enzym, maar aan een lagere evenwichtsconcentratie van het product.

Manjón, A., Iborra, J.L., Arocas, A. 1991. Short-chain flavour ester synthesis by immobilized lipase in organic media. Biotechnol. Lett. 13: 339-344.

3. Hoewel de enzymatische activiteit en stabiliteit bij de hydrolyse van olijfolie in omgekeerde micellen hoger is dan in een emulsiesysteem, zal het hiermee behaalde voordeel niet opwegen tegen de gevolgen die dit heeft voor de opwerking.

Praseres, D.M.F., Garcia, F.A.P., Cabral, J.M.S. 1992. Kinetics and stability of a Chromobacterium viscosum lipase in reversed micellar and aqueous media. J. Chem. Tech. Biotechnol. 53: 159-164.
Praseres, D.M.F., Garcia, F.A.P., Cabral, J.M.S. 1993. An ultrafiltration membrane bioreactor for the lipolysis of olive oil in reversed micellar media. Biotechnol. Bioeng. 41: 761-770.

4. Het patenteren van een enzymatische suikerestersynthese in een waterig systeem door Seino et al., is tot nu toe als zodanig overbodig gebleken doordat het systeem niet reproduceerbaar is.

Seino, H., Uchibori, T., Inamasu, S., Nishitani, T. 1983. Verfahren zur herstellung von zucker- order zuckeralkohol-fettsäureestern. Duits patent DE 3430944 A1.

5. De opschudding die het ontdekken van wetenschappelijke fraude meestal veroorzaakt, toont aan dat het boek "Betrayers of the truth" niet genoeg gelezen wordt.

Broad, W., Wade, N. 1982. Betrayers of the truth, fraud and deceit in the halls of science. Simon and Schuster, New York.

6. Het uitzetten van wild in Nederland moet met het huidige wegennet en wagenpark, fel bekritiseerd worden door dierenbeschermingsorganisaties.

7. Het spreekwoord "als twee druppels water op elkaar lijken" is vooral voor de huidige automodellen van toepassing.

8. De grote problemen die studenten hebben met het toepassen van massabalansen doen het ergste vrezen voor de plannen om ter voorkoming van overbemesting een mineraalhuishouding verplicht te stellen.

9. Het taalgebruik in juridische documenten is een waarborg voor de werkgelegenheid in de juridische sector.

10. Een PC in huiselijke omgeving is ideaal om eenvoudige taken op complexe wijze uit te voeren.

11. In vele proefschriften wordt in het nawoord melding gemaakt van het feit dat je nooit in je ééntje kunt promoveren; dit wordt echter zelden in praktijk gebracht.

Stellingen behorend bij het proefschrift: "Enzymatic synthesis of polyol esters in aqueous-organic two-phase systems".

Anja Janssen, 11 juni 1993

*"We dance round in a ring and suppose*
*But the Secret sits in the middle and knows."*
R. Frost
From "The Secret Sits"

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# CONTENTS

# chapter 1

# INTRODUCTION

Lipase-catalyzed reactions are receiving increasingly attention in several fields of industry, such as food, chemical and pharmaceutical. Several reasons can be given. Generally, enzymatic synthesis is more selective than chemical synthesis. Selectivity is useful if one of the substrates contains, for example, more than one hydroxyl group. In a chemical process no distinction can be made between the different hydroxyl groups, which results in a mixture of reaction products, while enzymes can distinguish between primary and secundary hydroxyl groups. Furthermore, enzymes are active under mild reaction conditions. This is of special interest if high temperatures should be avoided, for example, at reactions with carbohydrates or unsaturated fatty acids, which will discolor at high temperatures. In addition, foods can be considered to be natural, if the additives that are used are synthesized with an enzyme as catalyst.

In this chapter, an overview is given of recent developments in lipase-catalyzed esterification. The esterification of carbohydrates and fatty acids and the use of organic solvents in enzymatic synthesis are discussed in more detail.

## LIPASE-CATALYZED ESTERIFICATION

Lipases belong to the enzymatic class of hydrolases and catalyse both hydrolysis and synthesis of esters. This thesis is focussed on lipase-catalyzed esterification. Esterification is the reaction between an alcohol with one or more hydroxyl groups and a carboxylic acid,

such as fatty acids. Although the possibility to catalyse the esterification of glycerol and fatty acid with lipase is already known for a very long time,[24] only for the last two decades this topic is more extensively studied. In 1977, Tsujisaka et al.[60] described the esterification of glycerol and fatty acid by four types of lipases. They showed that not only fatty acids, but also dicarboxylic and aromatic acids were recognized as substrate by some lipases. Nowadays, a broad variety of esters can be synthesized enzymatically. Besides esterification, also examples of inter- and transesterification are discussed. Interesterification is the reaction between two esters with exchange of fatty acids. Transesterification is the reaction between an ester and a fatty acid (also called acidolysis) or between an ester and an alcohol (also called alcoholysis). Lipase-catalyzed hydrolysis is not discussed here. For this subject, the reader is referred to reviews of John et al.[25] and Mukherjee,[46] that are dealing with applications of lipase catalysis in general.

**Synthesis of fatty acid-based surfactants**

Interest is growing in lipase-catalyzed synthesis of emulsifying agents such as monoacylglycerol, carbohydrate esters and amino acid esters. These esters have gained in importance since they are food grade and biodegradable.[32] Fatty acid derivatives are applied in several fields of industry for example the food, cosmetic, detergent, and textile auxiliary industry.

Monoacylglycerols can be obtained by alcoholysis of glycerol and triacylglycerols,[23,39,40] or by esterification of glycerol and fatty acid.[62,66,68] Monoacylglycerol has better emulsifying properties than a mixture of acylglycerols and for this reason it is desirable to obtain monoacylglycerol at high purity. The most useful method is to prevent the reaction from reaching equilibrium by removing the product (monoacylglycerol) from the reaction medium. This can be done by crystallization[39,40] or adsorption on silica[62].

An efficient process for the enzymatic carbohydrate ester synthesis is described by Björkling et al.[5] They developed a solvent-free process in which alkyl-glucoside and long-chain fatty acids were mixed with a heat-stable, immobilized lipase derived from a strain of *Candida antarctica*. High monoester yields were obtained when the reaction was performed at 70 °C under reduced pressure. Carbohydrate ester synthesis is discussed in more detail in the next section of this chapter.

Lipase-catalyzed esterification of the amino acid L-homoserine and fatty acid has been observed in an aqueous-organic two-phase system[47]. The emulsifying activity of the product is found to be higher than for nonionic surfactants. The same type of surfactants is synthesized by the transesterification of soybean oil and lysine.[42] In this case an amine instead of an alcohol is used as substrate.

**Synthesis of flavors**

Lipase-catalyzed esterification is described for the synthesis of short-chain flavor esters, such as isoamyl acetate[31] and ethyl butyrate,[18,67] which are found in the aroma of banana and strawberry, respectively. The ability of lipase to distinguish between enantiomers is often used for the synthesis of flavor esters. For example, the production of chiral hydroxyacid esters, which are constituents of various tropical fruits, is described.[12] Besides production of an enantiomeric ester, stereoselective esterification is also used for the resolution of racemic alcohols, for example *d,l*-menthol.[30,56]

Usually alcohol and acid are two separate molecules, however, if both alcohol and acid are present in one molecule, intramolecular esterification can take place. An example is the lipase-catalyzed lactonization reaction of hydroxy acids to macrocyclic mono- and oligolactones.[3] Macrocyclic lactones are aromatic substances that are used in perfumes.

**Synthesis of edible oil equivalents**

The synthesis of triacylglycerols with characteristic properties, such as a specified melting range, is useful for the food industry. A well-known example is the production of cocoabutter equivalents, which is prepared by acidolysis of palm oil and stearic acid with a 1,3-specific lipase.[33] The composition of the esterified product is almost the same as the composition of cocoabutter. The interesterification of butterfat is catalyzed by a non-specific lipase.[26] Fatty acids are randomly distributed among the triacylglycerols of the reaction product, which results in a product with a lower melting range as compared to the untreated butterfat. To prevent hydrolysis of triacylglycerols during interesterification or acidolysis, the water content of the reaction mixture has to be low.

It is also possible to produce triacylglycerols by esterifcation of glycerol and fatty acid. It is important to remove the by-product water thus keeping the water activity of the system low, in order to obtain pure triacylglycerol instead of a mixture of mono-, di-, and

triacylglycerols. This can be done by spontaneous evaporation, molecular sieves, vacuum or dry air bubbling.[13] By using a pervaporation system it is even possible to integrate the reaction surface and the water removal surface.[63]

**Monomer and polymer synthesis**

The synthesis of most of the above mentioned esters is useful in the food, flavor and detergents industry. However, interest for enzymatic synthesis is growing also in the chemical industry. Lipase-catalyzed esterification of diols and acrylic or methacrylic acids to produce monomers for the plastic industry has been shown to be technically feasible.[20,59] Also the enzymatic synthesis of polymers is described.[10] Alkyds (unsaturated polyesters) were prepared by lipase-catalyzed polytransesterifications of diesters of fumaric acid and 1,4-butanediol.[16,17] Polycarbonate, which is of commercial importance as high performance plastic, has been synthesized through the condensation reaction between diphenyl carbonate and a number of bifunctional alcohols.[1]

**Synthesis of amides**

As already mentioned for the synthesis of N- $\epsilon$ -oleyllysine,[42] except of an alcohol, also an amine can be a substrate for lipase. Margolin and Klibanov[35] showed the feasibility of peptide synthesis. The lipase-catalyzed synthesis of N-lauryloleylamide[41] and several other fatty amides[4] has been reported. Fatty amides, which have a high melting point and which are physically and chemically quite stable, find many applications in the textile, paper, wood, metal, rubber, plastic and coating industry. Fatty hydroxamic acids were synthesized by the reaction of hydroxyl amine with fatty acid.[54] These chelating agents are used in analytical chemistry, therapeutics and agronomy.

## ENZYMATIC SYNTHESIS OF CARBOHYDRATE ESTERS

In the previous section, an overview was given of possible lipase-catalyzed esterification reactions. The synthesis of a broad variety of esters and their applications was described. However, parameters that affect the product concentration, such as choice of enzyme, activity and stability of the enzyme, substrate concentration and choice of

solvent were not considered. In this section, these aspects are discussed for the esterification of carbohydrates and fatty acids. The choice of solvent is discussed in more detail in the next section.

For the enzymatic esterification of fatty acids and carbohydrates, an appropriate reaction medium has to be chosen. Carbohydrates are solid substances at the reaction temperature and only polar solvents can be used to dissolve reasonable amounts of these carbohydrates. Pyridine,[7,57] dimethylformamide,[2,6,50] and 2-methyl-2-butanol[27] are found to be suitable solvents. In these solvents, the carbohydrate and the fatty acid do dissolve, however, enzymes do not. This means that the reaction mixture consists of a suspension of enzyme particles in the solvent. This type of reaction system is used for the alcoholysis of various plant and animal oils with sugar alcohols.[7] Other examples are the alcoholysis of activated fatty acids with monosaccharides[57] and disaccharides,[6,50] and the esterification of oleic acid with glucose[2] and fructose.[27] Besides lipases, the proteases subtilisin[6,50] and alkylated trypsin[2] are used for carbohydrate ester synthesis.

Another approach in enzymatic esterification of carbohydrates is to modify the carbohydrate in order to increase the solubility in organic solvents. Examples are described in literature of mono- and disaccharides with one blocked hydroxyl group that are soluble in solvents such as acetone, tetrahydrofuran, and methylene chloride.[6,58] Furthermore, acetylated sugars are found to be soluble in less polar solvents such as chloroform and diethyl ether.[9]

Specificity is often mentioned as an advantage of using an enzyme instead of a chemical catalyst. In the studies mentioned before,[6,7,50,57] both lipase and subtilisin, exhibit a strong preference towards the primary hydroxyl groups of the carbohydrate. In case of monosaccharides with a blocked primairy hydroxyl group, a preference towards C-2 or C-3 hydroxyl groups exists, depending on the source of lipase and the monosaccharide used.[58] An overview of the selectivity of enzymatic acylation of carbohydrates is given by Riva and Secundo.[51]

Selective esterification of carbohydrates is shown in the studies mentioned before,[6,7,50,57,58] however, yields and/or reaction rates are very low. In addition, the solvents that are used in these studies are not accepted for food applications. For large-scale processing, a higher yield and reaction rate is desired. Furthermore, the reuse and stability of the enzyme has to be taken into account and the use of toxic solvents should be avoided. Part of these conditions are fulfilled in the process of Björkling et al.[5] and Fregapane et al.[15] Björkling et al.[5] studied the esterification of alkyl-glucosides with

long-chain fatty acid. This process was already described in the previous section. Fregapane et al.[15] showed the lipase-catalyzed esterification of sugar acetals and fatty acids at 75 °C. In this process, catalyzed by immobilized lipase of *Mucor miehei* (Lipozyme), mono- and diesters are formed and yields of 50-90% were obtained. The product of the reaction had to be subjected to a mild acid-hydrolysis in order to remove the acetal group. In both papers the stability of the lipase was not discussed. This might be important, because of the high reaction temperatures that were used.

The use of lipase as catalyst for the carbohydrate ester synthesis is an attractive option, because of the mild reaction conditions and selective catalysis. Until now reported reaction rates are low and modification of the carbohydrate is necessary for the processes that seem to be the most suitable ones for large-scale processing.[5,15]

## ORGANIC SOLVENTS

In the previous section, the use of organic solvents in enzymatic esterification is already mentioned. Many years ago, the idea that enzymes are only active in aqueous solutions was found to be wrong. Many enzymes have been shown to be active in the presence of organic solvents. This greatly enhanced the possibilities for enzyme-catalyzed synthesis. Nonpolar substrates can be used, which are immiscible with water and synthesis reactions (reverse hydrolysis) are possible. In the first part of this section, an overview is given of some aspects of the use of enzymes in aqueous-organic solvent mixtures, aqueous-organic two-phase systems, and anhydrous organic solvents. In the second part, the effect of organic solvents on the equilibrium position in aqueous-organic two-phase systems is discussed in more detail.

### Organic solvents in enzymatic synthesis

*Aqueous-organic solvent mixtures*

In aqueous-organic solvent mixtures, a water-miscible solvent, called cosolvent, is added to an aqueous solution in order to increase the solubility of nonpolar substrates and to reduce the water activity. These reaction systems are found to be useful for synthesis reactions such as the synthesis of peptides,[8,49] D-mandelonitrile,[65] and the antibiotic

penicillin G.[14] Small amounts of cosolvents, such as acetonitrile, tetrahydrofurane, acetone, and methanol can be added without affecting the activity and stability of the enzyme. In some cases even an increase in the enzyme activity is reported at low cosolvent concentrations.[19] A high cosolvent concentration will certainly lead to a loss of activity of the enzyme. The dependence of enzyme activity, expressed in terms of the maximal reaction rate velocity ($v_m$), on the cosolvent concentration is found to have a threshold character.[43] The threshold concentration is the concentration of the cosolvent at which the enzyme has lost half of its activity. The threshold concentration can be predicted by the denaturation capacity.[28] The latter is a new criterion for selection of organic solvents as reaction media in biocatalysis. Methods to stabilize enzymes against the denaturing effects of cosolvents, such as multi-point interaction with a support material and selective chemical modification are recently reviewed.[44,45]

*Aqueous-organic two-phase systems*

Bio-organic synthesis is often performed in a reaction system, consisting of two phases; an aqueous phase and an organic phase. The organic phase is composed of the organic solvent and serves as a reservoir for the nonpolar reactants. The enzyme is located in the aqueous phase, except for lipase which usually prefers the interface between the aqueous and organic phase. Two types of two-phase system can be distinguised, the emulsion-type system and the trapped aqueous phase system.[21] In an emulsion-type system the volumes of the organic and the aqueous phase are of the same order of magnitude, resulting in a water-in-oil or an oil-in-water emulsion. In a trapped aqueous phase system, the aqueous phase is very small and can be restricted to the pores of the catalyst particle. Although the water content of trapped aqueous phase systems can be very low, the water activity is still close to 1. The possibility to solubilize the enzyme in a microemulsion,[36] which consists of small water droplets stabilized by surfactants, in a bulk organic solvent is left out of consideration in this chapter.

The choice of the organic solvent will affect the activity and stability of the enzyme. Attempts are made to elucidate the solvent properties which are important for their use in enzymatic synthesis. Several solvent parameters, such as the Hildebrand solubility parameter, dielectric constant and log *P*, have been used to describe the activity of immobilized bacterial cells in different solvents.[29] Log *P*, which is the logarithm of the partition coefficient in the octanol-water two-phase system, is found to give the best correlation. It was shown that nonpolar solvents, having a log *P* value of 4 or more, were

suitable for microbial synthesis.[29] Log *P* can also be used to correlate enzymatic activity with the polarity of the medium.[48] Recently, log *P* in combination with the electron acceptance index or the polarizability of the solvent showed a good correlation with the initial esterification activity of lipase.[61] Schneider[53] introduced the three-dimensional solubility parameter approach to predict enzyme activity in all kind of nonaqueous systems. He expected this approach to be more universal because polar, dispersive and hydrogen bonding interactions are taken into account. However, more data are necessary to approve the usefulness of this approach.

In addition to correlation of log *P* with enzyme activity, log *P* also correlates with enzyme stability.[48] Nonpolar solvents gave better operational stability than polar solvents. Furthermore, the denaturation capacity,[28] which is already discussed for the aqueous organic solvent mixtures, can be applied to immiscible organic solvents in two-phase systems.

*Anhydrous organic solvents*

Anhydrous organic solvent systems can be compared with the trapped aqueous phase systems. The difference is that the water content is reduced until no discrete aqueous phase is present anymore. This results in a water activity far below 1. The enzyme, which is usually freeze dried before addition to the anhydrous organic solvent, is not soluble in this system and is present as suspended catalyst particles. The amount of water required for enzyme activity is found to be much less than that needed to form a monolayer on the surface of the enzyme.[70,71] The enzyme has a very rigid conformation at these conditions. Due to this rigidity, the enzyme activity can be enhanced by the use of ligands, for example competitive inhibitors.[52] If a ligand is added to the enzyme solution before freeze drying, the enzyme is much more active in an anhydrous reaction medium than the enzyme freeze dried without such a ligand. In the presence of a ligand, the enzyme is locked in a conformation resembling the enzyme-substrate complex. However, addition of small amounts of water to the reaction medium loosens up the protein molecules and irreversibly destroys the 'enzyme memory'.

Another phenomenon that is due to the rigidity of the enzyme in anhydrous organic solvents is the increased thermostability.[69] The reduced mobility of the protein molecule hinders partial unfolding which is the first step of the inactivation process. Furthermore, processes which lead to the irreversible inactivation of the enzyme require water and will be prevented in anhydrous organic solvents.

**Solvent effects on equilibrium**

*Dilute systems*

The equilibrium position of a reaction is described by the equilibrium constant. For a reaction

$$A + B \rightleftharpoons C + H_2O$$

the equilibrium constant $K$ is given by

$$K = \frac{a_C \cdot a_{H_2O}}{a_A \cdot a_B} \tag{1}$$

where $a_i$ is the thermodynamic activity of component $i$. The activity is related to the molal concentration (mole.kg$^{-1}$) or to the mole fraction (mole.mole$^{-1}$) of a component by the activity coefficient. If activity coefficients are normalized according to Henry's law, the activity coefficients in dilute solutions will be nearly one for the solutes as well as for the solvent. For dilute aqueous solutions, the molal concentration in mole.kg$^{-1}$ is equal to the molar concentration in kmole.m$^{-3}$. This means that the equilibrium constant for an ideal dilute aqueous solution ($K_w$) becomes

$$K_w = \frac{[C]_w \cdot [H_2O]_w}{[A]_w \cdot [B]_w} \tag{2}$$

where $[i]_w$ is the molar concentration of component $i$ in the aqueous phase.

In two-phase systems, the reactants are distributed between the aqueous and the organic phase (figure 1). This partitioning is dependent on the characteristics of the component, and the aqueous and the organic phase and is quantified by partition coefficients. The partitioning of the reactants will affect the reactant concentrations at equilibrium. In this section, models for the prediction of the equilibrium position in two-phase systems are reviewed.

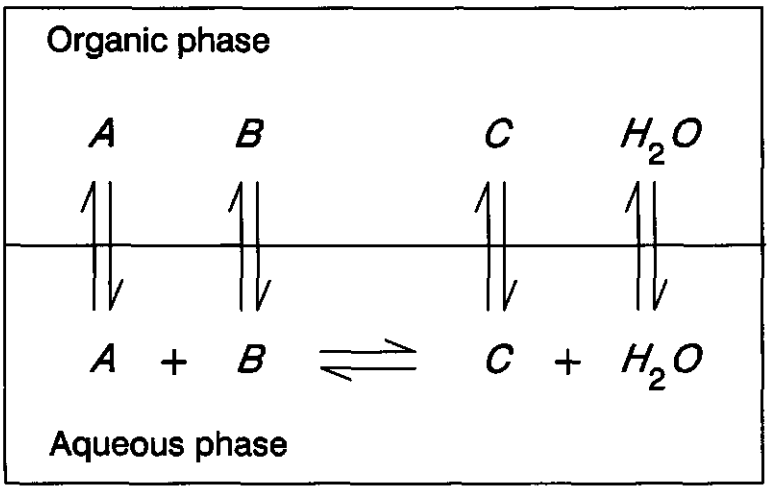


**Figure 1:** Enzyme catalysis in an aqueous-organic two-phase system (adapted from Eggers et al.[11]).

More than a decade ago, Martinek et al.[37,38] developed a model, where the equilibrium position in a two-phase system is compared with the equilibrium position in a one-phase system, with water as the reaction medium. In this model the biphasic product concentration, which is the total amount of product divided by the total volume of the biphasic system, is related to the partition coefficients of substrates and products and to the volume ratio of both phases. A requirement is that both phases are dilute, which means that the substrate and product concentrations are low. In that case the equilibrium constant can be expressed as concentrations rather than as activities (equation (2)).

In the model of Martinek an apparent equilibrium constant ($K_{bi}$) is defined

$$K_{bi} = \frac{[C]_{bi} \cdot [H_2O]_{bi}}{[A]_{bi} \cdot [B]_{bi}} \quad (3)$$

where $[i]_{bi}$ is the biphasic concentration of component *i*. Based on a mass balance, the biphasic concentration can be expressed by

$$[i]_{bi} = \frac{[i]_w \cdot (1 + P_i \cdot \alpha)}{1 + \alpha} \quad (4)$$

$P_i$ is the partition coefficient, given by

$$P_i = \frac{[i]_{org}}{[i]_w} \quad (5)$$

where $[i]_{org}$ and $[i]_w$ are the concentration of component *i* in the organic and aqueous phase, respectively. The volume ratio $\alpha$ is defined as

$$\alpha = \frac{V_{org}}{V_w} \quad (6)$$

where $V_{org}$ is the volume of the organic phase and $V_w$ is the volume of the aqueous phase. Combining equations (3) and (4) gives

$$K_{bi} = K_w \cdot \frac{(1 + \alpha \cdot P_C)(1 + \alpha \cdot P_{H_2O})}{(1 + \alpha \cdot P_A)(1 + \alpha \cdot P_B)} \quad (7)$$

where $K_w$ is the equilibrium constant for an ideal dilute aqueous solution as defined in equation (2).

For enzymatic reactions it is often preferred to express the amount of water in mole fractions and the amount of the other reactants in concentrations.[55] The advantage is that in dilute aqueous solutions, the water activity is close to one, since both the activity coefficient and the mole fraction of water approximate one. With the assumption that the water activity is one, equation (1) becomes

$$K_w^* = \frac{[C]_w \cdot a_{H_2O}}{[A]_w \cdot [B]_w} = \frac{[C]_w}{[A]_w \cdot [B]_w} \tag{8}$$

The equilibrium constants, where the amount of water is expressed as mole fraction and the amounts of other reactants are expressed as concentrations, are marked with the superscript *. The dimension of these equilibrium constants is $m^3.kmole^{-1}$ and its value will be different from that of the equilibrium constants, where the water concentration is used.

Eggers et al.[11] followed this approach and proposed to use an apparent equilibrium constant that is only a function of the concentrations of $A$, $B$, and $C$

$$K_{bi}^* = \frac{[C]_{bi}}{[A]_{bi} \cdot [B]_{bi}} \tag{9}$$

Analog to the model of Martinek, mass balances are used to express the equilibrium constant as a function of the partition coefficients and the volume ratio

$$K_{bi}^* = K_w^* \cdot \frac{(1 + \alpha \cdot P_C)(1 + \alpha)}{(1 + \alpha \cdot P_A)(1 + \alpha \cdot P_B)} \tag{10}$$

$K_{bi}/K_w$ and $K_{bi}^*/K_w^*$ are related by

$$K_{bi}/K_w = K_{bi}^*/K_w^* \cdot \frac{1 + \alpha \cdot P_{H_2O}}{1 + \alpha} \tag{11}$$

The esterification of 1-propanol and butanoic acid in a two-phase system of water and hexane is used to show the differences between the model of Martinek (equation (7)) and the model of Eggers (equation (10)). The partition coefficients are estimated by using the UNIFAC group contribution method and the UNIFAC parameter table of Magnussen et al.[34] This reaction is chosen as an example, since the partition coefficients of the substrates are low and the partition coefficient of the ester is high. This indicates that the substrate concentrations will be relatively high in the aqueous phase, while the product (ester) will be extracted to the organic phase. The ratios of $K_{bi}/K_w$ and $K_{bi}^*/K_w^*$ are used as a measure of the equilibrium position in a two-phase system as compared to

that in an aqueous solution. In figure 2 the logarithms of $K_{bi}/K_w$ and $K_{bi}^*/K_w^*$ are plotted as a function of the logarithm of the volume ratio $\alpha$. The equation of Martinek (figure 2a) predicts an optimum in $K_{bi}/K_w$ at a volume ratio of 1. The equation of Eggers (figure 2b) predicts an increase in $K_{bi}^*/K_w^*$ by increasing the volume ratio, indicating that a higher degree of esterification is obtained at higher values of $\alpha$. If $P_{H_2O} \ll 1$, one can derive from equation (11) that at low values of $\alpha$ ($\alpha \ll 1$), $K_{bi}/K_w$ and $K_{bi}^*/K_w^*$ are equal, which also can be seen in figures 2a and 2b. Furthermore, at high values of $\alpha$ ($\alpha \gg 1$ and $P_{H_2O} \ll 1$), $1/(1+\alpha)$ decreases, which results in a decrease of $K_{bi}/K_w$ with regard to $K_{bi}^*/K_w^*$. This trend also can be seen in figures 2a and 2b.

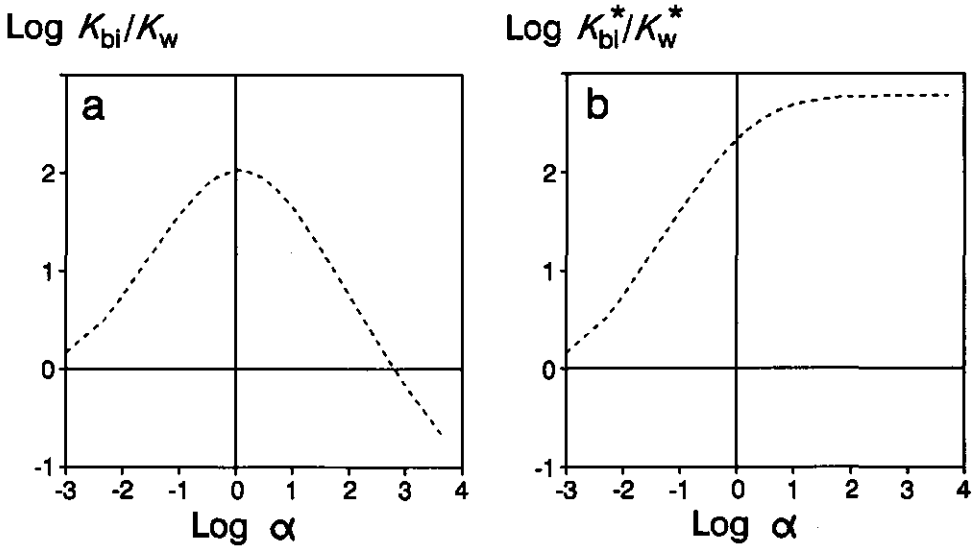


**Figure 2:** Prediction of equilibrium data as a function of log $\alpha$ for the esterification of 1-propanol and butanoic acid in a water-hexane two-phase system. Partition coefficients are 0.35 (propanol), 2.2 (butanoic acid), 460 (propyl butanoate) and $1.2 \times 10^{-4}$ (water). Part a is calculated from equation (7) and part b is calculated from equation (10).

In contrast with the apparent equilibrium constant, the equilibrium constants for an ideal dilute aqueous solution $K_w$ and $K_w^*$ are independent of $\alpha$. If $K_w$ or $K_w^*$, and the initial biphasic substrates concentrations are known, the biphasic product concentration as a function of $\alpha$ can be calculated from mass balances and either equation (7) or equation (10). As was discussed by Eggers et al.,[11] the biphasic product concentration is a more useful parameter than $K_{bi}/K_w$ or $K_{bi}^*/K_w^*$ for the comparison of two-phase reaction systems. The biphasic product concentrations as a function of $\alpha$, calculated with the equations of Martinek et al.,[38] and Eggers et al.,[11] respectively, are shown in figure 3 and the curves are found to be exactly the same. Figure 3 shows that the maximum in the plot

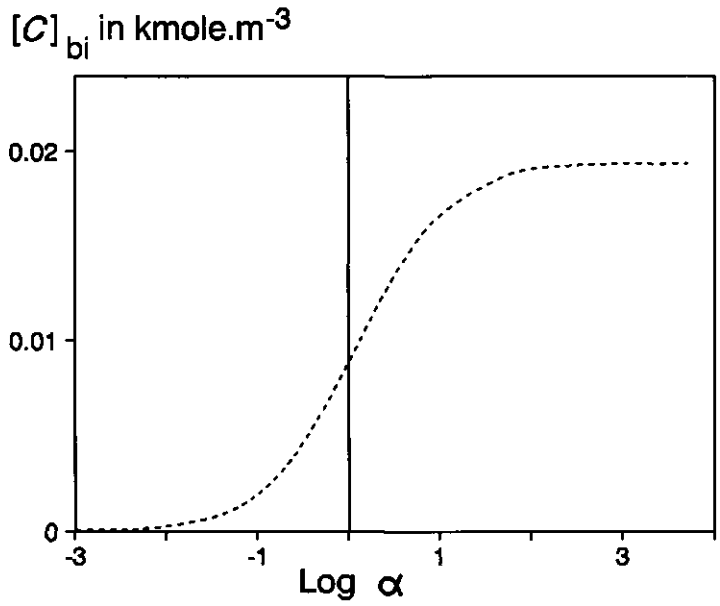


**Figure 3:** Prediction of the biphasic product concentration in hexane as a function of log $\alpha$ with the equations of Martinek et al.[38] or Eggers et al.[11]
Initial concentrations: 0.1 kmole.m$^{-3}$ butanoic acid and 0.1 kmole.m$^{-3}$ 1-propanol; biphasic volume = 2.10$^{-3}$ m$^3$; $K_w$ = 0.278, and $K_w^*$ = 0.005 m$^3$.kmole$^{-1}$; $P_A$ = 0.35, $P_B$ = 2.2, $P_C$ = 460 and $P_{H_2O}$ = 1.2×10$^{-4}$.

of log $K_{bi}/K_w$ versus log $\alpha$ (figure 2a) is not an indication of a maximum biphasic product concentration. However, the course of figure 2b, where log $K_{bi}^*/K_w^*$ is plotted versus log $\alpha$, is the same as the course of figure 3, where $[C]_{bi}$ is plotted versus log $\alpha$. Due to this correlation, it is more convenient to use the equations of Eggers et al.[11]

Eggers et al.[11] argued that it was incorrect to include the partition coefficient of water as Martinek et al.[38] did. They showed a figure in which they compared an experimentally $K_{bi}$, determined by Martinek et al., with $K_{bi}^*$ that is calculated according to equation (3). However, $K_{bi}$ and $K_{bi}^*$ as well as $K_w$ and $K_w^*$ are essentially different

$$K_{bi}^* = \frac{K_{bi}}{[H_2O]_{bi}} \quad \text{and} \quad K_w^* = \frac{K_w}{[H_2O]_w} \tag{12}$$

This means that Eggers et al.[11] incorrectly compared $K_{bi}$ with $K_{bi}^*$. Furthermore, in the calculation of $K_{bi}^*$, Eggers et al.[11] have used $K_w$, obtained from Martinek et al.[38] instead of $K_w^*$. The correct $K_{bi}^*$ and $K_w^*$ can be obtained by division of the experimental $K_{bi}$ and $K_w$ of Martinek et al.[38] by $[H_2O]_{bi}$ and $[H_2O]_w$, respectively, as proposed in equation (12). This results in an experimental $K_{bi}^*$, which is in good agreement with the calculated $K_{bi}^*$, according to equation (10) as is shown in figure 4.

If partition coefficients are known in several solvents, equation (10) can also be used to predict the effect of solvents on the apparent equilibrium constant $K_{bi}^*$. Partition coefficients of 1-propanol, butanoic acid, propyl butanoate and water in several solvents are estimated by using the UNIFAC group contribution method. These partition coefficients, and $K_{bi}^*/K_w^*$, calculated with equation (10) are shown in table I. It can be expected that for the dilute two-phase systems of table I, $K_w^*$ has a constant value. Therefore, an increase in $K_{bi}^*/K_w^*$ indicates an increase in $K_{bi}^*$. Equation (9) shows that an increase in $K_{bi}^*$ results in an increase in $[C]_{bi}$ at constant initial biphasic concentrations of A and B. Then it can be concluded from the data in table I that esterification is favorable in solvents with low partition coefficients for the substrates, 1-propanol and butanoic acid and a high partition coefficient for the ester. The latter condition is not very clear from table I, since the values of all partition coefficients for the ester are high.

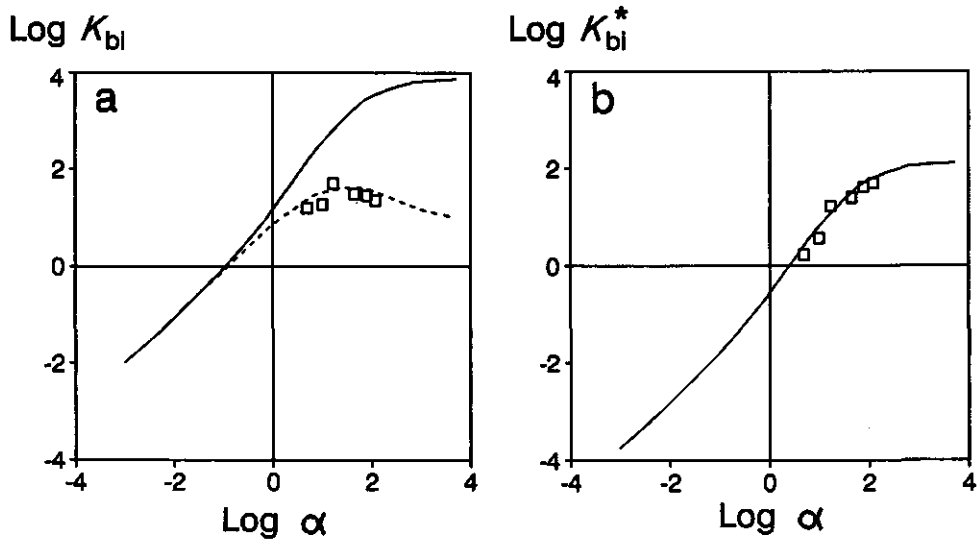


**Figure 4:** Reproduction of equilibrium data for the synthesis of N-benzoyl-L-phenylalanine ethyl ester in a water-chloroform reaction system.
Part a is taken from Eggers et al.[11]: The symbols are the experimental values of $K_{bi}$ from Martinek et al.,[38] the dotted and the solid lines were calculated with equation (7) and (10), respectively, using $P_A$ = 0.11, $P_B$ = 0.01, $P_C$ = 4100, $P_{H_2O}$ = 0.0012 and $K_w$ = 0.002 (obtained from Martinek et al.[38]).
Part b is a recalculation: The symbols are the experimental values of $K_{bi}$ from Martinek et al.,[38] divided by $[H_2O]_{bi}$. For the calculation of the curve, equation (10) was used with $K_w^*$ = 3.6 · $10^{-5}$ $m^3.kmole^{-1}$ (= $K_w/[H_2O]_w$).

Halling[22] followed another approach for the prediction of solvent effects on reaction equilibria in dilute systems. He proposed to define separate equilibrium constants for the aqueous phase ($K_w$) and the organic phase ($K_{org}$). Thus for the reaction of figure 1

$$K_w = \frac{[C]_w \cdot [H_2O]_w}{[A]_w \cdot [B]_w}; \qquad K_{org} = \frac{[C]_{org} \cdot [H_2O]_{org}}{[A]_{org} \cdot [B]_{org}} \tag{13}$$

**Table I:** Partition coefficients and equilibrium data for the esterification of 1-propanol and butanoic acid at a phase volume ratio ($\alpha$) of 1.

| Solvent | Partition coefficients[a] for | | | | $K_{bi}^*/K_w^*$ eq. (10) |
|---|---|---|---|---|---|
| | Propanol | Butanoic acid | Ester | Water | |
| 2-Methyl-2-butanol | 17 | 6.9 | 1200 | 0.14 | 17 |
| Octanol | 17 | 5.1 | 1500 | 0.043 | 27 |
| Tetrachloromethane | 6.6 | 1.5 | 600 | 0.00017 | 63 |
| Chloroform | 15 | 3.5 | 5000 | 0.0014 | 139 |
| Diethyl ether | 8.4 | 4.7 | 4500 | 0.012 | 168 |
| Isooctane | 0.3 | 1.9 | 360 | 0.00012 | 192 |
| Hexane | 0.35 | 2.2 | 460 | 0.00012 | 213 |
| Benzene | 0.28 | 1.8 | 1700 | 0.00065 | 949 |

Note:

[a] Partition coefficients are estimated by using the UNIFAC group contribution method and the UNIFAC parameter table of Magnussen et al.[34]

To compare the equilibrium position in a two-phase system with that of an aqueous solution, the ratio of the two equilibrium constants can be used

$$K_{org}/K_w = \frac{[C]_{org}\cdot[H_2O]_{org}}{[C]_w\cdot[H_2O]_w}\cdot\frac{[A]_w\cdot[B]_w}{[A]_{org}\cdot[B]_{org}} = \frac{P_C\cdot P_{H_2O}}{P_A\cdot P_B} \qquad (14)$$

The change of $K_{org}/K_w$ on changing the organic solvent can now be calculated if the partition coefficients are known for every reactant. It is convenient to rewrite equation (14) as

$$\log K_{org}/K_w = \log P_C + \log P_{H_2O} - \log P_A - \log P_B \qquad (15)$$

For the determination of the logarithm of the partition coefficients group contribution relations are available. These relations assume that molecules are divided

into functional groups. Every group has a certain value, and the sum of these values is the logarithm of the partition coefficient. Many groups in reactant *A* and *B* will not be changed by the reaction and these groups will also be present in the product *C*. This means that $\log K_{org}/K_w$ is only dependent on the values of the groups that change during the reaction. For example, for an esterification reaction, $\log K_{org}/K_w$ is determined by the hydroxyl, carbonyl, water and ester groups

$$\text{Log}\, K_{org}/K_w = \text{"}-COO-\text{"}\ \text{group} + \text{"}H_2O\text{"}\ \text{group} - \text{"}-COOH\text{"}\ \text{group} - \text{"}-OH\text{"}\ \text{group} \quad (16)$$

Halling[22] concludes that if only the reacting groups will have an effect on $\log K_{org}/K_w$, the equilibrium position in different solvents is equal for all reactions of the same type.

Valivety et al.[61] studied the effect of solvents on an esterification reaction in a reaction system that consists predominantly of organic solvent. However, the water activity of this system is still close to 1. They proposed to use a practically useful equilibrium constant ($K_0^*$)

$$K_0^* = K^* \cdot \frac{\gamma_A \cdot \gamma_B}{\gamma_C} = \frac{[C]_{org} \cdot a_{H_2O}}{[A]_{org} \cdot [B]_{org}} \quad (17)$$

where the equilibrium constant $K^*$ is a quotient of activities as defined in equation (1) and $\gamma_i$ is the activity coefficient of component *i*. The superscript * in $K^*$ and $K_0^*$ indicates that the amount of water is expressed as mole fraction, while the amounts of *A*, *B*, and *C* are expressed as concentrations.

The different methods to predict the effect of solvents on the equilibrium position are compared in table II. Data of the esterification of 1-propanol and butanoic acid calculated according to equation (10) of Eggers et al.[11] (data from table I), are compared with data for aliphatic esterification reactions and data of the esterification of ethanol and acetic acid (obtained from Halling[22]). Furthermore, experimental data for the esterification of dodecanol and decanoic acid, obtained from Valivety et al.[61], are shown. No experimental data are available in the solvent octanol, because this solvent is also a substrate for the lipase-catalyzed esterification. Although $K_{bi}^*/K_w^*$ is not the same as

$K_{org}/K_w$ and is certainly different from $K_0^*$, the logarithms of these variables are an indication of the degree of esterification in a certain solvent. Therefore, the relative values of the logarithm of $K_{bi}^*/K_w^*$, $K_{org}/K_w$, and $K_0^*$, respectively, are compared.

**Table II:** Equilibrium data for some esterification reactions in several solvents.

| Solvent | Logarithm of | | | |
|---|---|---|---|---|
| | $K_{bi}^*/K_w^*$ [a] eq. (10) | $K_{org}/K_w$ [b] eq. (16) | $K_{org}/K_w$ [c] eq. (15) | $K_0^*$ [d] eq. (17) |
| Octanol | 1.43 | 1.16 | 1.21 | - |
| Tetrachloromethane | 1.80 | 5.05 | 4.87 | 2.86 |
| Chloroform | 2.14 | - | 4.25 | 2.18 |
| Diethyl ether | 2.23 | 1.56 | 1.84 | 1.26 |
| Isooctane | 2.28 | - | - | 3.20 |
| Hexane | 2.33 | - | 5.45 | 2.85 |
| Benzene | 2.98 | 4.52 | 4.68 | 2.52 |

Notes:

[a] $K_{bi}^*/K_w^*$ for esterification of 1-propanol and butanoic acid. Data are from table I.

[b] $K_{org}/K_w$ for aliphatic esterification reactions. Data are from Halling.[22]

[c] $K_{org}/K_w$ for esterification of ethanol and acetic acid. Data are from Halling.[22]

[d] $K_0^*$ for esterification of dodecanol and decanoic acid. Data are from Valivety et al.[61]

Table II shows that octanol is in all cases the least favorable solvent for esterification. The nonpolar solvents, isooctane, hexane and benzene, are clearly good solvents for esterification. In all prediction methods, these solvents showed relatively high values. In tetrachloromethane high values are obtained for $K_{org}/K_w$ and $K_0^*$, however, $K_{bi}^*/K_w^*$ is relatively low. The opposite is found for diethyl ether, a relatively high value for $K_{bi}^*/K_w^*$ and low values for $K_{org}/K_w$ and $K_0^*$. The values of chloroform are in all cases somewhere in the middle, which means that this is an intermediate solvent for esterification.

The values that are presented in table II are obtained from experiments and different calculation methods, but also from different esterification reactions. The results in this table show that in different solvents, the relative equilibrium positions for these esterification reactions are in most cases the same. This is in agreement with the findings of Halling.[22]

*Nondilute systems*

For nonideal systems, activities instead of concentrations must be used. The activity of component i ($a_i$) is related to the concentration through the activity coefficient ($\gamma_i$)

$$a_i = \gamma_i \cdot [i] \tag{18}$$

The water activity ($a_w$) is often related to the mole fraction ($x_w$) and the activity coefficient

$$a_w = \gamma_w \cdot x_w \tag{19}$$

In two-phase systems at equilibrium, the activity of component *i* in the aqueous phase is equal to the activity of that component in the organic phase. If activities and activity coefficients instead of concentrations are used in the mass balance, the expression for the biphasic concentration becomes

$$[i]_{bi} = \frac{a_i}{(1+\alpha)} \cdot \left( \frac{1}{(\gamma_i)_w} + \frac{\alpha}{(\gamma_i)_o} \right) \tag{20}$$

Straathof et al.[55] showed that for nondilute reaction systems, equation (10) for the apparent equilibrium constant can be described as

$$K^*_{bi} = \frac{[C]_{bi} \cdot a_{H_2O}}{[A]_{bi} \cdot [B]_{bi}} = K^* \cdot \frac{\left( \frac{1}{(\gamma_C)_w} + \frac{\alpha}{(\gamma_C)_{org}} \right) \cdot (1+\alpha)}{\left( \frac{1}{(\gamma_A)_w} + \frac{\alpha}{(\gamma_A)_{org}} \right) \cdot \left( \frac{1}{(\gamma_B)_w} + \frac{\alpha}{(\gamma_B)_{org}} \right)} \tag{21}$$

where the equilibrium constant $K^*$ is a quotient of activities as defined in equation (1). The supercript * in $K^*$ indicates that the amount of water is expressed as mole fraction, while the amounts of $A$, $B$, and $C$ are expressed as concentrations. Equation (21) can be used for two-phase systems with a non-ideal aqueous phase, for example, esterification of glycerol and fatty acid.[64] To calculate $K_{bi}^*$, the water activity, equilibrium constant, and several activity coefficients have to be known. Especially the determination of the activity coefficients may lead to problems, since these values are dependent on the composition of the reaction medium. Subsequently, activity coefficients may change when the composition of the reaction medium changes. This has to be taken into account when using equation (21).

## AIMS OF THIS WORK

The use of lipase as a catalyst for the synthesis of carbohydrate esters is attractive. For large-scale processing, high reaction rates and high monoester yields are desired. In addition, direct use of substrates without any modification is preferred. As described in the section 'Enzymatic synthesis of carbohydrate esters', until now, modification of the carbohydrates is necessary for the processes that until now are the most suitable for large-scale processing.

Especially for esterification reactions with more than one product, knowledge of the equilibrium position of the reaction is of importance. Dilute reaction systems are not of practical importance. For industrial applications, high product concentrations are desired, which implicate the use of nondilute reaction systems. In literature only one model is described for the prediction of the equilibrium position in non-dilute two-phase systems. However, to use this model, activity coefficients have to be determined as a function of the composition of the reaction medium.

The aim of this thesis is to develop a reaction system for the lipase-catalyzed esterification of carbohydrates and fatty acids without modification of the substrates and in which high reaction rates can be obtained. Furthermore, it is the aim to predict the product concentrations at the reaction equilibrium in non-dilute two-phase systems and to gain a better insight into the factors that affect the equilibrium concentrations.

## OUTLINE OF THE THESIS

In this thesis the lipase-catalyzed esterification of polyols and fatty acids is discussed. Special attention is focussed on the equilibrium position in two-phase reaction systems.

In chapter 2 and 3, two different two-phase reaction systems for the synthesis of carbohydrate ester are presented. In one system, the solvent 2-pyrrolidone is used (chapter 2). Several aspects, such as the influence of water, the specificity and stability of the enzyme and partitioning of the solvent are discussed. Also the application of a two-phase membrane reactor is discussed. In the other system, the carbohydrate is dissolved in water (chapter 3). The reaction system consists of an organic phase, containing the fatty acid, and an aqueous phase, which is a saturated carbohydrate solution. The importance of the water activity is discussed. Furthermore, a membrane reactor is presented in which it is possible to keep the water activity low during the reaction.

Addition of hexadecane to the reaction system of chapter 3 was found to have an enormous effect on the ester concentration at equilibrium. Solvent effects are described in chapter 4 for the model reaction between glycerol and decanoic acid. Experimental data are compared with calculations of a computer program called TREP (Two-phase Reaction Equilibrium Prediction). This program is based on the UNIFAC group contribution method and mass balances, and is developed for nondilute reaction systems with a water activity below 1.

In chapter 5 the esterification of glycerol and several fatty acids is studied. Experimental results are compared with calculations with TREP. Besides equilibrium concentrations, also the effect of the solvent on initial reaction rates is discussed. In chapter 6 the usefulness of the program TREP is shown for the esterification of decanoic acid and several alcohols. The reaction systems with 1,3-propanediol and sorbitol are emulsion-type systems, while the systems with 1-dodecanol and 1-butanol are trapped-aqueous phase systems.

In the general discussion (chapter 7), calculations with the program TREP are compared with data as reviewed in chapter 1. Furthermore, the possibility to increase the reaction rate of sorbitol ester synthesis by increasing the temperature is discussed. Temperature effects on the equilibrium ester concentation are also presented. Furthermore, attempts to synthesize sucrose esters enzymatically and the elucidation of the structure of sorbitol di-, tri-, and tetraesters are described.

## NOMENCLATURE

| | | |
|---|---|---|
| $a$ | activity | (-) or ($kmole.m^{-3}$) |
| $[i]$ | concentration of component i | ($kmole.m^{-3}$) |
| $K$ | equilibrium constant | (-) |
| $K_{bi}$ | biphasic equilibrium constant | (-) |
| $K_{org}$ | equilibrium constant for dilute organic phase | (-) |
| $K_w$ | equilibrium constant for dilute aqueous solution | (-) |
| $K_{bi}^*$ | biphasic equilibrium constant | ($m^3.kmole^{-1}$) |
| $K_w^*$ | equilibrium constant for dilute aqueous solution | ($m^3.kmole^{-1}$) |
| $K_0^*$ | practically useful equilibrium constant | ($m^3.kmole^{-1}$) |
| $P$ | partition coefficient | ($M_{org}.M_w^{-1}$) |
| $V$ | volume | ($m^3$) |
| $x$ | mole fraction | (-) |
| $\alpha$ | volume ratio | (-) |
| $\gamma$ | activity coefficient | (-) |

**Subscripts:**

| | |
|---|---|
| bi | biphasic |
| i,A,B,C | components |
| org | organic phase |
| w | aqueous phase |

**Superscripts:**

| | |
|---|---|
| * | using water mole fraction instead of water concentration |

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# chapter 2

# ENZYMATIC SYNTHESIS OF CARBOHYDRATE ESTERS IN 2-PYRROLIDONE


## SUMMARY

The lipase-mediated esterification of sorbitol and fatty acid was investigated in a two-phase system with 2-pyrrolidone as cosolvent for sorbitol. The lipase from *Chromobacterium viscosum* showed an initial esterification rate of 1.4 mmole.g$^{-1}$.h$^{-1}$, and after 74 h, 80% of the initial sorbitol content was converted into sorbitol esters. With fructose or glucose as a substrate, initial esterification rates were 0.2 and 0.04 mmole.g$^{-1}$.h$^{-1}$, respectively; disaccharides were not reactive at all. The effects of the sorbitol, fatty acid, water, and 2-pyrrolidone concentrations on esterification activity were studied. An excess of fatty acid and a water concentration around 1 M were found to be necessary for optimum ester production. The polar organic cosolvent 2-pyrrolidone can inactivate the lipase. It is a suitable cosolvent for carbohydrates, provided that its concentration is low.

Esterification was also studied in a two-phase membrane reactor. The value of the enzyme-based initial reaction rate was half of the reaction rate in an emulsion system. The water activity in the membrane system was relatively high, which resulted in low product yields.



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## INTRODUCTION

Esters of carbohydrates and fatty acids can be used as surfactants in the food, detergent, and cosmetic industries. They are increasingly used, due to the fact they are not harmful to the environment since they have a good biological degradability and a low toxicity.[12] Until now, carbohydrate esters have been synthesized chemically. However, the chemical synthesis has some disadvantages, since, for example, coloration of the product may occur during heating. These problems might be circumvented by the use of enzymatic esterification in a well-defined reaction medium.

Usually, enzymatic ester synthesis requires an organic solvent; however, carbohydrates are poorly soluble in the common organic solvents, such as octane or chloroform. Appropriate solvents for carbohydrates are, among others, pyridine and dimethylformamide. Chopineau et al.[4] have studied the transesterification reaction between a number of sugar alcohols and various plant and animal oils in dry pyridine. The reaction is catalysed by porcine pancreatic and *Chromobacterium viscosum* lipases. These enzymes are insoluble in pyridine, which means that the reaction mixture is a suspension of enzyme particles in a solution of substrates in organic solvent. Hence the reaction is catalysed at a solid-liquid interface. Dry pyridine is also used as solvent for the porcine pancreatic lipase-mediated transesterification reaction between trichloroethyl esters and monosaccharides.[16] Riva et al.[15] have studied the subtilisin-catalysed regioselective esterification between an activated fatty acid and carbohydrates with dimethylformamide as solvent.

Another approach in enzymatic esterification of carbohydrates is to modify the carbohydrates to increase the solubility in organic solvents. Monosaccharides with blocked C-6-hydroxyl groups are soluble in organic solvents such as acetone, tetrahydrofuran, and methylene chloride.[17] The transesterification reaction between trichloroethyl butyrate and these modified monosaccharides is catalysed by lipases from *Chromobacterium viscosum*, porcine pancreas, *Candida rugosa*, and *Aspergillus niger*. Carrea et al.[3] have studied the lipase-catalysed transesterification between activated lauric acid and a modified disaccharide, the 1-O-hexyl derivative of sucrose. This reaction is performed in dry acetone. Another possibility to make carbohydrates more lipophilic is acetylation.[5] Acetylated carbohydrates are soluble in benzene, toluene, chloroform, acetone, dioxane, diethylether, and carbon tetrachloride. The esterification of the acetylated sugar and a fatty acid is catalysed by immobilized lipase. Björkling et al.[1] have

studied the esterification of modified glucose and long-chain fatty acids in a solvent-free process. The reaction is performed at elevated temperature under reduced pressure. A heat-stable lipase of *Candida antarctica* is used.

For high reaction rates, it is necessary that the carbohydrate be dissolved, and therefore a polar organic medium is preferred. Laane et al.[11] have shown a correlation between the stability of an enzyme in an organic solvent and the log *P* value of this solvent. The log *P* value is defined as the logarithm of the partition coefficient of a given compound in the octanol-water two-phase system. Apolar solvents with log $P>4$ give a high enzyme stability, and the more polar solvents, having a log $P<2$, exhibit a low enzyme stability. Solvents for carbohydrates are essentially polar, with log *P* values below 2, and should therefore be unsuited for enzymatic reactions. However, there are exceptions to these rules, as was shown by the work of Klibanov,[4,15,16,17] which was mentioned before. Furthermore, Guagliardi et al.[7] have studied the stability and activity of a thermostable malic enzyme in monophasic systems of water and polar organic solvents. They report an increase in enzyme stability with an increase in polarity of the incubation mixture. This indicates that the enzyme stability/solvent polarity relationship is reversed when using water/polar organic solvent solutions. An explanation for this phenomenon was given by Gekko and Timasheff[6] in their studies on the stability of proteins in glycerol-water mixtures. They reported a stabilization effect of glycerol in water, due to preferential hydration of the protein. They assume that a thermodynamically unfavorable interaction tends to minimize the surface of contact between proteins and glycerol, thus stabilizing the native structure of the proteins.

Not all polar solvents are suited as cosolvents for esterification reactions. Glycerol and other alcohols contain hydroxyl groups and may compete with the substrate. Others, like pyridine and dimethylformamide, are very toxic and therefore not suitable. A new polar cosolvent for carbohydrates in enzymatic synthesis is 2-pyrrolidone. The log *P* value of 2-pyrrolidone is -0.9, calculated according to Rekker.[14] In industry, 2-pyrrolidone is used as a solvent for polymers, sorbitol, glycerol, and sugars. It is known to be noncorrosive, to have good chemical stability, and to be miscible with a number of solvents, for example water, ethanol, chloroform and benzene.[19] In this paper, the suitability of 2-pyrrolidone for carbohydrate esterification will be investigated. Special attention will be paid to enzyme stability in the reaction medium.

It is well known that the use of a two-phase membrane reactor in enzymatic reactions can have advantages, when compared with the reaction performed in an emulsion system. The membrane serves as the separation interface between the two phases, thus avoiding an energy-demanding phase separation. The membrane also can be used as an immobilization carrier of the enzyme.

Immobilization of lipase is performed on hydrophobic[8,9] as well as hydrophilic membranes.[13,18] Both membrane systems can be used both for hydrolysis of oils and for esterification of glycerol and fatty acids. However, at high glycerol concentrations, a hydrophilic membrane device is preferred for esterification.[18] In this paper the suitability of this membrane reactor for the reaction under study will be investigated.

## MATERIALS AND METHODS

### Materials

Lipases were obtained as follows: *Chromobacterium viscosum*, *Aspergillus niger*, and porcine pancreatic lipase from Biocatalysts Ltd. (UK); *Pseudomonas fluorescens* (lipase P) from Amano; *Candida rugosa* (type OF-360) from Meito Sangyo (Japan), and *Mucor miehei* from Novo (Denmark).

The carbohydrates used in this work, D-sorbitol, D-fructose, D-glucose monohydrate, and sucrose (all p.a. quality), were obtained from Merck (Germany). Oleic acid was also obtained from Merck (Germany) and consisted of a mixture of fatty acids: 1%-2% C14, 5% C16, 5%-6% C16:1, 1% C18, 72% C18:1, 9%-11% C18:2, 1% C18:3, 2% C20. The mixture of sucrose esters (DK-F110) was obtained from Suiker Unie Research (Holland). All other chemicals were of p.a. quality and obtained from Merck (Germany).

The hollow fiber membrane module was purchased from Organon (Holland). The fibers made of cellulose (Cuprophan™, Enka, Germany) had an internal diameter of 0.2 mm and a wall thickness of 8 $\mu$m; the total membrane area was 0.77 m$^2$.

### Reaction

The carbohydrate was dissolved in 2-pyrrolidone. In a typical experiment, 0.13 g sorbitol, 0.5 g 2-pyrrolidone, 1.3 g fatty acid, 25 mg lipase from *Chromobacterium viscosum* and 10 $\mu$l 0.1 M sodium phosphate buffer, pH 7.0, were mixed in 10-ml stoppered glass

bottles. The bottles were shaken by an end-over-end incubation (100 rpm) at 40 °C. Prior to HPLC analysis, the mixture was centrifuged and a 100-$\mu$l sample was taken from the fatty acid phase.

**Initial esterification rate**

The reaction mixture consisted of 1.3 g carbohydrate, 10 g 2-pyrrolidone, 27 g fatty acid, 0.5 g lipase from *Chromobacterium viscosum*, and 0.2 ml 0.1 M sodium phosphate buffer, pH 7.0. This solution was mixed thoroughly at 40 °C, and at regular time intervals, samples were taken out from the fatty acid phase and analyzed by HPLC. The initial esterification is defined as the amount of fatty acid (millimoles) bound to carbohydrate per gram crude lipase per hour.

**HPLC analysis**

The determination of the fatty acid phase, containing fatty acid, carbohydrate esters, and a part of the 2-pyrrolidone, was performed by HPLC using two size exclusion columns (PLgel 30 cm, Polymer Laboratories), placed in serial order. The columns were eluted with tetrahydrofuran at a flow rate of 1.0 ml.min$^{-1}$ and the effluent was monitored with a refractive index detector. Chromatograms were processed on a Spectra-Physics SP4290 integrator. In this analysis, oleic acid, which consisted of a mixture of fatty acids, showed one peak with a retention time of 13.5 min. The ester concentration is expressed as moles of ester per mole of fatty acid at t=0, unless stated otherwise.

**Measurement of water**

The water content of 2-pyrrolidone, sorbitol, fatty acid, and lipase was determined with a Mettler DL18 Karl Fischer Titrator (Mettler, Switzerland).[22] The initial water concentration of the reaction mixture was calculated with these values. To determine the water activity of the reaction mixtures, the relative humidity at 40 °C was measured with a Rotronic Hygroskop DT (Rotronic AG, Switzerland).

**Purification of the product**

The esterification reaction between sorbitol and fatty acid was carried out as described. After 64 h, the enzyme was removed by centrifugation. The reaction product (2.0 g) was applied to a silica column (Merck: Kieselgel 60, 0.040-0.063 mm) and eluted with chloroform/methanol (93/7, v/v). The effluent was fractionated and monitored by

HPLC. Fractions containing product were pooled and the solvent was evaporated *in vacuo*. The yield of this purification was 24 mg monoester (circa 1%). The structure of the purified product was confirmed by spectrometric methods. The infrared spectrum (Philips PU 9700; $CHCl_3$) showed a strong band at 1730 $cm^{-1}$, indicative of a carboxylic ester. The $^1$H-NMR spectrum (Bruker AC 200E; $CD_3OD$, TMS as internal reference) showed peaks of fatty acid ($\delta$ 0.85 t [$CH_3$], 1.28 br s [$CH_2$], $\delta$ 1.61 br s [$\underline{CH_2}CH_2CO$], 2.35 t [$\underline{CH_2}CO$]) and of sorbitol [$\delta$ 3.6 - 4.2, m]. The $^{13}$C-chemical shifts of the carbohydrate part of the C1-ester were (Bruker AC 200E operating at 50.3270 MHz; $CD_3OD$, internal reference TMS; differences with corresponding signals of sorbitol are given in parentheses): 67.1 [C1, $\Delta\delta$ = +3.0 ppm], 70.9 [C2, $\Delta\delta$ = -2.7 ppm], 71.3 [C3], 73.3 [C4], 75.4 [C5], and 65.1 [C6]. The chemical shifts of the C6-ester were: 64.5 [C1], 73.7 [C2], 71.3 [C3], 73.3 [C4], 72.9 [C5, $\Delta\delta$ = -2.1 ppm], and 27.8 [C6, $\Delta\delta$ = +3.0 ppm].

**Stability of lipase in 2-pyrrolidone**

Twenty-five milligrams of lipase from *Chromobacterium viscosum* was added to a solution of 0.1 M phosphate buffer in water (pH 7) and 2-pyrrolidone (total volume 25 ml) in stoppered glass bottles. The bottles were shaken on a reciprocal shaker (150 rpm) at 40 °C and samples were taken out at regular time intervals. The lipase activity was determined by adding the sample to 50 ml of an emulsion containing 2% (v/v) tributyrin, 0.1% (w/v) arabic gum, and 2 mM maleic acid, pH 6. The pH was kept at 6 by addition of 0.01 M NaOH. One unit of lipase activity was defined as the amount of lipase requiring the addition of 1 $\mu$mole of NaOH per minute.

**Partition coefficient of 2-pyrrolidone**

To determine the partition coefficient of 2-pyrrolidone, several solutions of 0.1 M sodium phosphate buffer (pH 7) and 2-pyrrolidone were prepared. Sorbitol was added to these 2-pyrrolidone/buffer solutions at a concentration of 0, 1.0 and 1.5 M, respectively. One ml of the buffer/2-pyrrolidone solution and 1 ml fatty acid were added to each other and the mixture was shaken vigorously for 15 min. The phases were separated by centrifugation. Concentrations of fatty acid and 2-pyrrolidone were determined by HPLC analysis.

**Membrane bioreactor**

The internal circuit of the hollow fiber membrane was filled with fatty acid and the external circuit was filled with water. The initial volume of the fatty acid phase was 100 ± 10 ml, circulating at 1.2 $l.h^{-1}$; the initial volume of the water phase was 150 ± 10 ml, circulating at 3.9 $l.h^{-1}$. The immobilization of 1 g lipase from *Chromobacterium viscosum* at the inner fiber side was carried out as follows: Lipase was dissolved in water and centrifuged to remove cell debris. The clear solution was dispersed in the fatty acid phase, and for 16 h ultrafiltrated from the inner fiber side towards the external circuit, thus immobilizing the lipase on the inner fiber side.[13,18] After immobilization, the fatty acid phase was replaced. The reaction was started by changing the water phase for a solution containing 640 g. $l^{-1}$ 2-pyrrolidone, 200 $g.l^{-1}$ sorbitol, and 300 $g.l^{-1}$ buffer (0.1 M sodium phosphate in water, pH 7). The water content of both circuits was determined by Karl-Fischer titration. The concentrations of fatty acid, esters, and 2-pyrrolidone were determined by HPLC analysis. The initial esterification rate in the membrane reactor was measured at 30 °C.

## RESULTS AND DISCUSSION

**Emulsion system**

The reaction system contains carbohydrate, dissolved in 2-pyrrolidone, fatty acid, a small amount of water, and lipase. This system separates into two phases. One phase contains the carbohydrate, water, and 2-pyrrolidone, from now on called the water phase. The other phase contains the fatty acid, 2-pyrrolidone, and a trace amount of water, from now on called the fatty acid phase. The partition of 2-pyrrolidone over both phases will be discussed under 'Solvent'. In this emulsion system, the volume ratio of the fatty acid phase and water phase is 10 up to 15. The lipase is present as suspended particles; thus, the reaction is assumed to take place at the solid-liquid interface.

**Enzyme**

Lipases were screened for esterification activity in the above-described two-phase system. No reaction was detected with the lipases from the porcine pancreas, *Candida*

*rugosa*, *Mucor miehei*, and *Aspergillus niger*. Only the lipases from *Chromobacterium viscosum* and *Pseudomonas fluorescens* were active in 2-pyrrolidone, and since the former lipase showed 8-10 times the activity of the latter, it was selected for further studies.

**Analysis**

When the products from the reaction between sorbitol and fatty acid, catalyzed by *Chromobacterium viscosum* lipase, were analyzed, the HPLC chromatogram of the reaction mixture showed the peaks of fatty acid and 2-pyrrolidone with retention times of 13.5 and 16.9 minutes, respectively. Peaks with retention times of 11.5, 11.9, and 12.5 minutes are also present. These are presumed to be the peaks of tri-, di-, and monoesters. The chromatogram is compared with a chromatogram of DK F110, a commercially available mixture of sucrose fatty acid esters,[10] and in this chromatogram the peaks of the mono-, di-, and triesters have the same retention times. To confirm the structure of these compounds, the monoester of sorbitol and fatty acid is purified by silica gel column chromatography. The HPLC chromatogram of the purified product shows one peak with a retention time of 12.5 minutes.

The structure is established by the IR- and NMR-spectra. The infrared absorption spectrum shows strong absorption at 1730 $cm^{-1}$, indicative of an ester bond. In the $^{1}H$-NMR spectrum, signals of both the fatty acid part and the sorbitol part of the molecule are clearly visible. $^{13}C$-NMR proved to be the best technique for the elucidation of the structure of the product, which appeared to be a mixture of two isomers. Using the characteristic downfield shift of $C_{\alpha}$ and upfield shift of $C_{\beta}$ upon esterification of sugars and sugar alcohols,[2,20] it was established that the mixture consisted of 1-monoacyl- and 6-monoacylsorbitol. The 1-ester was the major product. In the $^{13}C$-NMR spectrum, no signals are visible derived from C-C double bonds, which indicates that the fatty acid part consists of saturated fatty acids. It is known that *Chromobacterium viscosum* lipase exhibits a preference towards primary hydroxyl groups of the alcohol.[4,16] However, no literature data are available about a fatty acid specificity of this lipase. Whether or not lipase from *Chromobacterium viscosum* is more reactive towards saturated fatty acids will be a subject for further investigation.

**Kinetics and specificity**

Figure 1 shows the time course of esterification of fatty acid with sorbitol in 2-pyrrolidone using lipase from *Chromobacterium viscosum*. The initial enzyme-based esterification rate is 1.4 mmole $g^{-1}.h^{-1}$. After 74 h, the esterification has proceeded to values, expressed as moles of ester per mole fatty acid at t=0, of 0.060 for the monoester, 0.047 for the diester, and 0.006 for the triester. Eighty percent of the initial sorbitol content is converted and the reaction still proceeds. No reaction is found in the absence of lipase. The water activity of this system is 0.20.

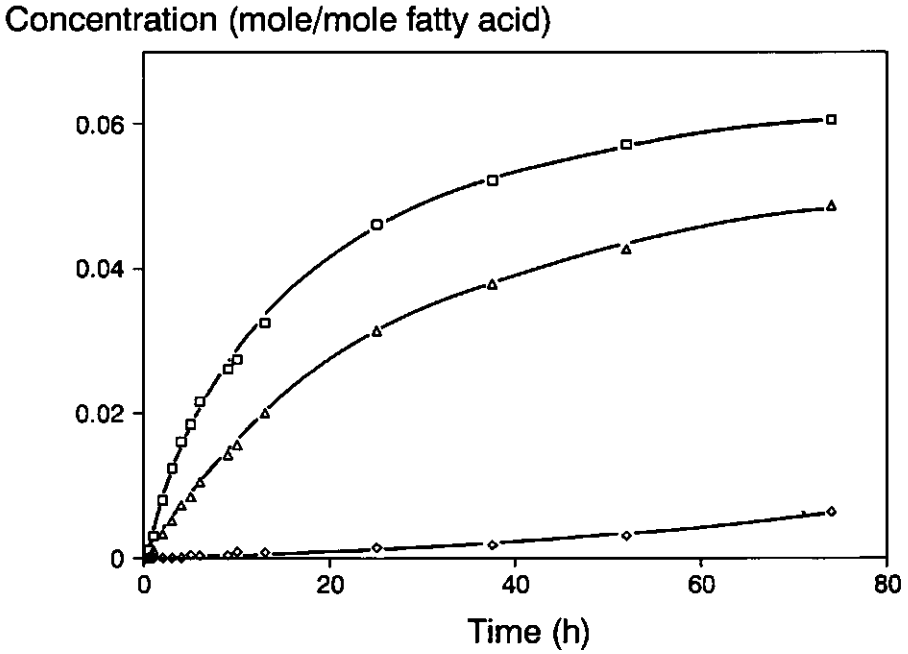


**Figure 1:** Time course for the esterification of fatty acid and D-sorbitol.
The reaction mixture contained 2.5 g sorbitol, 10 g 2-pyrrolidone, 27 g fatty acid, 0.5 g lipase (*Chromobacterium viscosum*), and 0.2 ml 0.1 M sodium phosphate buffer at pH 7.0. Sorbitol mono- (□), sorbitol di- (△), and sorbitol triester (◊).

**Table I:** Comparison of enzyme activity in esterification reactions.

| Reaction | Enzyme | Esterification rate ($mmole.g^{-1}.h^{-1}$) | References |
|---|---|---|---|
| Sugar alcohols and triolein in pyridine | Porcine pancreatic lipase | 0.0007 - 0.0026 | Chopineau et al.[4] |
| Monosaccharides and trichloroethylester in pyridine | Porcine pancreatic lipase | 0.014 - 0.044 | Therisod et al.[16] |
| 6-O-butyrylglucose and trichloroethylester in tetrahydrofuran | Lipase from *Chromobacterium viscosum* | 0.07 | Therisod et al.[17] |
| Monosaccharides and trichloroethylbutyrate in dimethylformamide | Subtilisin | 0.09 | Riva et al.[15] |
| Disaccharides and trichloroethylbutyrate in dimethylformamide | Subtilisin | 0.03 - 0.19 | Riva et al.[15] |
| Sucrose and trifluoroethylbutyrate in dimethylformamide | Protease N | 0.03 | Carrea et al.[3] |
| Hexanoylsucrose and trifluoroethyl laurate in acetone | Lipase from *Chromobacterium viscosum* | 0.002 | Carrea et al.[3] |
| Alkyl-D-glucopyranoside and dodecanoic acid (solvent-free) | Lipase from *Candida antarctica* | 0.8 - 15.6 | Björkling et al.[1] |
| Sorbitol/monosaccharides and fatty acid in 2-pyrrolidone | Lipase from *Chromobacterium viscosum* | 0.04 - 1.4 | Present report |

The initial esterification rate is also determined with mono- and disaccharides as substrate. For fructose and glucose as substrate, the rates are 0.20 and 0.04 mmole.$g^{-1}.h^{-1}$, respectively. With fructose, mono- as well as diesters are formed, whereas with glucose only monoesters are formed. Together with the results obtained with sorbitol, this is a strong indication that the enzyme has a preference for primary hydroxyl groups. With disaccharides as substrates, no esterification can be detected after 2 weeks of incubation. Esterification rates as measured in this investigation and from literature data are given in table I. Quantitative conclusions cannot be drawn, because of different reaction conditions and different purities of lipase. Qualitatively, the data show that the enzymatic esterification of carbohydrates and fatty acids is very slow. When compared with the other solvents, with 2-pyrrolidone the esterification rates are relatively high, up to one order of magnitude.

**Substrate concentration**

Since the reaction rate is relatively high with sorbitol, this is chosen as the model compound to study the esterification reaction with 2-pyrrolidone as cosolvent. The correlation between the substrate concentration and the amount of mono-, di-, and triester formed in 70 h is shown in figures 2a and 2b. In figure 2a, the ester concentration is expressed as moles of ester per mole of fatty acid at $t=0$, whereas in figure 2b the ester concentration is expressed as moles of ester per mole of sorbitol at $t=0$.

The concentrations of mono-, di-, and triester increase at increasing overall sorbitol concentrations (figure 2a). In these experiments, sorbitol concentrations higher than 0.38 M are not possible, since this is the maximum solubility of sorbitol in 2-pyrrolidone. The ratio of mono-, di-, and triester is not very dependent on the sorbitol concentration. Figure 2b shows a dependence of the ratio of mono-, di-, and triesters on the fatty acid concentration. The monoester concentration shows a maximum between 2.3 and 2.7 M fatty acid, while the maximum of the diester concentration is situated at higher fatty acid concentrations (2.6 - 2.8 M). The triester concentration increases slowly above a fatty acid concentration of 2 M. Figure 2b also shows that there is hardly any product formation below an overall fatty acid concentration of 1.5 M. Compared to sorbitol, a relatively high fatty acid concentration is necessary for ester production. This is probably due to inactivation of the enzyme by 2-pyrrolidone, since the overall 2-pyrrolidone concentration increases with decreasing fatty acid concentration. This will be discussed under 'Stability'.

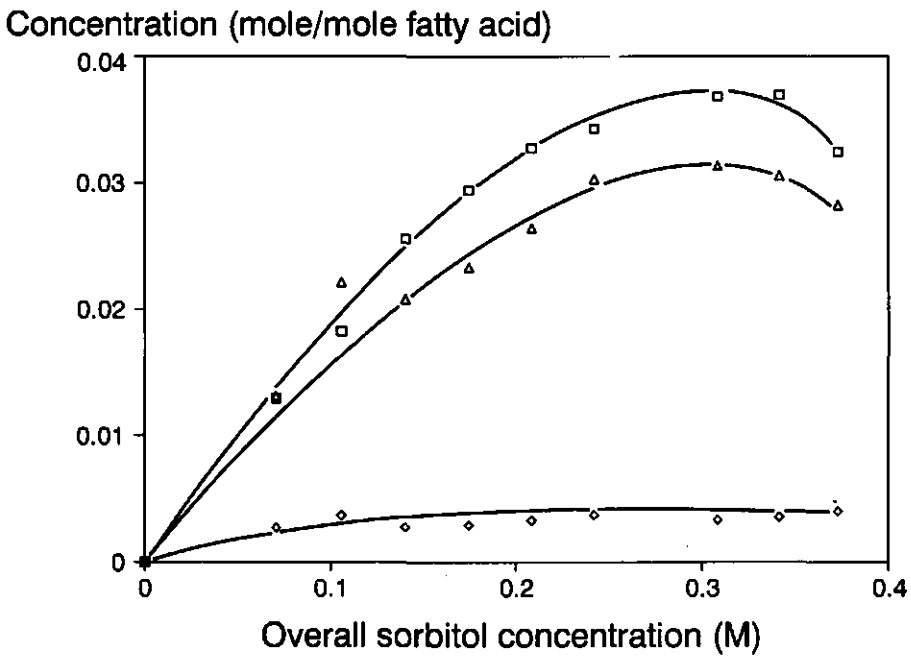


**Figure 2a:** Ester concentration as a function of the overall sorbitol concentration.
The reaction was carried out with 1.3 g fatty acid, a varying amount of sorbitol, 0.5 g 2-pyrrolidone, 10 μl sodium phosphate buffer at pH 7.0, and 25 mg lipase (*Chromobacterium viscosum*). The samples were incubated for 70 h at 40 °C. The ester concentration is calculated as moles of ester per mole of fatty acid (at t=0). Sorbitol mono- (□), sorbitol di- (△), and sorbitol triester (◇).

**Influence of water**

Figure 3 shows the effect of the water content on the amount of ester produced in 70 h. A strong dependence of enzyme activity on water concentration can be seen. At very low water concentrations (i.e. below 0.2 M), no activity is detected, indicating that a certain amount of water is essential to maintain conformation and activity of the enzyme.[21] The maximum ester production is obtained at an overall initial water concentration of 0.4-1.0 M. At water concentrations above 1.0 M, ester production

decreases, since the equilibrium favors hydrolysis and low ester concentrations. The overall water concentration increases during the synthesis reaction, but the maximum increase in these experiments is only 0.14 M. The ratio of mono-, di-, and triesters is not very dependent on the water content (data not shown).

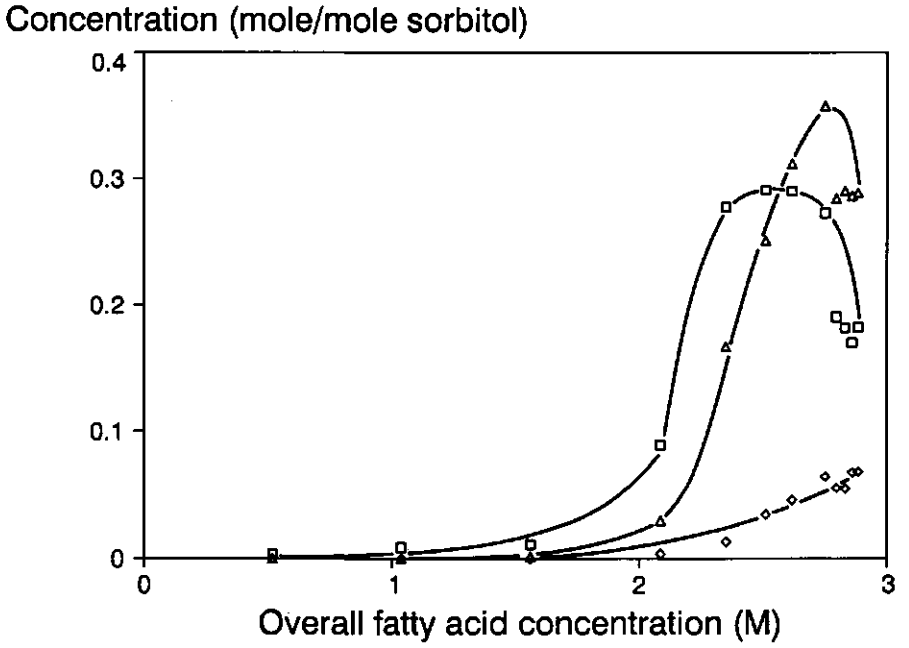


**Figure 2b:** Ester concentration as a function of the overall fatty acid concentration.
The reaction was carried out with 0.13 g sorbitol, a varying amount of fatty acid, 0.5 g 2-pyrrolidone, 10 $\mu$l sodium phosphate buffer at pH 7.0, and 25 mg lipase (*Chromobacterium viscosum*). The samples were incubated for 70 h at 40 °C. The ester concentration is calculated as moles of ester per mole of sorbitol (at t=0). Sorbitol mono- (□), sorbitol di- (△), and sorbitol triester (◇).

**Stability**

The effect of 2-pyrrolidone on the conversion of sorbitol is investigated (figure 4). To dissolve sorbitol, a minimum amount of 2-pyrrolidone (2 M) is necessary. Above a 2-pyrrolidone concentration of 3 M, ester production rapidly decreases, probably due to inactivation of lipase. At low 2-pyrrolidone concentrations, the enzyme is preferentially

hydrated[6]; however, at higher concentrations the enzyme loses the essential water layer. The water activity is reduced from $a_w$ = 0.3 at 2 M 2-pyrrolidone to $a_w$ = 0.2 at 4 M 2-pyrrolidone. Increasing the water activity of a reaction mixture with a 2-pyrrolidone concentration of 3 M by addition of water results in an ester concentration of 0.060 mole.mole$^{-1}$, formed within 70 h. This implies that the inactivation is reversible.

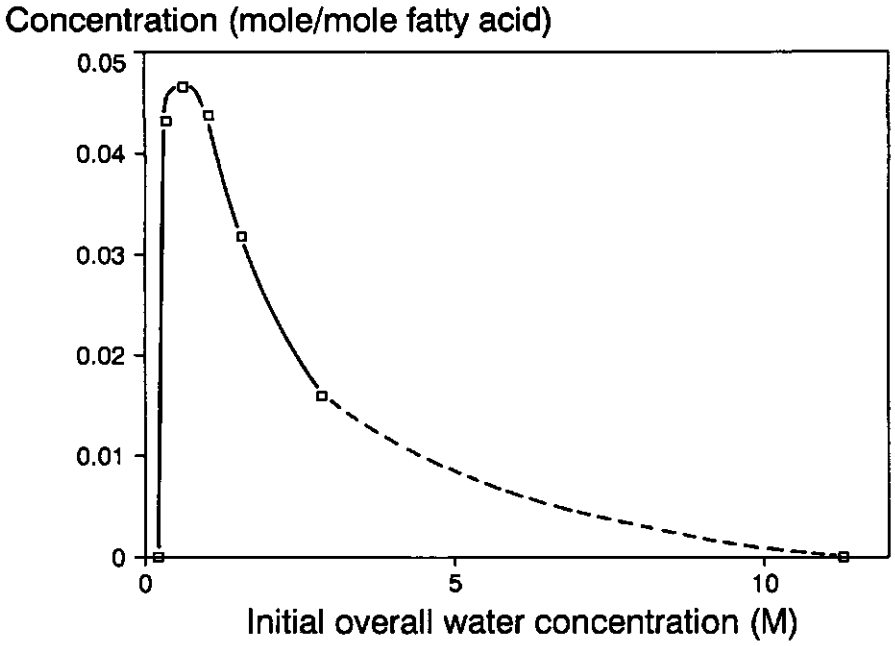


**Figure 3:** Effect of water on the esterification of fatty acid and D-sorbitol.
The reaction was carried out with 0.13 g sorbitol, 0.5 g 2-pyrrolidone, 1.3 g fatty acid, 50 mg lipase (*Chromobacterium viscosum*), and 0.1 M sodium phosphate buffer at pH 7.0. The initial water concentration was determined by Karl-Fischer titration. The samples were incubated for 70 h at 40 °C. The ester concentration is calculated as moles of ester (mono-, di-, and triester) per mole of fatty acid (at t=0).

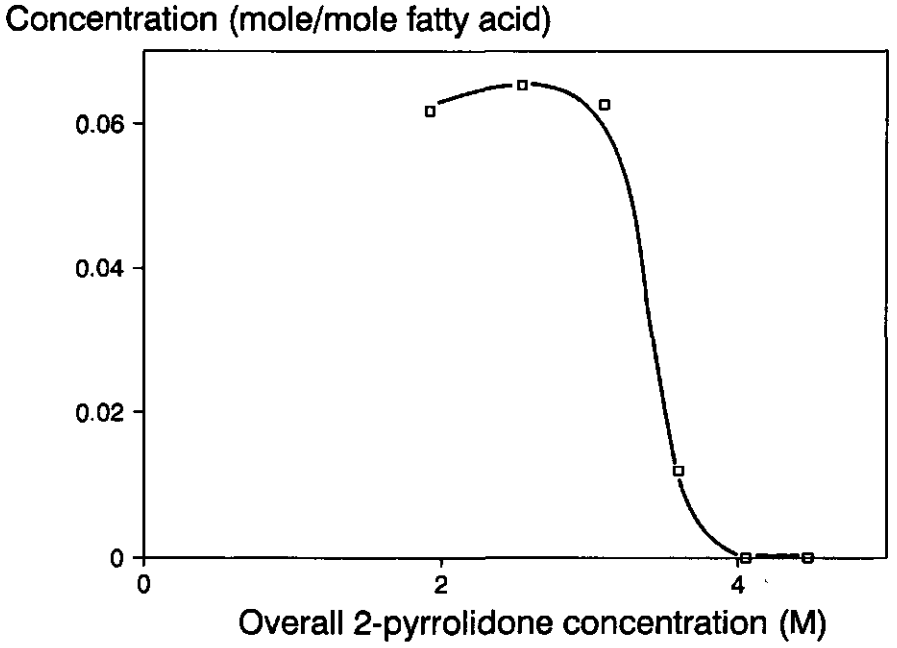


**Figure 4:** Effect of increasing concentration of 2-pyrrolidone on the esterification. The reaction was carried out with 0.8 g sorbitol, 1.3 g fatty acid, 25 mg lipase (*Chromobacterium viscosum*), and 10 µl sodium phosphate buffer at pH 7.0. The samples were incubated for 70 h at 40 °C. The ester concentration is calculated as moles of ester (mono-, di-, and triester) per mole of fatty acid (at t=0).

The results on the stability of lipase in monophasic solutions of water and 2-pyrrolidone are shown in figure 5. When lipase is incubated in water (0 M 2-pyrrolidone, $a_w$ = 1), an initial increase in activity is observed, followed by a slight decrease. After 300 h, the activity is about 70%. This increase in activity is even higher with incubation of lipase in 2.6 M 2-pyrrolidone ($a_w$ = 0.9). The activating effect of small amounts of polar solvents was also observed by Guagliardi et al.,[7] when incubating *Sulfolobus solfataricus* malic enzyme in solutions of water and alcohols. In 5.3 M 2-pyrrolidone ($a_w$ = 0.8), lipase

activity decreases down to about 50%. When incubating lipase in 7.9, 10.5, and 13.2 M 2-pyrrolidone, no activity is detected after 95, 3, and 1 h, respectively (the latter two data are not shown). The $a_w$ values are 0.7, 0.5, and 0.1, respectively.

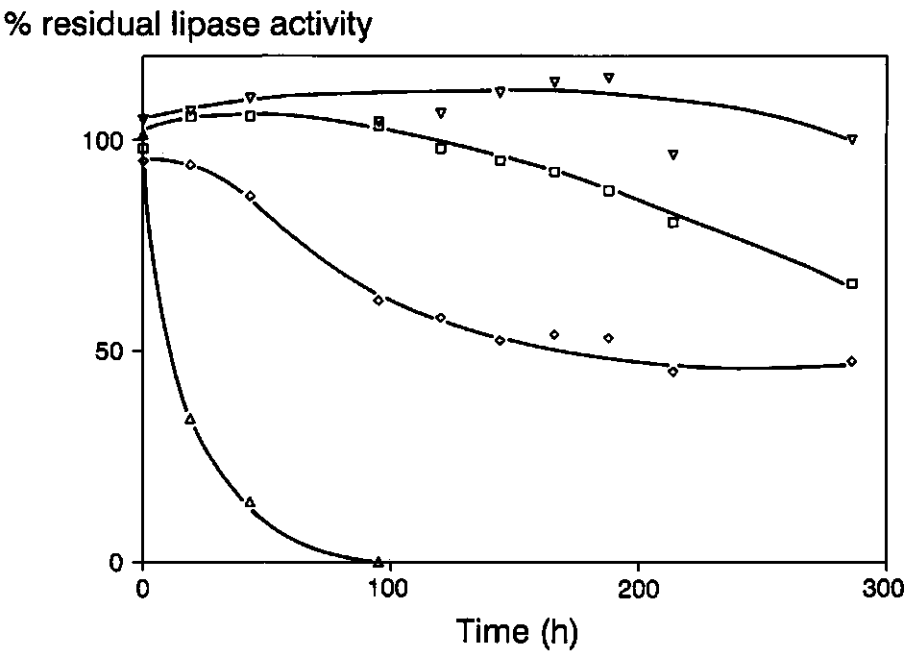


**Figure 5:** Effect of 2-pyrrolidone on the stability of *Chromobacterium viscosum* lipase. Lipase was incubated at 40 °C in mixtures of 0.1 M sodium phosphate at pH 7 in water and 2-pyrrolidone. At the times indicated, the residual lipase activity was measured by tributyrin assay. Concentrations of 2-pyrrolidone were: 0 M (□); 2.6 M (▽); 5.3 M (◇); 7.9 M (△).

In the reaction mixture, containing fatty acid, sorbitol, 2-pyrrolidone, and buffer, the product concentration rapidly decreases above an overall 2-pyrrolidone concentration of 3 M. When the overall 2-pyrrolidone concentration is 3 M, the concentration in the water phase is about 9 M. For inactivation, the concentration of 2-pyrrolidone in the water

phase rather than its overall concentration should be considered. Thus, inactivation takes place at 2-pyrrolidone concentrations in the water phase above 9 M, and this is in agreement with the data obtained in the monophasic water/2-pyrrolidone solutions, as discussed in figure 5. A correlation between water activity and enzyme inactivation is not found. In the reaction mixture, an overall concentration of 3 M corresponds with $a_w \sim 0.20$, whereas in the monophasic water/2-pyrrolidone solutions, a concentration of 9 M corresponds to $a_w \sim 0.6$.

**Solvent**

To develop a reactor for the production of carbohydrate esters in 2-pyrrolidone, it is important to study the behavior of 2-pyrrolidone in the two-phase reaction system. Therefore, the partition coefficient is determined. The partition coefficient of 2-pyrrolidone is defined as the concentration in the fatty acid phase divided by the concentration in the water phase. This partition coefficient is dependent on the composition of the phases, since we are dealing with nonideal solutions. The results are given in figure 6. Without sorbitol in the system, the partition coefficient decreases from 0.40 to 0.17 when the water concentration in the water phase is increased. Addition of sorbitol to the system enhances the partition coefficient slightly. An explanation would be that the polarity of the water phase decreases with addition of sorbitol; this means a lower difference in polarity between the two phases, resulting in a partition of 2-pyrrolidone in favor of the fatty acid phase.

**Membrane reactor**

Since the reaction system consists of two phases, a two-phase membrane reactor might be a suitable reactor configuration. In this investigation, we used a hydrophilic hollow-fiber membrane reactor containing lipase immobilized at the inner fiber side. The experiment is started by circulating fatty acid in the internal circuit and a solution of sorbitol, 2-pyrrolidone, and buffer in the external circuit. There is a rapid diffusion of 2-pyrrolidone from the external to the internal circuit until equilibrium is reached. Equilibrium values are in agreement with the results obtained with the partition experiments (figure 6).

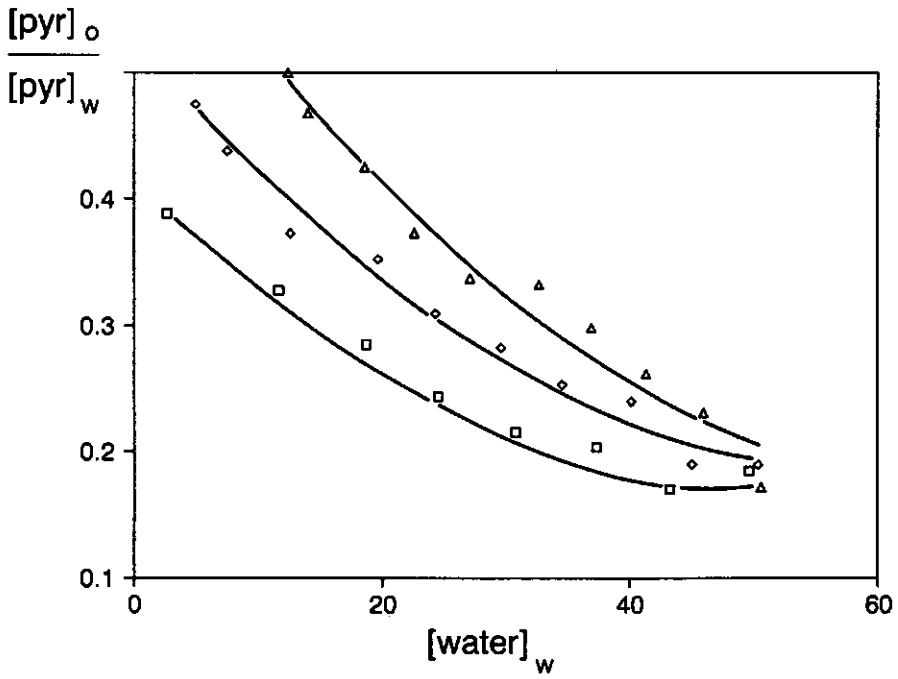


**Figure 6:** Partition coefficient of 2-pyrrolidone plotted versus water concentration in the water phase (expressed in M). The water phase has index *w* and the fatty acid phase has index *o*. It should be noted that the volume ratio of fatty acid and water phase is not constant in these experiments. Overall sorbitol concentrations were: 0 M (□); 0.5 M (◊); 0.75 M (△).

The results of a typical membrane bioreactor experiment are shown in figure 7. The concentrations of mono- and diester increase with time; accumulation of triester is not detected. After 400 h, the concentrations of mono- and diesters are 0.008 and 0.002 mole.mole$^{-1}$ fatty acid (at t=0), respectively. These ester concentrations are low compared to the concentrations obtained with the emulsion system in figure 1. However, they cannot be compared directly, because the composition of the two phases in the membrane reactor experiment is different from that in the batch experiment, as reported in figure 1. Therefore the water activity in the membrane experiment ($a_w$ = 0.7) is different from that in the emulsion experiment ($a_w$ = 0.2), shifting the equilibrium from

esterification to hydrolysis. The compositions for the membrane reactor had to be different, because of operating problems due to the high viscosity of the water phase under the conditions of the emulsion reactor.

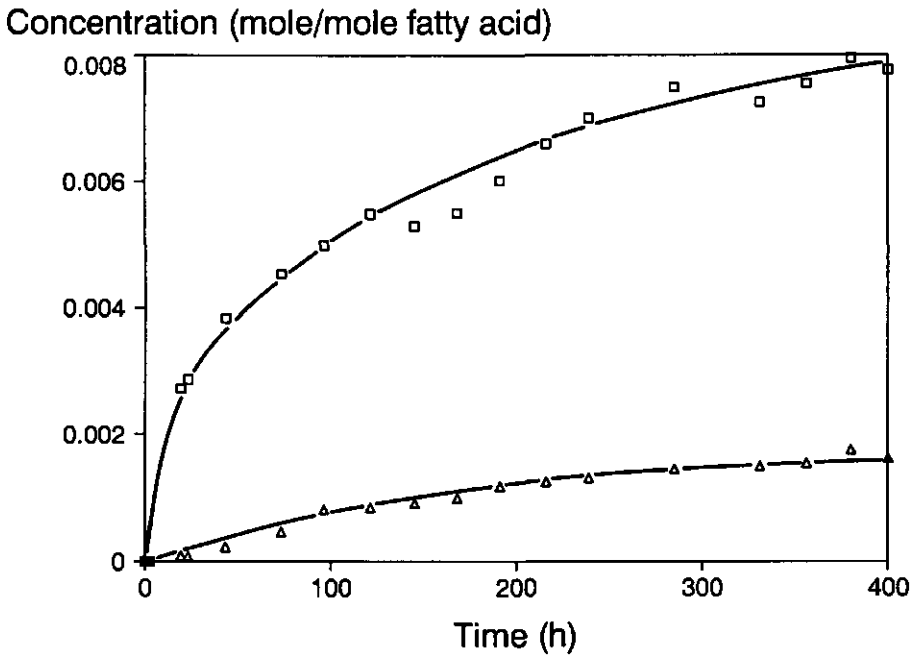


**Figure 7:** Esterification of sorbitol and fatty acid in a membrane reactor.
The experiment was performed with the following overall concentrations: 1.3 M fatty acid, 4.5 M 2-pyrrolidone, 0.7 M sorbitol and 10.0 M water. The concentrations in the fatty acid phase were 2.5 M fatty acid, 2.3 M 2-pyrrolidone, and 1.9 M water; the concentrations in the water phase were 6.7 M 2-pyrrolidone, 18 M water, and 1.3 M sorbitol. The concentrations of sorbitol monoester (□) and sorbitol diester (△) in the fatty acid phase are shown. Sorbitol triester was not detected.

The enzyme-based initial esterification rate of the membrane experiment is 0.7 mmole.$g^{-1}$.$h^{-1}$, based on a crude lipase load of 75 mg.$m^{-2}$.[18] This initial reaction rate is half the reaction rate in a batch experiment (figure 1), indicating that the enzyme in a membrane reactor has an activity of the same order of magnitude as in an emulsion

reactor. The same results are reported by Van der Padt.[18] Thus, a membrane reactor can be suitable for this type of conversion, but since the reaction conditions are not optimum, further studies are needed to improve the yield.

## ACKNOWLEDGEMENTS

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# chapter 3

# ENZYMATIC SYNTHESIS OF CARBOHYDRATE ESTERS IN AQUEOUS MEDIA


## SUMMARY

In a two-phase system of D-sorbitol in water and decanoic acid the esterification is catalyzed by lipase from *Candida rugosa*. The initial esterification rate is 3.0 mmole.$g^{-1}$.$h^{-1}$ and a mixture of mono-, di-, tri-, and tetraesters is formed. The water activity of the reaction system is dependent on the sorbitol mole fraction in the aqueous phase. An increase in the ester concentrations is obtained by a decrease of the water activity. By addition of solid sorbitol, the water activity is kept low during the reaction. The ester concentrations at equilibrium are a factor of 5 higher with decanoic acid as a substrate than with oleic acid as a substrate. In a two-phase membrane reactor the initial esterification rate is 6.8 mmole.$g^{-1}$.$h^{-1}$. After 570 hours this reaction rate is only reduced by 15%, which indicates a good stability of lipase in this membrane system.



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## INTRODUCTION

Interest is growing in the enzymatic esterification of carbohydrates and fatty acids. Carbohydrate esters can be used as surfactants in food, detergent and cosmetic industry.[2] Several papers have reported the esterification of carbohydrates and fatty acids or modified fatty acids, catalyzed by lipase or protease with polar solvents such as pyridine and dimethylformamide as reaction medium.[1,6,7] Alternatively, 2-pyrrolidone is a suitable solvent for carbohydrates as is discussed in chapter 2. However, such solvents are not accepted for food applications.

In this chapter the enzymatic esterification of carbohydrates and fatty acids in a two-phase system with water as the polar solvent is discussed. The water phase consists of a fully saturated carbohydrate solution and the organic phase consists of fatty acid. Also applications of a two-phase membrane reactor are investigated. This type of reactor can have advantages compared to a stirred tank reactor, since the membrane serves as separator of the two phases as well as immobilization carrier of the enzyme.[4,8]

## MATERIALS AND METHODS

### Materials

Lipases were obtained from Biocatalysts Ltd. (UK). All chemicals were of p.a. quality and obtained from Merck (FRG). The hollow fiber membrane module was purchased from Organon (Holland). The fibers made of cellulose (Cuprophan™, Enka, FRG) had an internal diameter of 0.2 mm and a wall thickness of 8 $\mu$m, the total membrane area was 0.77 m$^2$.

### Emulsion system

For the determination of the time course of the reaction, 35 g decanoic acid, 12 g sorbitol, 0.1 M sodium phosphate buffer of pH = 7.0 and 0.2 g lipase from *Candida rugosa* were mixed in a 0.3 l vessel. The emulsion was mixed by a six bladed turbine impeller (500 rpm) at 35 °C. At regular time intervals, samples were taken out and phases were separated by centrifugation. The organic phase was analyzed by HPLC. Other experiments were performed in 10 ml stoppered glass bottles, which were shaken by an

end-over-end incubator (100 rpm) at 35 °C. For the screening of lipases the reaction mixture consisted of 1.8 g decanoic acid, 0.3 g sorbitol, 0.15 ml 0.1 M sodium phosphate buffer (pH=7) and 25 mg lipase.

**Membrane reactor**

The immobilization of 1 g lipase from *Candida rugosa* at the inner fiber side of the membrane reactor was carried out as described by Van der Padt et al.[8]. In this procedure lipase was dissolved in water and subsequently dispersed in the organic phase of the internal circuit of the hollow fiber membrane. The organic phase consisted of a mixture of decanoic acid and hexadecane (5:1 w/w). The water was ultrafiltrated during 16 hours from the internal towards the external circuit, thus immobilizing lipase on the inner fiber side. After immobilization of lipase the organic phase was replaced by 120 ± 10 ml of a mixture of decanoic acid and hexadecane (5:1 w/w). The reaction was started by filling the external circuit with 120 ± 10 ml of a fully saturated sorbitol solution. This water phase contained an excess of sorbitol and was filtered before it was pumped in the membrane reactor. The organic and water phases were circulated with 1.2 $l.h^{-1}$ and 1.8 $l.h^{-1}$, respectively. The experimental set-up is schematically shown in figure 1. The reaction was performed at 30 °C. Samples were taken from the organic phase and analyzed by HPLC.

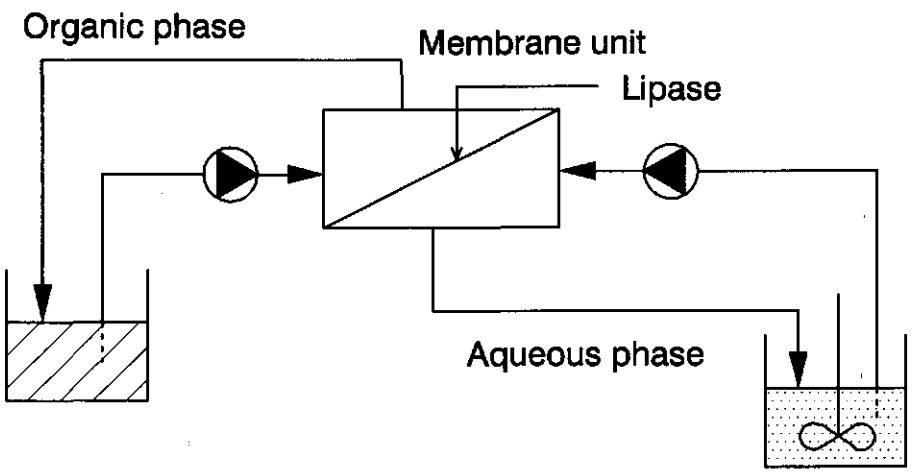


**Figure 1** : The experimental set-up of the membrane reactor.

**Analysis**

The carbohydrate ester concentration was determined by HPLC, using two serial size exclusion columns (PLgel 30 cm, Polymer Laboratories). The columns were eluted with tetrahydrofuran and a refractive index detector was used. The system was calibrated using decanoic acid and the retention times of mono-, di-, tri- and tetraesters corresponded with the retention times expected on the basis of the molecular weights. The ester concentrations were expressed as mole fractions. In the organic phase the sum of the mole fractions of fatty acid, mono-, di-, tri-, and tetraesters is 1.

## RESULTS AND DISCUSSION

Several lipases were screened with respect to their ability to catalyze the esterification between decanoic acid and sorbitol (table I). After 48 hours of incubation, the ester concentration per gram of crude enzyme was highest for the lipase from *Candida rugosa*. This lipase was used for further investigation.

**Table I**: Esterification of decanoic acid and D-sorbitol, catalyzed by lipase.

| Lipase source | Lipase activity[1] ($U.mg^{-1}$) | Ester concentration[2] (after 48 hours of incubation) ($mole.mole^{-1}$ organic phase) |
|---|---|---|
| *Candida rugosa* | 85.7 | 0.027 |
| *Chromobacterium viscosum* | 53.0 | 0.011 |
| Porcine pancreatic | unknown | - |
| *Aspergillus niger* | 1.6 | 0.014 |
| *Pseudomonas fluoresens* | 8.5 | - |
| *Rhizopus delemar* | 7.2 | 0.012 |

1) One unit of lipase activity is defined as the release of 1 μmole of free fatty acids from olive oil per minute at 37 °C and pH=7.8. Values as given by the manufacturer.

2) The ester concentration is the sum of the mono-, di- and triester concentrations.

Figure 2 shows that mono-, di- as well as triesters are formed. In this emulsion system the equilibrium is reached after circa 300 hours, when 34% of the initial sorbitol content is converted. At equilibrium the concentrations of mono-, di- and triester are 0.037, 0.050 and 0.034 mole.mole$^{-1}$ organic phase, respectively. The initial esterification rate, which is defined as the amount of fatty acid bound to carbohydrate per gram crude lipase per hour, is 3.0 mmole.g$^{-1}$.h$^{-1}$.

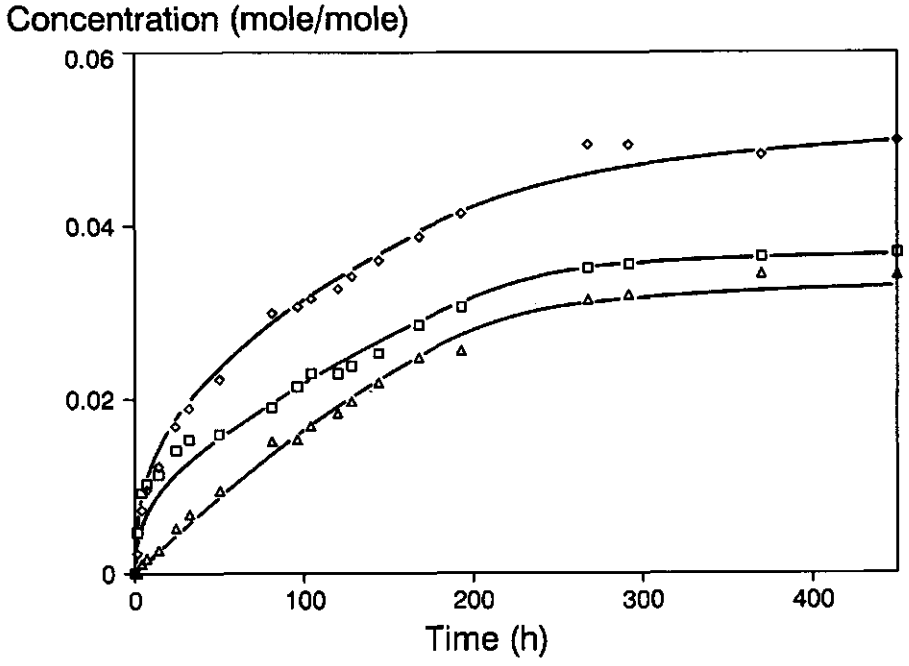


**Figure 2:** The synthesis of sorbitol mono- (□), sorbitol di- (◊) and sorbitol triester (△). The reaction mixture contained 35 g decanoic acid, 12 g sorbitol, 4 ml 0.1 M sodium phosphate buffer of pH=7.0 and 0.2 g lipase (*Candida rugosa*).

The equilibrium value of the esterification reaction can be expected to be strongly dependent on the water content, since water is a product of the reaction. In figure 3 the time course of the ester concentration is shown for several mole fractions sorbitol in the

aqueous phase. The ester concentration is the sum of the mono-, di- and triester concentrations. At the experiment with the low mole fraction sorbitol and subsequently a high water activity, the equilibrium is reached within 50 hours and the ester concentration

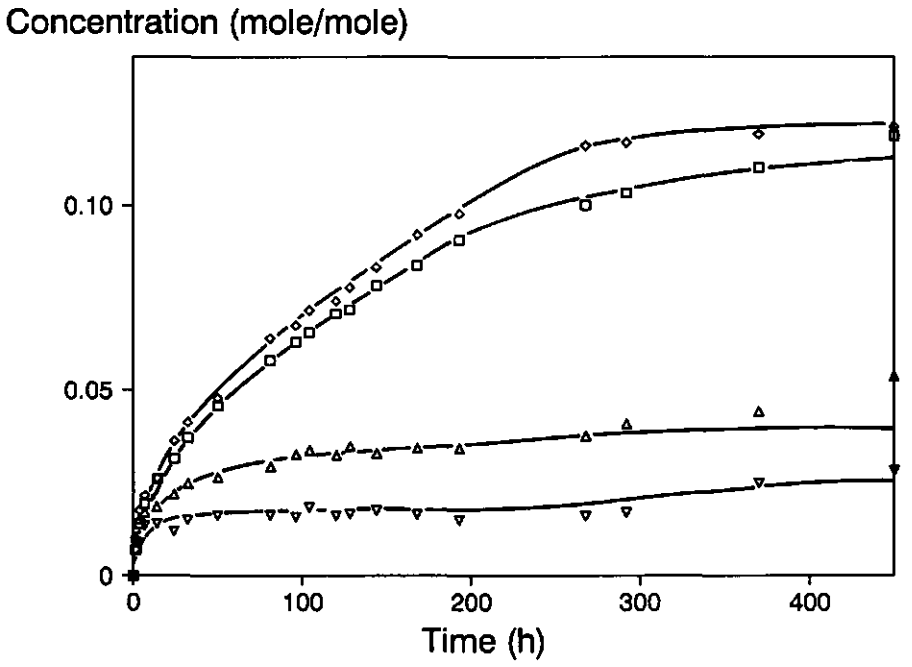


**Figure 3:** The effect of water on the esterification of decanoic acid and sorbitol.
The reaction was carried out with 35 g decanoic acid, 12 g sorbitol, 200 mg lipase (*Candida rugosa*) and 2, 4, 8, and 16 ml 0.1 M sodium phosphate buffer. The initial sorbitol mole fractions in the aqueous phase were 0.37 (□), 0.23 (◇), 0.13 (△), and 0.07 (▽). The water activity of these experiments is presented in table II.

is low. At a mole fraction sorbitol of 0.13, the ester concentration at equilibrium is slightly higher. At a mole fraction of 0.23, 300 hours are needed to reach the equilibrium and the ester concentrations are 3 times higher compared to the experiment with a sorbitol mole fraction of 0.13. When the sorbitol concentration is raised even more, after 450 hours of incubation, the equilibrium is not yet reached. In table II, more results of these

experiments are shown, such as water activities and mono-, di- and triester concentrations. During the experiments the water activity increased, which is caused by consumption of sorbitol and production of water. Water activities below 0.71 cannot be reached at the applied reaction temperature of 35 °C, since 0.71 represents the water activity of a fully saturated sorbitol solution. In the experiment with a mole fraction sorbitol of 0.23, the water phase is fully saturated with sorbitol and an excess of sorbitol is present at the start of the experiment. Due to consumption of sorbitol and production of water during the reaction, the solid sorbitol is disappeared and at equilibrium the water activity of the water phase is increased to 0.83. In the experiment with a mole fraction sorbitol of 0.37, even after 450 hours of incubation solid sorbitol is present in the aqueous phase. Subsequently the water activity is still 0.71.

**Table II**: The effect of the mole fraction sorbitol and the water activity in the water phase on the esterification of decanoic acid and sorbitol.[1)]

| Mole fraction sorbitol | Water activity [2)] | | Concentration at t=450 h (mole.mole$^{-1}$ organic phase) | | |
|---|---|---|---|---|---|
| (t=0 h) | (t=0 h) | (t=450 h) | Monoester | Diester | Triester |
| 0.37 | 0.71 | 0.71 | 0.031 | 0.047 | 0.040 |
| 0.23 | 0.71 | 0.83 | 0.037 | 0.050 | 0.034 |
| 0.13 | 0.84 | 0.87 | 0.010 | 0.016 | 0.013 |
| 0.07 | 0.92 | 0.93 | 0.006 | 0.007 | 0.004 |

1) The time courses of these experiments are plotted in figure 3.

2) The water activity is calculated according the method of Norrish.[3]

In figure 4, results are shown from experiments with a large aqueous phase as compared to the experiments of table II and figure 3. In this figure, the maximum increase in water activity during the experiment is 0.03. From figure 4, it is clear that the ester concentrations increase upon decreasing the water activity. Furthermore, small amounts of tetraester are formed in these experiments.

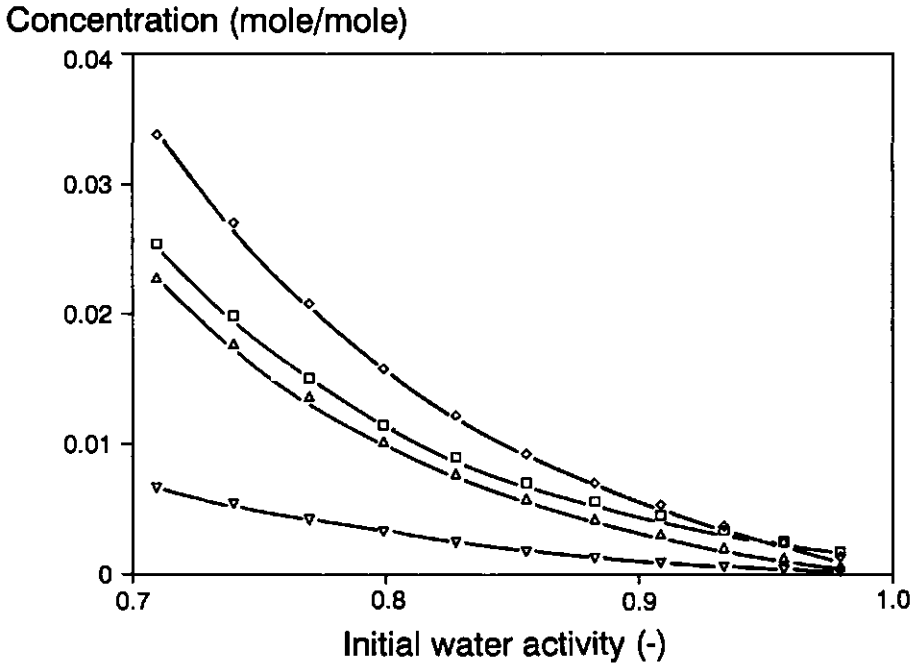


**Figure 4**: The ester concentration at equilibrium as a function of the initial water activity. The reaction was carried out with 20 mmole decanoic acid, 91 mmole sorbitol/water solution and 25 mg lipase (*Candida rugosa*). The samples were incubated for 600 hours. Sorbitol mono- (□), sorbitol di- (◊) sorbitol tri- (△) and sorbitol tetraester (▽).

Tetraesters were not detected in the experiments of figures 2-3 and table I-II, and this is probably due to the HPLC analysis method that is used. The experiments of figure 4 and 5 are analyzed with a new HPLC column. Although the column is of the same type as the old column that was used for the previous experiments of this chapter, the new column seems to give a better resolution. By using the old column, a shoulder was observed in the chromatogram at the triester peak. However, in the chromatogram that is obtained with the new column, a small peak with a lower retention time than the triester peak is clearly visible. This is assumed to be the tetraester peak. In the experiments of figure 4 and 5, the tetraester concentration is shown. It can be assumed, that small

amounts of tetraester were also present in all other experiments. This means that the presented triester concentrations in figure 2 and table II, in fact, are composed of triester and a small amount of tetraester. In chapter 7, the purification of sorbitol tetraester from a reaction mixture and the elucidation of its structure are described.

In the studies of Seino et al.[5], the lipase catalyzed esterification of oleic acid and various carbohydrates is reported. They have found conversions of 60 - 70% after 72 hours of incubation in a system consisting predominantly of water, for which reason the water activity of their system can be assumed to be close to 1. These results are in contradiction with our results of figure 4, where almost no esters are formed at water activities close to one.

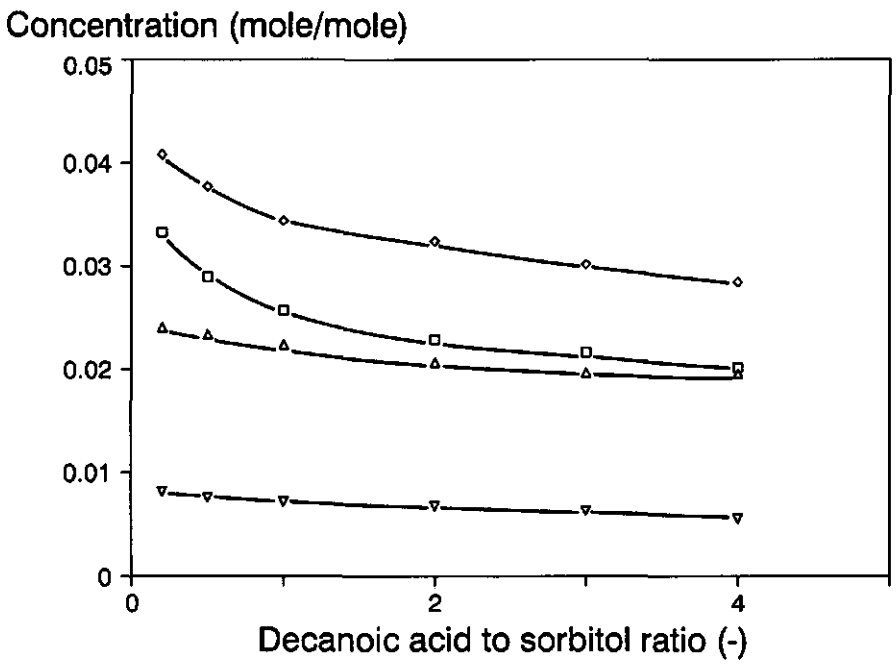


**Figure 5a:** The ester concentration as a function of the sorbitol to decanoic acid ratio. The reaction was carried out with 10 mmole sorbitol, 36 mmole water, decanoic acid and 25 mg lipase (*Candida rugosa*). The samples were incubated for 500 hours. Sorbitol mono- (□), sorbitol di- (◇) sorbitol tri- (△) and sorbitol tetraester (▽).

In the emulsion experiments of figures 5a and 5b, the effect of the fatty acid concentration on the ester concentrations at equilibrium is shown for two different fatty acids. Here, the initial water activity is kept constant and the amount of fatty acid is varied. These experiments are carried out with decanoic acid and oleic acid. It is remarkable that the oleic acid ester concentrations in figure 5b, are about a factor 5 lower than the

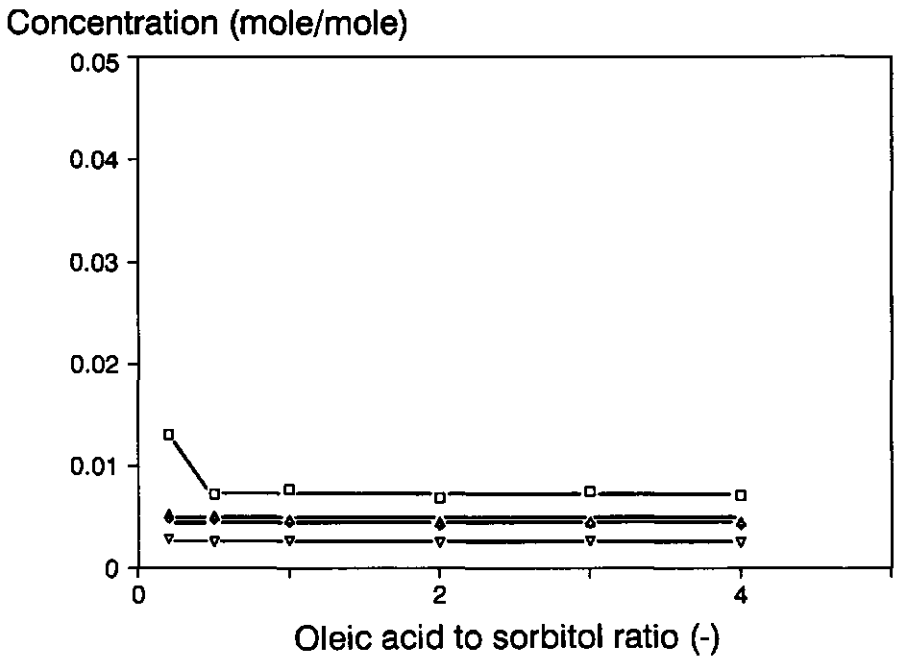


**Figure 5b:** The ester concentration as a function of the sorbitol to oleic acid ratio.
The reaction was carried out with 10 mmole sorbitol, 36 mmole water, decanoic acid or oleic acid and 25 mg lipase (*Candida rugosa*). The samples were incubated for 500 hours. Sorbitol mono- (□), sorbitol di- (◊) sorbitol tri- (△) and sorbitol tetraester (▽).

decanoic acid ester concentrations in figure 5a. Lower oleic acid ester concentrations in comparison with the esters of decanoic acid are also observed in the esterification of glycerol (see chapter 5). However, the oleic acid ester concentrations are less than a factor 2 lower than the decanoic acid ester concentrations for these reactions. Oleic acid

is a very nonpolar solvent (log P = 7.7), which is probable not suitable to dissolve the polar sorbitol esters. This is in agreement with the results of chapter 6, where it is shown that addition of nonpolar solvents such as decane resulted in extremely low sorbitol ester concentrations.

Furthermore, figures 5a and 5b shows that the mono-, di-, tri- and tetraester concentrations are not very dependent on the sorbitol to fatty acid ratio. This is a difference with esterification of glycerol and decanoic acid (chapter 4), where the mole fraction of mono-, di-, and triesters at equilibrium is clearly affected by the initial glycerol to fatty acid ratio. For the esterification of glycerol and decanoic acid, higher ester concentrations at equilibrium were obtained than for the esterification of sorbitol and decanoic acid. Due to the low sorbitol ester concentrations, the thermodynamic activities of water, sorbitol and fatty acid will only change slightly during the reaction. This means that at equilibrium these activities will be more or less the same for experiments with different initial sorbitol to fatty acid ratios. As a consequence, the ester concentrations at equilibrium will be also the same.

The enzymatic esterification of carbohydrates and fatty acids or activated fatty acids is generally very slow. As is discussed in chapter 2, the esterification of sorbitol and fatty acid in 2-pyrrolidone, catalyzed by lipase from *Chromobacterium viscosum* (specific acitivity: 53 olive oil units.mg$^{-1}$ crude lipase) resulted in an esterification rate of 1.4 mmole.g$^{-1}$.h$^{-1}$. Therisod et al.[7] have found esterification rates in the range of 0.014 - 0.044 mmole.g$^{-1}$.h$^{-1}$ for the transesterification of monosaccharides and trichloroethylester in pyridine, catalyzed by porcine pancreatic lipase (specific activity: 11 olive oil units.mg$^{-1}$ crude lipase).

In this study the esterifications with D-fructose, D-glucose and sucrose as substrate instead of sorbitol were also investigated. The esterification rates with the monosaccharides fructose and glucose as substrate were found to be 0.3 and 0.02 mmole.g$^{-1}$.h$^{-1}$, respectively. The reaction conditions were equal to the conditions described in figure 2. It can be seen that the esterification rates of the monosaccharides and sorbitol in the two phase system are good, when compared to literature data. However, no esterification has been detected in this system with the disaccharide sucrose as substrate.

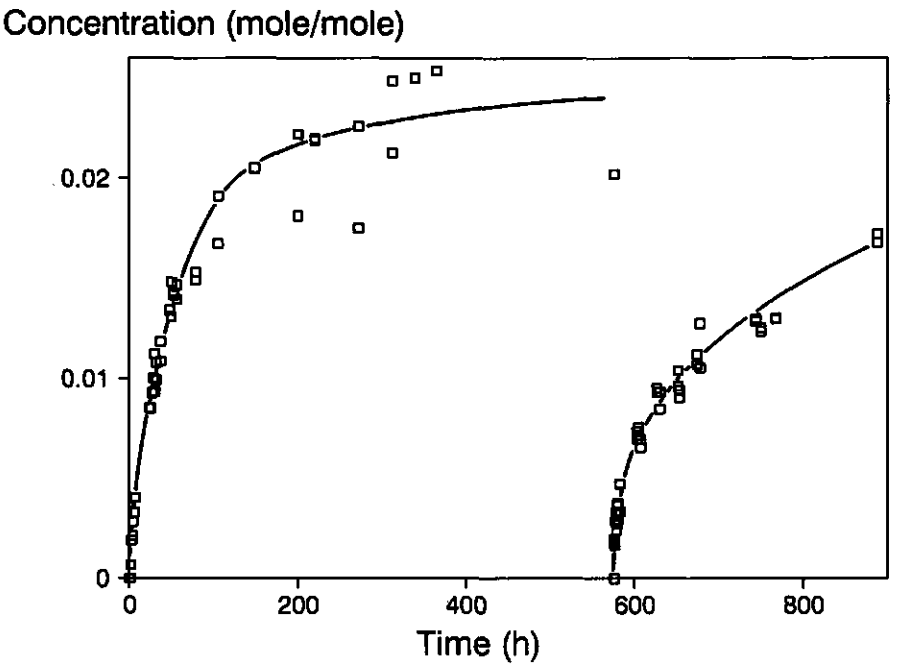


**Figure 6**: Two batches of the esterification of sorbitol and decanoic acid in a membrane reactor. The concentration is the sum of the mono-, di- and triester concentrations.

The esterification of decanoic acid and sorbitol in a two-phase membrane reactor was studied. Lipase from *Candida rugosa* was immobilized at the inner fiber side of a hydrophilic hollow fiber membrane. The organic phase, containing decanoic acid and hexadecane, was circulated at the inner fiber side. Hexadecane was added to the organic phase to prevent the precipitation of decanoic acid. The water phase, which was circulated at the shell side, was fully saturated with sorbitol and an excess of sorbitol was added to prevent an increase in water activity during the reaction, due to sorbitol consumption and water production.

The ester concentration in the organic phase, plotted versus time, is shown in figure 6. The concentrations of mono-, di- and triester at equilibrium (300 - 570 hours) are 0.006, 0.009 and 0.008 mole.mole$^{-1}$ organic phase, respectively (data not shown). The ester concentrations are about 5 times lower compared to the emulsion system of figure 2. This will be due to the solvent hexadecane in the organic phase. In an emulsion system with the same concentration of hexadecane in the organic phase, the same ester concentrations at equilibrium were detected. In chapter 6, it is shown that nonpolar solvents, such as hexadecane, are very poor solvents for the synthesis of sorbitol ester. Higher ester concentrations would be obtained when a polar solvent was used.

The initial esterification rate in the membrane system is calculated to be 6.8 mmole.g$^{-1}$.h$^{-1}$, based on a crude lipase load of 75 mg.m$^{-2}$.[8] In an emulsion system with hexadecane in the organic phase, the initial esterification rate is 0.9 mmole.g$^{-1}$.h$^{-1}$. Besides an expression of the initial reaction rate per gram crude enzyme, it is also possible to express the initial reaction rate per m$^3$ organic phase. For the membrane system, this volumetric initial reaction rate is 3.3 mole per m$^3$ organic phase per hour and for the emulsion system this reaction rate is 7.2 mole.m$^{-3}$.h$^{-1}$. A higher enzyme based initial reaction rate and a lower volumetric initial reaction rate in a membrane reactor compared to an emulsion system are reported before for the esterification of glycerol and decanoic acid.[8]

To study the stability of lipase in this membrane system both the organic and water phase were replaced after 570 hours. The initial esterification rate was determined again (figure 6) and found to be reduced from 6.8 to 5.8 mmole.g$^{-1}$.h$^{-1}$. This indicates that the stability of lipase is fairly good.

## CONCLUSIONS

Esterification of sorbitol and decanoic acid in a two-phase system is possible and the reaction rates are high, compared to literature data. In this reaction system, esterification is also detected with fructose and glucose as a substrate, but not with the disaccharide sucrose as a substrate. The water activity of the reaction system, which is dependent on the carbohydrate mole fraction in the aqueous phase, is found to be an important parameter for the sorbitol ester concentrations at equilibrium. The sorbitol ester

concentrations are also dependent on the polarity of the fatty acid that is used as a substrate. With the nonpolar oleic acid as a substrate, ester concentrations at equilibrium are a factor of 5 lower than with the more polar decanoic acid as a substrate. The two-phase membrane reactor seems to be a suitable type of reactor for this esterification reaction. In this type of reactor, the water activity of the aqueous phase can be kept constant during the experiment and the stability of lipase is good. After 3 weeks, the lipase activity is only reduced with 15%.

## ACKNOWLEDGEMENTS

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# chapter 4

# THE EFFECT OF ORGANIC SOLVENTS ON THE EQUILIBRIUM POSITION OF ENZYMATIC ACYLGLYCEROL SYNTHESIS


## SUMMARY

The effect of organic solvents on the equilibrium position of lipase-catalyzed esterification of glycerol and decanoic acid has been investigated. The reaction is carried out in an aqueous-organic two-phase system. In polar solvents, high mole fractions of monoacylglycerol and low mole fractions of triacylglycerol are measured, while in nonpolar solvents, the measured differences in the mole fractions of mono-, di-, and triacylglycerols are less. There is a good correlation between the ester mole fractions at equilibrium and the log $P$ of the solvent (partition coefficient in *n*-octanol-water), however, only if the group of tertiary alcohols is excluded. In the plot of the ester mole fractions as a function of the logarithm of the solubility of water in the organic solvent, the tertiary alcohols can be included; however, in this case other deviations appear.

For the prediction of the effect of organic solvents on the ester mole fractions at reaction equilibrium in nondilute reaction systems with a water activity below 1, the program TREP (Two-phase Reaction Equilibrium Prediction) is developed, which is based on the UNIFAC group contribution method. With this model the equilibrium data



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are essentially predicted from basic thermodynamic data. The required equilibrium constants are estimated from experiments without an organic solvent in the reaction medium. The mole fractions calculated by TREP show the same trends as the experimentally measured mole fractions; however, some variation is observed in the absolute values. These deviations may be due to inaccuracies in the UNIFAC group contribution method. TREP is found to be a correct method to predict within some limits the ester mole fractions at equilibrium for all mixtures of solvents, substrates, and products.

The production of monoesters can be enhanced in reaction systems with a sufficient high concentration of a polar solvent. In experiments with a triglyme-to-decanoic acid ratio of 5, almost no di- and triesters can be detected at equilibrium.

## INTRODUCTION

The use of organic solvents in enzymatic synthesis is now generally accepted. Because of the great variety of organic solvents, attempts are made to establish rules for the choice of organic solvents. Important parameters are the activity and stability of the enzyme in organic solvents. In general, hydrophobic solvents are preferred, since it is known that retention of enzyme activity is favorable in these solvents. Brink and Tramper[1] used the Hildebrand solubility parameter to relate biocatalytic activity and solvent hydrophobicity. However, a better correlation was found by Laane et al.[9], who used the log $P$ value, which is the partition coefficient of a given compound in the octanol-water two-phase system. Recently, two new parameters were introduced. Schneider[15] suggested using a three-dimensional solubility parameter approach to predict enzyme activity in nonaqueous systems. In this approach, polar and dispersive as well as hydrogen bonding interactions are taken into account. It is possible that the three-dimensional solubility parameter approach is more universal for the prediction of enzyme activity in all kinds of nonaqueous systems, however, more data are necessary to prove the usefulness of this approach. Khmelnitsky et al.[8] have introduced the denaturation capacity. This parameter can be used to predict the threshold concentration, i.e., the concentration of the organic solvent at which half inactivation of the enzyme is observed. For predictions using the denaturation capacity scale, the threshold concentrations of two reference solvents for the enzyme concerned have to be known from literature or experiments.

Besides the effect of solvents on the activity and stability of the enzyme, there is also a solvent effect on the equilibrium position of reactions. Substrates and products will partition between both phases in aqueous-organic two-phase systems. The partition coefficient of a compound is usually defined as the concentration in the organic phase divided by the concentration in the aqueous phase. Martinek et al.[11] have shown quantitatively that the overall product concentration in aqueous-organic two-phase systems can be related to the partition coefficients of substrates as well as products and to the volume ratio of both phases. To obtain high overall product concentrations, it is essential to use an organic solvent for which the partition coefficient of the product is high. This results in an efficient extraction of the product to the organic phase and a higher conversion will be obtained. Eggers et al.[3] further developed this theory.

Halling[6] suggests a method to predict the effect of solvents on the equilibrium position in dilute systems by using a relation between the reaction equilibrium constants and the partition coefficients of all reactants. Group contribution correlations are used to predict the partition coefficients, and a correlation of the ratio of partition coefficients with the solubility of water in the organic solvent is shown. Valivety et al.[16] studied the esterification of dodecanol and decanoic acid in a predominantly organic system, with a water activity close to 1. They defined a concentration-based equilibrium constant, which consists of the equilibrium constant multiplied by the activity coefficients of alcohol and acid and divided by that of the ester. This practical equilibrium constant depends on the type of solvent, and a good correlation is found with the solubility of water in the solvent. They showed that esterification is favored in nonpolar solvents. This approach is only valid for reaction systems where the water activity is close to 1 and the activity coefficients are approximately constant. However, in many esterification reactions polar substrates, such as glycerol, are used. These systems usually consist of two phases, and to obtain high product concentrations at equilibrium, high glycerol and low water concentrations are necessary. This results in a low water activity. For the prediction of solvent effects on the equilibrium position in these systems, the approaches mentioned in the literature [3,6,11,16] cannot be used since they are only valid for dilute systems and/or systems with a water activity close to 1.

The aim of this chapter is to develop a thermodynamically based theory for prediction of the effect of solvents on esterification in aqueous-organic two-phase reaction systems with a low water activity. The prediction of the ester concentrations at equilibrium is based on calculation of the activity coefficients in both phases by using the

UNIFAC group contribution method and will be compared with the experimental results. The esterification of glycerol and decanoic acid is used as a model reaction. The measured ester concentrations will also be discussed in view of the empirical correlations of log $P$ and the solubility of water in the solvent. Furthermore, the results will be used to gain a better understanding of the conditions favorable for production of an excess of monoacylglycerols.

## THEORY

For a reaction, where water is one of the products,

$$A + B \rightleftharpoons C + H_2O$$

the reaction equilibrium can be described by

$$K = \frac{a_C \cdot a_{H_2O}}{a_A \cdot a_B} \tag{1}$$

where $a_i$ is the thermodynamic activity of component $i$ and $K$ is the equilibrium constant. The latter is constant for a given temperature. The activity is expressed as the product of the mole fraction ($x_i$) and the activity coefficient ($\gamma_i$). For nondilute solutions, the activity coefficient is a function of the mole fractions of all components in the system. Equation (1) can be written as

$$K = \frac{x_C \cdot x_{H_2O}}{x_A \cdot x_B} \cdot \frac{\gamma_C \cdot \gamma_{H_2O}}{\gamma_A \cdot \gamma_B} \tag{2}$$

Equation (1) shows that high product activities can be obtained when substrate activities are high, which means large excesses of the substrates $A$ and $B$ in the reaction mixture. Also, lowering of the water activity will result in an increase of the product activity and subsequently an increase of the product mole fraction at equilibrium. The water activity can be lowered, for example, by addition of a water-miscible solvent or by a high mole fraction of hydrophilic components, such as salts and sugars.

Furthermore, the addition of an organic solvent will affect the concentrations of the other components at equilibrium. The overall concentrations will be reduced by dilution, which is an unwanted effect. Besides dilution, the solvent will change the activity coefficients of the reaction components. From equation (2) it is clear that a change in the activity coefficients will cause a change in the equilibrium mole fractions. This change can be both positive as well as negative, dependent on the characteristics of the solvent.

For reactions in two-phase systems, the partition of the components over the phases has to be taken into account. At equilibrium, the activity of component *i* in phase 1 is equal to the activity of component *i* in phase 2:

$$a_i^{I} = a_i^{II} \tag{3}$$

$$x_i^{I} \cdot \gamma_i^{I} = x_i^{II} \cdot \gamma_i^{II} \tag{4}$$

In this article the esterification of glycerol and decanoic acid is studied. This reaction consists of three consecutive reaction steps. These reaction steps and the equilibrium constants concerned are defined as follows:

$$\text{Glycerol + fatty acid} \rightleftharpoons \text{monoester + } H_2O \qquad K_M = \frac{a_M \cdot a_{H_2O}}{a_G \cdot a_F} \tag{5}$$

$$\text{Monoester + fatty acid} \rightleftharpoons \text{diester + } H_2O \qquad K_D = \frac{a_D \cdot a_{H_2O}}{a_M \cdot a_F} \tag{6}$$

$$\text{Diester + fatty acid} \rightleftharpoons \text{triester + } H_2O \qquad K_T = \frac{a_T \cdot a_{H_2O}}{a_D \cdot a_F} \tag{7}$$

where *G* is glycerol, *F* is fatty acid, *M* is monoester, *D* is diester, and *T* is triester.

For the calculation of the effect of organic solvents on the equilibrium concentrations, it is important to determine the activity coefficients. For multicomponent mixtures, activity coefficients can be estimated by using binary data. These data can be extrapolated to multicomponent activity coefficients using, e.g., the UNIQUAC approach. However, the number of components of interest is very large and binary data are often not available. Therefore, it is convenient that computational estimates are available for liquid-liquid systems such as the UNIFAC group contribution method.[4]

Furthermore, the equilibrium constants have to be known. In this study, these values are determined experimentally in a reaction system without a solvent: The reactant concentrations at equilibrium are measured and the activity coefficients are calculated using UNIFAC. The equilibrium constants then can be calculated with equation (2). With these equilibrium constants and the initial amounts, ester concentrations at equilibrium can be predicted for any reaction system with a solvent. For this purpose a computer program is developed.

## MATERIALS AND METHODS

### Materials

Lipase from *Chromobacterium viscosum* was obtained from Biocatalysts Ltd. (United Kingdom). The enzyme had a specific activity of 53 U/mg solid (determined by Biocatalysts with olive oil as a substrate). 1-Monodecanoylglycerol, 1,3-didecanoylglycerol, and tridecanoylglycerol were obtained from Sigma Chemical Company (USA). Glycerol, toluene, chloroform, 1-nonene, and 2-methyl-2-pentanol were all of 99% purity from Janssen Chimica (Belgium). 3-Ethyl-3-pentanol (97%), 2-methyl-2-hexanol (97%), 2,6-dimethyl-4-heptanol (90%), and 3-methyl-3-pentanol (99%) were obtained from Aldrich Chemie (Belgium). Hexane was high-performance liquid chromatography (HPLC) grade from Rathburn. Decanoic acid (98%), isopropyl ether (98%), pentyl ether (95%), isoamyl ether (95%), and 2,4-dimethyl-3-pentanol (98%) were from Merck (Germany). Triglyme (triethylene glycol dimethyl ether), diglyme (diethylene glycol dimethyl ether), monoglyme (ethylene glycol dimethyl ether), and all other solvents had a purity of at least 99% and were also obtained from Merck.

### Reaction

Ten mmoles decanoic acid, 20 mmole glycerol, 20 mmole water, 10 mmole solvent, and 25 mg lipase were mixed in 10-mL stoppered glass bottles. The bottles were shaken by an end-over-end incubator (150 rpm) at 35 °C. After 200 - 300 h samples were taken from the organic phase and analyzed by HPLC.

**Analysis**

The fatty acid and ester concentrations were determined by HPLC using two size exclusion columns (PLgel 30 cm, Polymer Laboratories) placed in serial order. The columns were eluted with tetrahydrofuran at a flow rate of 1.0 $mL.min^{-1}$, and the effluent was monitored with a refractive index detector. The fatty acid and ester concentrations were calculated using the internal normalization method. The response factors were determined using a standard solution of decanoic acid, 1-monodecanoylglycerol, 1,3-didecanoylglycerol, and tridecanoylglycerol. Concentrations were expressed as mole fractions. In the organic phase, the sum of mole fractions of solvent, fatty acid, mono-, di-, and triacylglycerol is 1.

**Equilibrium**

Whether the equilibrium is reached or the reaction is stopped due to inactivation of the enzyme is investigated by:

*1. Addition of extra lipase after 200 h of incubation.* No significant change of the mole fractions was measured, which means that the reaction equilibrium is achieved within 200 h of incubation. Some experiments with nonpolar solvents and most experiments with polar solvents were controlled in this way.

*2. Starting the reaction with half the amount of decanoic acid (5 mmole), the other half is added after 200 h of incubation.* The mole fractions at equilibrium are supposed to be independent of the way it is reached, and for this reason, the equilibrium mole fractions have to be the same as in the experiment where 10 mmole of decanoic acid is added at the start of the experiment. Indeed, these experiments resulted in equilibrium mole fractions which usually deviate less than 5%, but in no case more than 10%. This control was used for the experiments with triglyme, ethyl ether, isopropyl ether, and toluene.

*3. Hydrolysis instead of esterification.* The reaction was performed with 3.3 mmole tridecanoylglycerol, 16.7 mmole glycerol, 30 mmole water, 10 mmole solvent, and 25 mg lipase. Reaction conditions were the same as described under Reaction. Both hydrolysis experiments led to mole fractions at equilibrium, which deviate less than 10% of the mole fractions of the esterification experiments. The equilibrium positions in triglyme and toluene were confirmed in this way.

## Calculations

*Activity coefficients*

For the estimation of activity coefficients the UNIFAC group contribution method was used.[4] Molecules are divided into functional groups and the properties of each group are considered as well as the interactions between all possible pairs of groups. In this article, the UNIFAC parameter table of Magnussen et al.[10] is used. This table is especially developed for the prediction of liquid-liquid equilibria, including the prediction in aqueous-organic two-phase systems at temperatures between 10 and 40 °C. The activity coefficients are calculated with reference to an ideal solution in the sense of Raoult's law.

*Phase equilibrium*

A computer program was developed that calculated the activities of all components and the composition of the two phases in equilibrium. Input variables include the temperature, the required UNIFAC parameters, the measured amounts of esters and decanoic acid in the organic phase, and the total amounts of water and glycerol at equilibrium. The program started with calculation of the activities of all components in both phases. The next step was transfer of a small amount of each component to the phase with the lowest activity. This was followed by recalculation of all activities in each phase. This procedure was repeated until the activity for each component in phase 1 equals the activity in phase 2 within the given accuracy [equation (3)]. The output of this computer program consisted of the mole fractions, activity coefficients, and activities of all components in both phases at the phase equilibrium. The equilibrium constants were calculated according to equations (5)-(7).

*Reaction equilibrium*

Another computer program was developed to calculate the mole fractions of reactants in a two-phase system in case both the reaction equilibrium and the phase equilibrium were achieved. This program is called TREP, Two-phase Reaction Equilibrium Prediction. Input variables were the equilibrium constants, temperature, initial amounts of all components, and the required UNIFAC parameters. The program started with the calculation of the phase equilibrium. This was followed by a small reaction step, according to the reaction scheme of equations (5)-(7). After each reaction step, the phase equilibrium is recalculated. This was repeated until the reaction

equilibrium is reached, which means that the calculated ratio of activities equals the input equilibrium constants within a given accuracy. The output of TREP consisted of the mole fractions, activity coefficients, and activities of all components in both phases at reaction and phase equilibrium.

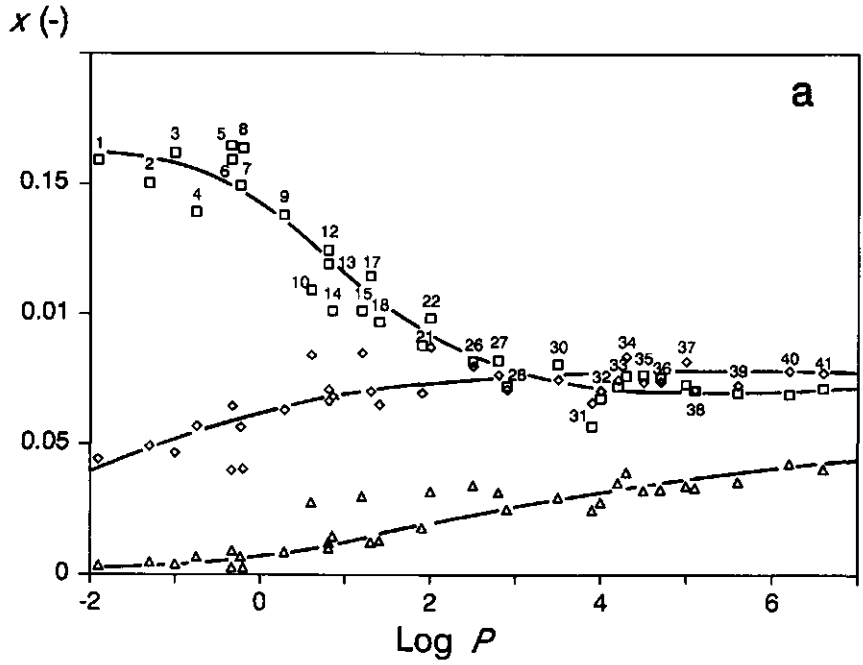
To compare the calculated mole fractions with the experimental mole fractions, the calculated mole fractions are corrected in such a way that the sum of mole fractions of solvent, fatty acid, mono-, di-, and triacylglycerol is 1.

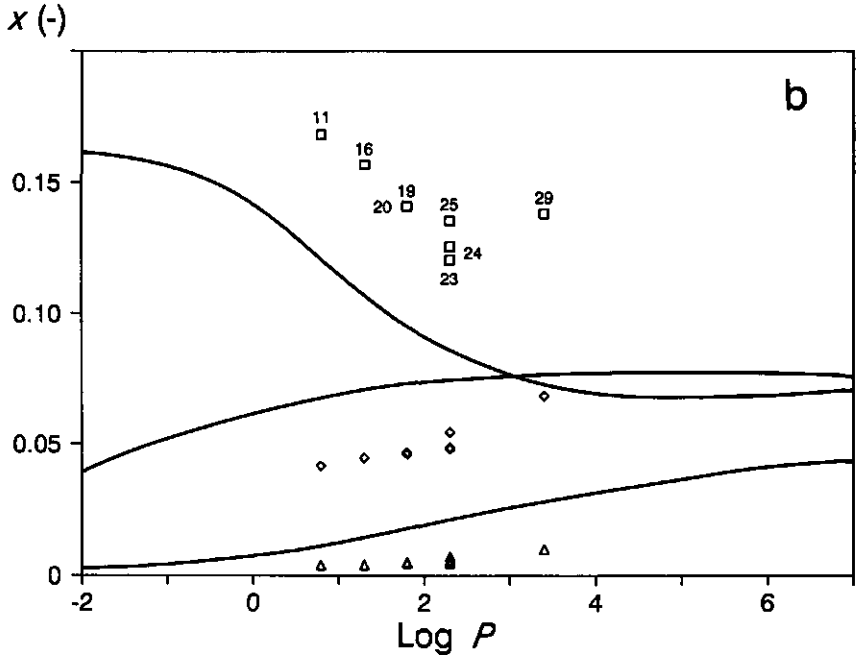
## RESULTS AND DISCUSSION

### Empirical correlations with log P and log $S_{w/o}$

The esterification of glycerol and decanoic acid is carried out in several solvents, including aliphatic and aromatic hydrocarbons, ethers, ketones, aldehydes, tertiary alcohols, nitriles, and halogenated hydrocarbons. The solvents used in this study and some of their characteristics are given in table I. This table includes also polar solvents, and it is known that the stability of enzymes in these solvents is not always good.[9] For this reason lipase from *Chromobacterium viscosum* is used, since it is known from previous studies[7] that the stability of this lipase in polar organic solvents is good. Experiments revealed that the reaction equilibrium is really achieved (see Materials and Methods, Equilibrium). Furthermore, experiments showed that the tertiary alcohols do not participate in the esterification reaction.

The ester mole fractions in the organic phase at equilibrium are plotted versus the log *P* value of the solvent (figure 1a,b). Tertiary alcohols are given separately in figure 1b, since they show a clearly different correlation with log *P*. Figure 1a clearly shows that the product mole fraction is influenced by log *P*. In a polar solvent, polar products such as monoesters are relatively better solvated; while nonpolar products such as triesters are better solvated by nonpolar solvents. This results in high mole fractions of monoester and low mole fractions of triester in polar solvents (log $P < 1$). Addition of solvents with decreasing polarity results in decreasing mole fractions of the monoester and increasing mole fractions of triester. The diester mole fraction is not very dependent on the solvent polarity. The addition of solvents to the reaction mixture gives the possibility of influencing the product distribution. However, log *P* is not the only parameter which


x (-)
a
0.15
0.01
0.05
0
-2
0
2
4
6
Log P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Log P

controls product distribution. From figure 1b, it is observed that the monoesters are more efficiently solvated by tertiary alcohols than is described by their log *P* values when compared with the other solvents.

Recently Valivety et al.[16] reported that the solubility of water in the solvent is a useful parameter for the selection of a solvent. Since the solubility of water is not known for all solvents used, the water solubility is calculated by using the UNIFAC group contribution method. The calculated solubility of water ($S^c_{w/o}$) is compared with the tabulated values of $S^t_{w/o}$ in table I and it is found that the values differ in most cases less than 10% of the average. Furthermore, it can be seen from table I that there is a reasonable correlation between log *P* and $S^c_{w/o}$. However, there are some exceptions, for example, dichloromethane and 1,2-dichloroethane. Also, a consistency with the whole group of tertiary alcohols is absent. To investigate whether or not the logarithm of the calculated solubility of water is a better parameter to predict the ester mole fractions at equilibrium, the data from figure 1 are plotted as a function of $S^c_{w/o}$ (figure 2). In this plot the tertiary alcohols cannot be recognized as a separate group, as with the log *P* correlation. Generally the same trends can be seen as in figure 1. Addition of a polar solvent, a solvent with a high solubility of water, results in a relatively high mole fraction of monoester, while the nonpolar solvents, having a low solubility of water, show relatively higher mole fractions of di- and triesters. However, data are more scattered in the relation with $S^c_{w/o}$ than in the relation with log *P*. In addition, $S^c_{w/o}$ cannot be used for the interesting group of polar solvents, which are completely miscible with water. For these two reasons we prefer to use log *P* in this study.

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**Figure 1:** Measured ester mole fraction in the organic phase as a function of log *P*.
The reaction was carried out with 10 mmole decanoic acid, 10 mmole solvent, 20 mmole glycerol, 20 mmole water, and 25 mg lipase (*Chromobacterium viscosum*). The samples were incubated for 300 h at 35 °C. Part (a) shows all solvents except of the tertiary alcohols; these are shown in part (b). The solvents are numbered as in table I. Monoacylglycerol (□), diacylglycerol (◇), and triacylglycerol (△). The lines represent the best fit of the experiments in part (a).

**Table I**: Characteristics of the organic solvents used in this study.

| | Solvent | log $P$ | log $S^{c}_{w/o}$ | log $S^{l}_{w/o}$ |
|---|---|---|---|---|
| 1. | Triglyme | -1.9 | -0.44 | |
| 2. | Diglyme | -1.3 | -0.47 | |
| 3. | *N,N*-Dimethylformamide | -1.0 | [a] | |
| 4. | Monoglyme | -0.75 | -0.47 | |
| 5. | 4-Hydroxy-4-methyl-2-pentanone | -0.34 | [a] | |
| 6. | Acetonitrile | -0.33 | [a] | |
| 7. | Acetone | -0.23 | [a] | |
| 8. | 1-Methyl-2-pyrrolidone | -0.20 | [b] | |
| 9. | 2-Butanone | 0.28 | -0.44 | -0.51 |
| 10. | Dichloromethane | 0.60 | -2.15 | -2.03 |
| 11. | 2-Methyl-2-propanol | 0.79 | [a] | |
| 12. | 2-Pentanone | 0.80 | -0.93 | -0.85 |
| 13. | 3-Pentanone | 0.80 | -1.28 | -0.95 |
| 14. | Ethyl ether | 0.85 | -1.19 | -1.24[c] |
| 15. | 1,2-Dichloroethane | 1.2 | -2.14 | -2.09 |
| 16. | 2-Methyl-2-butanol | 1.3 | -0.36 | -0.22 |
| 17. | 4-Methyl-2-pentanone | 1.3 | -1.18 | -1.02 |
| 18. | *tert*-butylmethyl ether | 1.4 | -1.02 | |
| 19. | 2-Methyl-2-pentanol | 1.8 | -0.44 | -0.41 |
| 20. | 3-Methyl-3-pentanol | 1.8 | -0.44 | |
| 21. | Isopropyl ether | 1.9 | -1.75 | -1.50 |
| 22. | Chloroform | 2.0 | -2.27 | -2.21 |
| 23. | 2,4-Dimethyl-3-pentanol | 2.3 | -0.52 | |
| 24. | 3-Ethyl-3-pentanol | 2.3 | -0.51 | |
| 25. | 2-Methyl-2-hexanol | 2.3 | -0.51 | |
| 26. | Toluene | 2.5 | -2.66 | -2.78[c] |
| 27. | Trifluorotrichloroethane | 2.8 | [b] | |
| 28. | Butyl ether | 2.9 | -1.50 | -1.87 |
| 29. | 2,6-Dimethyl-4-heptanol | 3.4 | -0.63 | |
| 30. | Hexane | 3.5 | -3.17 | -3.27[c] |
| 31. | Pentyl ether | 3.9 | -1.58 | |
| 32. | Isoamyl ether | 4.0 | -1.92 | |
| 33. | 1-Octene | 4.2 | -2.70 | |
| 34. | Phenyl ether | 4.3 | [b] | |
| 35. | Isooctane | 4.5 | -3.07 | -3.46[c] |

| | | | | |
|---|---|---|---|---|
| 36. | 1-Nonene | 4.7 | -2.7 | |
| 37. | Hexyl ether | 5.0 | -1.64 | |
| 38. | Nonane | 5.1 | -3.06 | |
| 39. | Decane | 5.6 | -3.03 | -3.24[c] |
| 40. | 1-Dodecene | 6.2 | -2.69 | |
| 41. | Dodecane | 6.6 | -2.98 | -3.21[c] |

*Note:* Log $P$ is the logarithm of the partition coefficient of a given compound in the octanol-water two-phase system, calculated according to Rekker.[13] Log $S_{w/o}$ is the logarithm of the saturated solubility of water in the solvent on a mole fraction basis. Log $S^c_{w/o}$ are values calculated by using the UNIFAC group contribution method ($T$ = 25 °C). Log $S^l_{w/o}$ are values taken from Riddick et al.[14] or from Valivety et al.[16]

[a] The solvent is miscible with water.

[b] The required UNIFAC parameters are not available.

[c] Solubility of water is taken from Valivety et al.[16]

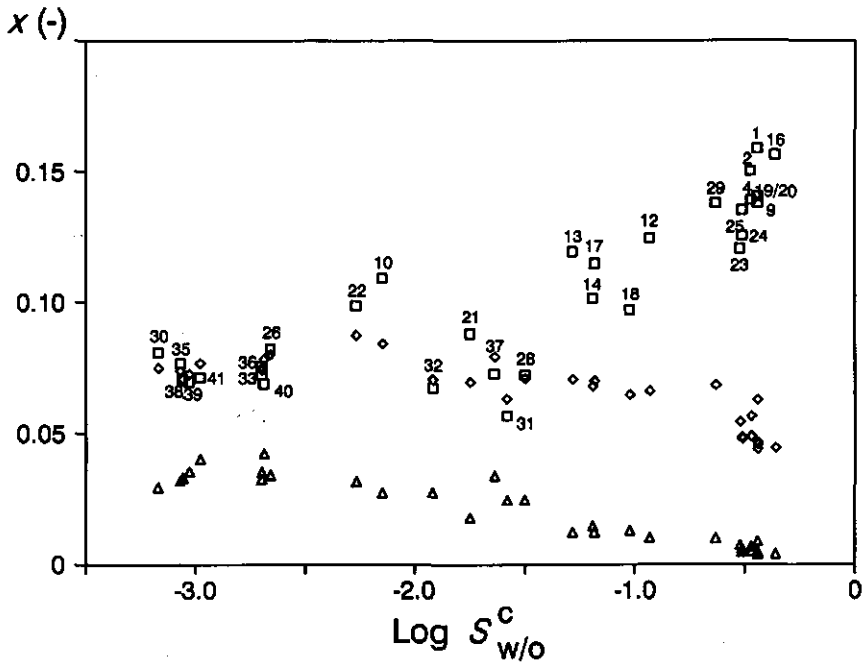



**Figure 2:** Ester mole fraction in the organic phase as a function of the solubility of water in the solvent. Data from experiments of figures 1a and 1b. Solvents are numbered as in table I. Monoacylglycerol (□), diacylglycerol (◊), and triacylglycerol (△).

The mole fraction of fatty acid in the organic phase is plotted versus log *P* (figure 3), and it can be seen that the fatty acid mole fraction has, within limits, a constant value, independent of the log *P* value. This means that the degree of esterification is not dependent on the choice of solvent. Valivety et al.[16] conclude that esterification is preferred in nonpolar solvents. They have studied the enzymatic synthesis of dodecyl decanoate, a reaction with a single, nonpolar product. In agreement with our results, this product is better solvated by nonpolar solvents and for this reason they report a higher degree of esterification in the nonpolar solvents. Figure 3 and 1, however, show that for esterification the polarity of the product determines which solvent is preferred, and multiproduct reactions can lead even to an independence of the total degree of esterification on the type of solvent.

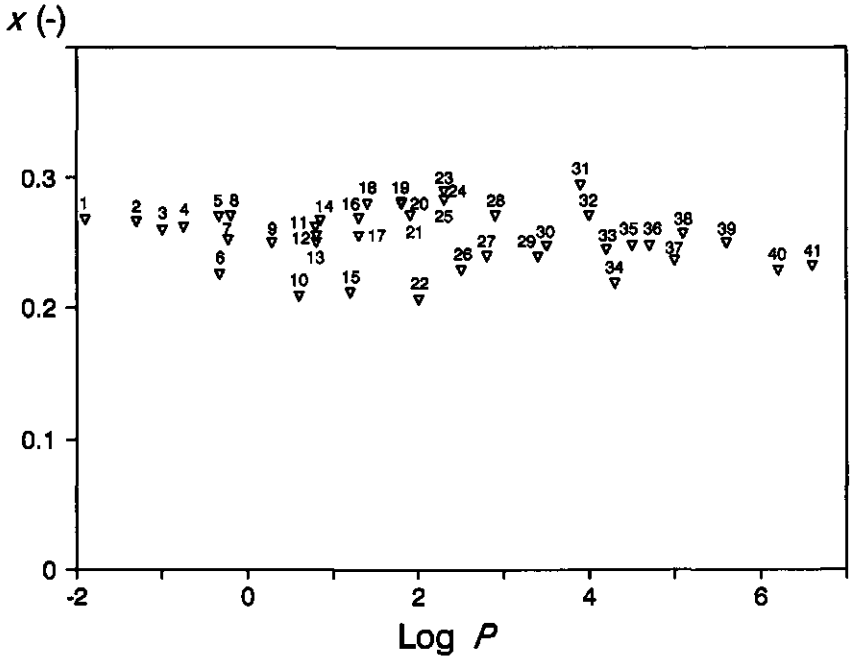



**Figure 3:** Fatty acid mole fraction in the organic phase as a function of log *P*.
Data from experiments of figures 1a and 1b. Solvents are numbered as in table I.

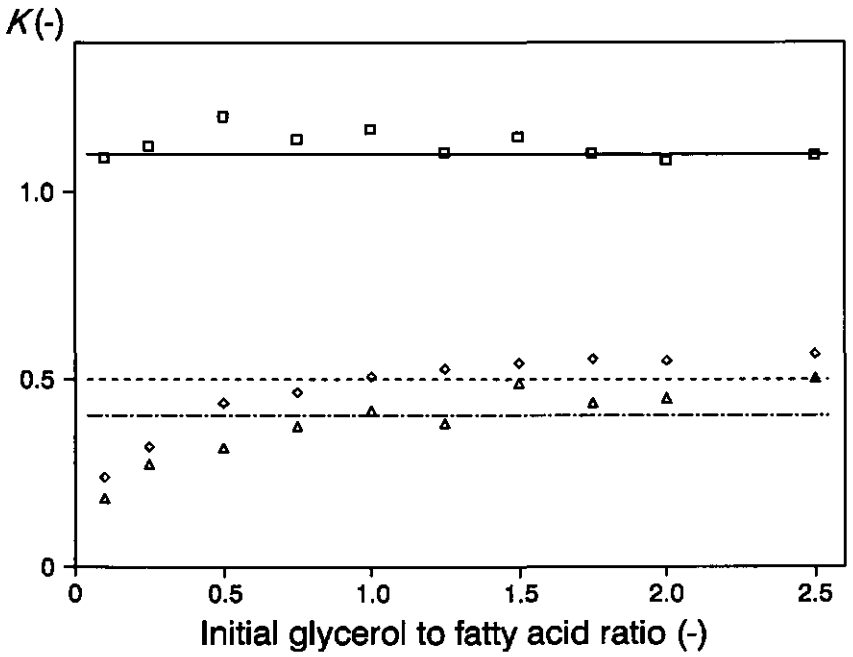



**Figure 4:** Equilibrium constants of mono- (□), di- (◊) and triacylglycerol (△) synthesis as a function of the glycerol-to-fatty acid ratio.
The reaction was carried out with 20 mmole decanoic acid, equal molar amounts of glycerol and water, and 25 mg lipase (*Chromobacterium viscosum*). The samples were incubated for 200 h at 35 °C. The lines represent the average values for the equilibrium constants.

**Determination of equilibrium constants**

The equilibrium constants are determined in experiments with a water/glycerol and a fatty acid phase but without the addition of an organic solvent. In these experiments the molar ratio of glycerol to fatty acid is varied, while the glycerol-to-water ratio is kept constant. The equilibrium mole fractions of the esters and fatty acid are measured in the organic phase. The mole fractions, activity coefficients, and activities in both phases at the phase equilibrium are calculated as described in Materials and Methods. From the activities, the equilibrium constants for the three consecutive reaction steps are calculated

and plotted versus the molar ratio of glycerol to fatty acid (figure 4). The equilibrium constants are expected to be constant for thermodynamic reasons. However, a decrease of $K_D$ and $K_T$ at low ratios can be seen in figure 4. In figure 5 the average $K$ values are used to describe the measured mole fractions at equilibrium from the same experiments. It can be seen that the calculation of the mole fractions is fairly good, indicating that the results are not extremely sensitive for the K values. In this study, the equilibrium constants of 1.1, 0.5, and 0.4 are used for the monoester, diester, and triester synthesis, respectively.

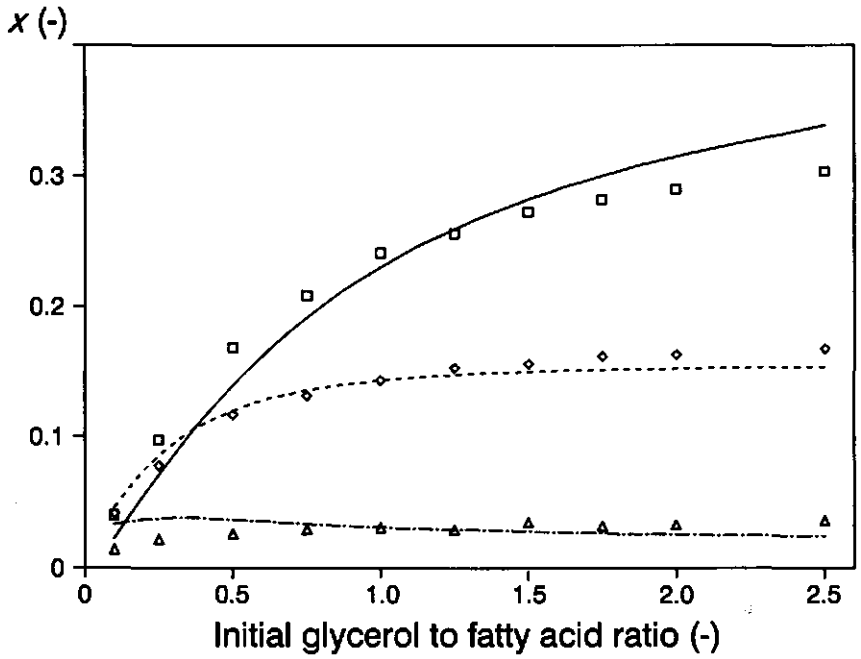



**Figure 5:** Measured and calculated ester mole fractions as a function of the glycerol-to-fatty acid ratio.
Experimental data are from figure 4. Input variables TREP: $K_M$=1.1, $K_D$=0.5 and $K_T$=0.4; $T$=308 K; Initial amounts are the same as in the experiments. Measured mono- (□), di- (◇), and triacylglycerol (△) mole fractions and calculated mono (——), di- (- - -), and triacylglycerol (- · - ·) mole fractions.

**Calculations with TREP**

The average values for the equilibrium constants are introduced into TREP, and the ester mole fractions for the experiments in figure 1 can be calculated. The calculations for the solvents dimethylformamide, acetonitrile, 1-methyl-2-pyrrolidone, 1,2-dichloroethane, trifluorotrichloroethane, and phenyl ether are missing, since not all the required group interaction parameters are known. The calculated ester mole fractions are plotted versus log *P* in figure 6, and the same trends can be seen as in figure 1. The high monoester mole fractions in tertiary alcohols are predicted from the calculations. Furthermore, it can be seen from figure 6 that in the alkenes, 1-octene, 1-nonene, and 1-dodecene, relatively high mole fractions of di- and triesters are calculated and in dichloromethane, a low monoester and a high di- and triester mole fraction is calculated.

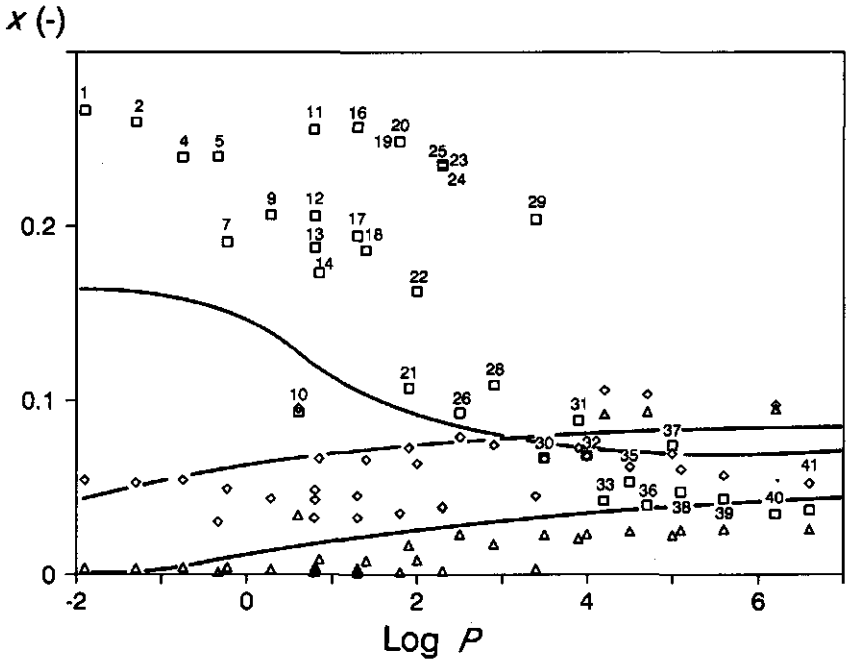



**Figure 6:** Calculated ester mole fractions as a function of log *P*.
Input variables TREP: $K_M$=1.1, $K_D$=0.5, and $K_T$=0.4; $T$=308 K; Initial amounts are 10 mmole decanoic acid, 10 mmole solvent, 20 mmole glycerol, and 20 mmole water. Solvents are numbered as in table I. The lines are taken from figure 1a. Monoacylglycerol (□), diacylglycerol (◇), and triacylglycerol (△).

The correlation between calculated and experimental mole fractions is shown in figure 7. The predicted values of the monoester mole fractions deviate less than a factor of 2 with the measured values. This deviation is generally in the upward direction for solvents with log $P$ below 4 and in the downward direction for solvents with log $P$ values above 4. The predictions of the diester mole fractions are fairly good; they deviate less than a factor of 1.5. The predictions of the triester mole fractions are generally too low. Deviations rise up to a factor of 4. However, this is at very low absolute concentrations, where relative deviation can easily be large. From figure 7 it can be seen that in hydrocarbons, for example decane and dodecane, the experimentally measured mole fractions are higher than the calculated mole fractions. This deviation is probably responsible for the higher $K$ values, as presented by Van der Padt et al.[17] They have investigated the same reaction with hexadecane as solvent, and the $K$ values are found to be 1.6, 0.8, and 0.6.

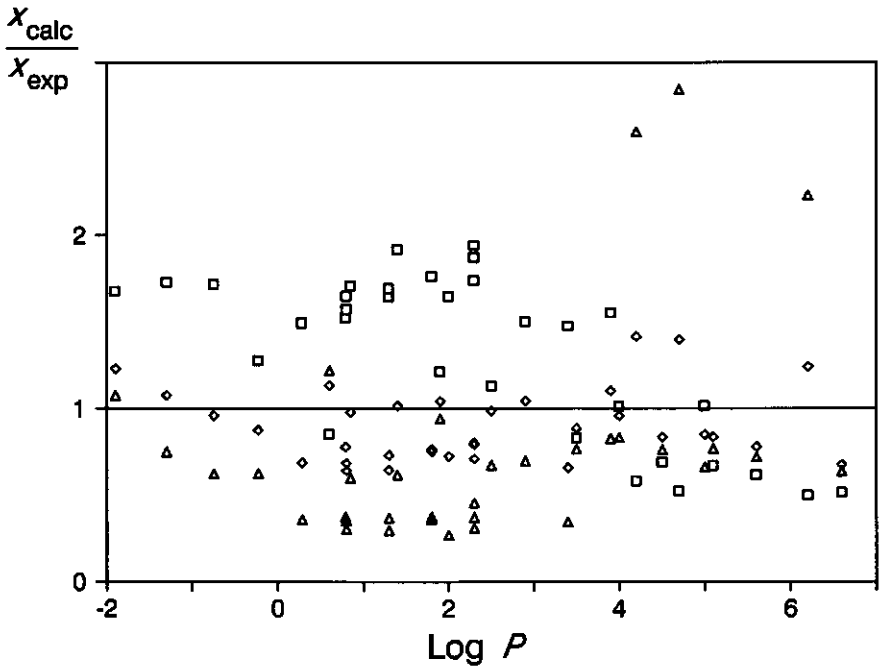



**Figure 7:** Calculated divided by measured ester mole fractions as a function of log $P$. Data are from figures 1 and 6. Monoacylglycerol (□), diacylglycerol (◇), and triacylglycerol (△).

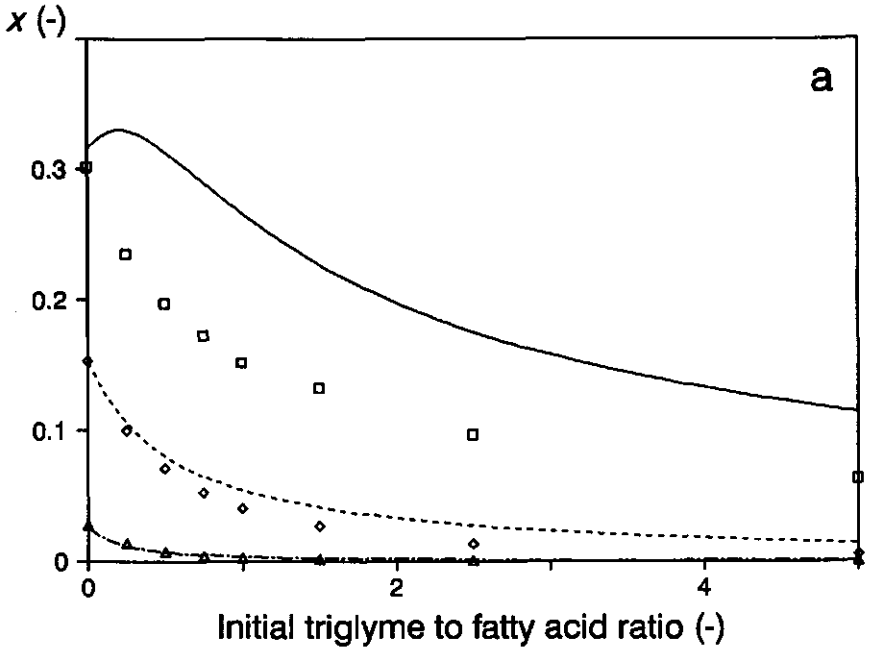
The UNIFAC method was originally developed to predict vapor-liquid equilibria. For the prediction of liquid-liquid equilibria, the UNIFAC method is less accurate. Therefore, it is necessary to use a parameter table which is suitable for prediction of liquid-liquid equilibria for the appropriate temperature range. The water and glycerol activities of water/glycerol mixtures are calculated by using the UNIFAC group contribution method as well as by using the formulas of Norrish.[12] The absolute differences between both calculation methods are less than 0.012, indicating that the prediction of simple mixtures with polar components is sufficient. For complex mixtures as used in our experiments, trends can be calculated; however, some variation is observed in prediction of the absolute values. Not only the substrate/product mixture has to be considered. Another aspect which has to be taken into account is the effect of the enzyme on the activity coefficient. This is left out of consideration to avoid complexicity.

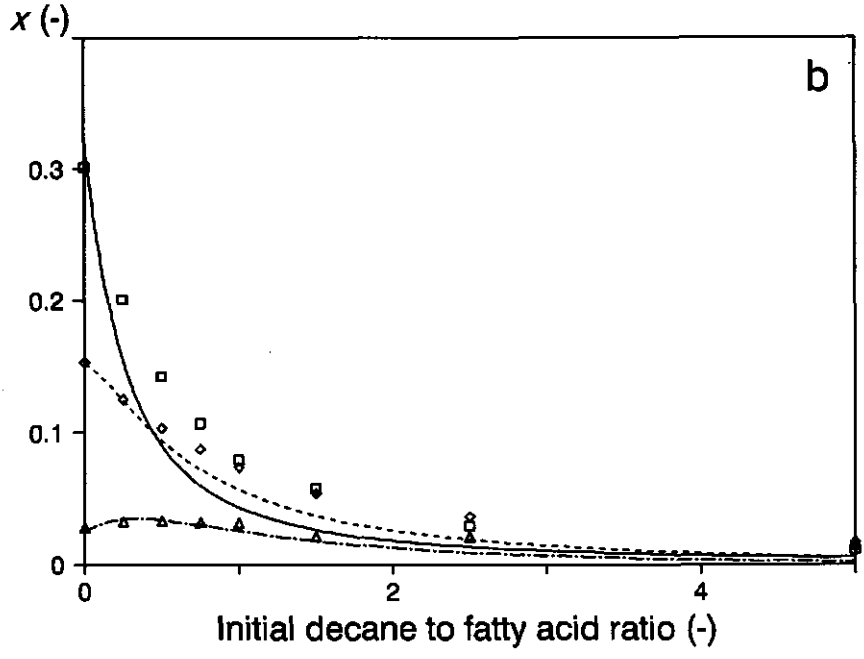
The UNIFAC group contribution method is also used by Bruce and Daugulis,[2] who studied the selection of appropriate organic solvents for an ethanol extractive fermentation process. They used UNIFAC to predict the partition coefficients of ethanol for several pure solvents and solvent mixtures. They concluded that experimental and predicted values are not exactly the same but the trends are in good agreement.

TREP is essentially a correct method for calculation of activity coefficients. For complex mixtures, its accuracy in predicting the exact quantitative values is acceptable at present. A more accurate group contribution method may yield a better agreement between experimental and calculated ester mole fractions. Yet with TREP and the measured $K$ value(s) for any reaction, it is possible to predict the mole fractions in any solvent within some margins. This means that a method is now available based on thermodynamic principles. This is clearly different from the correlations based on log $P$: For every reaction it is necessary to measure the mole fractions at equilibrium in 5 or 10 solvents to obtain the empirical correlation with log $P$.

**Consequences for monoester production**

Because TREP is based on thermodynamic principles, it is also possible to predict the influence of the solvent concentration on the equilibrium position of the esterification of glycerol and decanoic acid. This effect is studied by changing the molar ratio of solvent to decanoic acid. In figure 8 experimental and predicted ester mole fractions are shown for triglyme, a polar solvent with a log $P$ value of -1.9 as well as for decane, a nonpolar solvent with a log $P$ of 5.6. These figures show again a remarkable difference in the


x (-)
a
0.3
0.2
0.1
0
0
2
4
Initial triglyme to fatty acid ratio (-)


x (-)
b
0.3
0.2
0.1
0
0
2
4
Initial decane to fatty acid ratio (-)

equilibrium ester mole fractions in solvents with different polarity. In both solvents the ester mole fractions are reduced by an increasing solvent-to-fatty acid ratio. This reduction is not a linear dilution. In the nonpolar solvent decane, the decrease of the monoester mole fraction is faster than the decrease of the diester mole fraction. The triester mole fraction decreases only slowly, but in the experiments with the polar solvent triglyme, the relative decrease of the di- and triester mole fractions is faster than the decrease of the monoester mole fraction. These phenomena also can be explained by the solvation of the products by the solvents.

The calculations with TREP are represented by the lines in figure 8, and it can be seen that the predicted distribution of the ester mole fractions are in good agreement with the experimental results. There is some deviation in the absolute values of the predicted mole fractions. This deviation is again the highest for the monoester mole fractions and can be up to a factor of 2 in both solvents. Correlations based on the log $P$ of the solvent or the log $P$ of the decanoic acid/solvent mixtures cannot be used for prediction of the ester mole fractions at equilibrium. These examples show clearly the difference between empirical methods such as that of log $P$ and TREP, which is based on thermodynamic principles.

The results of figure 8a also show the conditions for the production of an excess of monoesters. At a triglyme-to-decanoic acid ratio of 5, at equilibrium the organic phase consists of three major components, monoester, decanoic acid, and solvent. No triester and only a very small amount of diester can be detected. Due to the high triglyme-to-decanoic acid ratio, the polarity of the organic phase is very high, which means that the

---

**Figure 8:** Measured and calculated ester mole fractions as a function of the molar ratio of triglyme (a) and decane (b) to decanoic acid.
The reaction was carried out with 10 mmole decanoic acid, 20 mmole glycerol, 20 mmole water, solvent, and 25 mg lipase (*Chromobacterium viscosum*). The samples were incubated for 200 h at 35 °C. Input variables TREP: $K_M$=1.1, $K_D$=0.5, and $K_T$=0.4; $T$=308 K; initial amounts are the same as in the experiments. Measured mono- (□), di- (◇), and triacylglycerol (△) mole fractions and calculated mono (——), di- (---), and triacylglycerol (– · – ·) mole fractions.

solvation of the monoester is very high. Addition of a certain concentration of a polar solvent is an efficient way to obtain monoesters as the only product in the reaction mixture. The concept of using polar organic solvents for the production of monoacylglycerol is also used by Graille.[5]

## CONCLUSIONS

It is shown that addition of an organic solvent to the reaction mixture is extremely important for equilibrium product distribution. For the production of monoesters, a polar solvent with a log *P* value below 1 is favorable, while for the production of triesters it is better to choose a nonpolar solvent with a high log *P* value. There is a clear correlation between the ester mole fractions at equilibrium and log *P* of the added solvent. Only the group of tertiary alcohols behaves differently.

To predict the effect of organic solvents on the ester mole fractions at equilibrium, the program TREP is developed. TREP is based on the UNIFAC group contribution model. With TREP it is possible to calculate the ester mole fractions at the reaction equilibrium under all conditions for all mixtures of solvents within some margins. It can be expected that this method also will work for the calculation of the product mole fractions of any other reaction in any solvent. The absolute accuracy is not yet 100%, but all trends are predicted. The differences may be due to deficiencies in the UNIFAC calculation method. It can be expected that improved group contribution methods will be developed in the future.

## ACKNOWLEDGEMENTS

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## NOMENCLATURE

| | | |
|---|---|---|
| $a$ | activity | (-) |
| $K$ | equilibrium constant | (-) |
| $P$ | partition coefficient in *n*-octanol-water | ($M_{org}.M_w^{-1}$) |
| $S^c_{w/o}$ | calculated solubility of water in organic solvent | (-) |
| $S^t_{w/o}$ | tabulated solubility of water in organic solvent | (-) |
| $x$ | mole fraction | (-) |
| $\gamma$ | activity coefficient | (-) |

**Subscripts:**

| | |
|---|---|
| calc | calculation |
| D | diester |
| exp | experimental |
| F | fatty acid |
| G | glycerol |
| M | monoester |
| T | triester |
| i,A,B,C | components |

**Superscripts:**

| | |
|---|---|
| I | phase 1 |
| II | phase 2 |

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# chapter 5

# SOLVENT EFFECTS ON LIPASE-CATALYSED ESTERIFICATION OF GLYCEROL AND FATTY ACIDS


## SUMMARY

The lipase-catalyzed acylglycerol synthesis with fatty acids of different chain length is studied. Measured ester mole fractions at equilibrium are compared with calculated mole fractions. For these calculations the computer program TREP (Two-phase Reaction Equilibrium Prediction) is used. This program is based on the UNIFAC group contribution method and is developed for nondilute two-phase reaction systems.

With one set of equilibrium constants, namely 1.3, 0.8 and 0.6 for monoester, diester, and triester synthesis, respectively, the equilibrium position of the reaction between glycerol and all saturated fatty acids with a chain length from 6 to 18 and oleic acid (cis-9-octadecenoic acid) can be calculated within some margins. Without addition of a solvent, the ester mole fractions at equilibrium are dependent on the fatty acid chain length. With the short-chain hexanoic acid, the monoester mole fraction is the highest ester mole fraction, while for the long-chain oleic acid, the diester mole fraction is the highest one. The ester mole fractions become independent on the chain length of the fatty acid with a solvent added in a sufficient high concentration. Both reactions, with saturated and unsaturated $C_{18}$ fatty acids, lead to the same equilibrium position.



This chapter is submitted for publication by the authors A.E.M. Janssen, A. Van der Padt and K. Van 't Riet.

The program TREP is found to make good predictions of the equilibrium amounts of ester and fatty acid. However, systematic deviations arise between measured and calculated amounts of water and glycerol in the organic phase. The calculated water and glycerol amounts are always lower than the measured ones. These deviations seem to be highest in nonpolar media and are probably due to deficiencies in the UNIFAC calculation method.

Some preliminary experiments show the effect of the choice of solvent on the reaction rates. In polar solvents, the monoester production rate is enhanced by a factor 1.5 as compared to the reaction rate in a system without solvent.

## INTRODUCTION

Lipase-catalyzed esterification of glycerol and fatty acid was already described in 1911.[6] However, this research topic is more extensively studied only for the last two decades. Several aspects of lipase-catalyzed acylglycerol synthesis are studied, such as source and specificity of the lipase, addition of an organic solvent, the role of water, immobilization, and the type of reactor. This and other aspects of lipase catalysis have recently been reviewed by John and Abraham,[7] and Mukherjee.[10]

This chapter is focussed on acylglycerol synthesis with fatty acids of different chain length. Only a few papers are known that describe acylglycerol synthesis with different chain length fatty acids. Tsujisaka et al.[14] studied the esterification of glycerol and $C_2$ - $C_{18}$ fatty acids, catalyzed by four lipases from moulds. With lipases from *Geotrichum candidum* and *Penicillium cyclopium*, acylglycerol synthesis was only detected with long-chain fatty acids. The lipases from *Aspergillus niger* and *Rhizopus delemar* also catalyzed the reaction with short and medium chain fatty acids. For *Aspergillus niger* lipase, the degree of synthesis after 16 h of reaction at 30 °C, was more or less the same for all fatty acids, except for acetic, tetradecanoic, hexadecanoic, and octadecanoic acid. The latter three fatty acids were in the solid state at the reaction temperature. The esterification of glycerol and $C_2$ - $C_{20}$ fatty acids, catalyzed by *Humicola lanuginosa* lipase, was studied by Ibrahim et al.[4] After 18 h of reaction at 45 °C, the degree of synthesis was more or less the same for the fatty acids with carbon numbers between $C_{12}$ and $C_{18}$. No acylglycerols were detected with $C_2$ - $C_8$ and $C_{20}$ fatty acids. Tahoun et al.[18] compared the degree of esterification for acids between $C_4$ and $C_{18}$, varying the number of double bonds

for $C_{18}$ from 0 to 2. The reaction was catalyzed by an intracellular lipase of *Aspergillus niger* at 30 °C. After 16 h of reaction, the degree of synthesis decreased with increasing chain length. Furthermore, higher degrees of synthesis were obtained with unsaturated fatty acids as compared with saturated fatty acid. In these three papers,[4,13,14] the degree of synthesis was measured after a certain reaction time and it can be expected that the reaction equilibrium was not always reached. If so, in these papers the degree of synthesis is an indication mainly of the specificity of the enzyme. No literature is available on the effect of the fatty acid chain length on the ester concentrations at the reaction equilibrium.

Predictions on the equilibrium position of a reaction in an aqueous-organic two-phase system were made by Martinek et al.[9] They showed a dependence of the overall product concentration on the partition coefficients of all reactants and the volume ratio of both phases. Halling[3] also used partition coefficients to make predictions about the shift of the equilibrium concentrations in different solvents. He argued that the effect of different solvents on the equilibrium position should be the same for all reactions of a given type, for example all esterification reactions. Furthermore, Halling[3] showed a correlation between the predicted equilibrium position and the solubility of water in the solvent. This correlation was confirmed by the experimental results of the esterification of dodecanol and decanoic acid.[15]

Prediction of the equilibrium position, by the methods as mentioned in literature,[3,9,15] is only valid for dilute reaction systems with a water activity close to 1. These requirements are certainly not fulfilled for the esterification of glycerol and fatty acid in a two-phase system, where the water activity is far below 1. For these nondilute reaction systems with a water activity below 1, the computer program TREP (Two-phase Reaction Equilibrium Prediction) was developed.[5] Measured and calculated results of the esterification of glycerol and decanoic acid in several organic solvents, were found to be in good agreement. It is the objective of this chapter to reveal the influence of fatty acid chain length on the equilibrium position of the esterification with glycerol.

## THEORY

In two-phase systems, all components partition between both phases. At the phase equilibrium, the thermodynamic activity of component $i$ in phase 1 ($a_i^{I}$), equals the thermodynamic activity of that component in the other phase ($a_i^{II}$)

$$a_i^{I} = a_i^{II} \tag{1}$$

The activity can be expressed as the product of the mole fraction ($x_i$) and the activity coefficient ($\gamma_i$). Then equation (1) becomes

$$x_i^{I} \cdot \gamma_i^{I} = x_i^{II} \cdot \gamma_i^{II} \tag{2}$$

For nondilute solutions, the activity coefficient is dependent on the mole fractions of all components in the system. The UNIFAC group contribution method can be used for calculation of activity coefficients.[2]

The esterification of glycerol and fatty acid consists of three consecutive reaction steps:


Three equilibrium constants can be defined, an equilibrium constant for monoester synthesis ($K_M$), one for diester synthesis ($K_D$) and one for triester synthesis ($K_T$):

$$K_M = \frac{a_M \cdot a_{H_2O}}{a_C \cdot a_F} \tag{3}$$

$$K_D = \frac{a_D \cdot a_{H_2O}}{a_M \cdot a_F} \tag{4}$$

$$K_T = \frac{a_T \cdot a_{H_2O}}{a_D \cdot a_F} \tag{5}$$

where *G* is glycerol, *F* is fatty acid, *M* is monoester, *D* is diester and *T* is triester.

The program TREP,[5] Two-phase Reaction Equilibrium Prediction, is developed to calculate the mole fractions of all reactants at phase and reaction equilibrium. In this program, activity coefficients are calculated by using the UNIFAC group contribution method. TREP can be used for every reaction for which the equilibrium constant(s), temperature, initial number of moles of all components, and the required UNIFAC parameters are known.

## MATERIALS AND METHODS

### Materials

Lipase from *Chromobacterium viscosum* was obtained from Biocatalysts Ltd. (UK). The enzyme had a specific activity of 29 U/mg solid (determined by Biocatalysts with olive oil as a substrate). The fatty acids, hexanoic acid (99%), octanoic acid (99%), dodecanoic acid (95%), and tetradecanoic acid (95%) were obtained from Janssen Chimica (Belgium). Decanoic acid (98%), hexadecanoic acid (96%), octadecanoic acid (97%), and cis-9-octadecenoic acid (oleic acid, 72%) were from Merck (Germany). Monoacylglycerols, diacylglycerols and triacylglycerols of the fatty acids mentioned before, were obtained from Sigma Chemical Company (USA), with the exception of monohexanoylglycerol, which was not available.

Glycerol, toluene and chloroform were of 99% purity from Janssen Chimica. Isopropyl ether (98%), isoamyl ether (95%) and hexyl ether (97%) were from Merck. Triglyme (triethylene glycol dimethyl ether), diglyme (diethylene glycol dimethyl ether), monoglyme (ethylene glycol dimethyl ether), and all other solvents had a purity of at least 99% and were obtained from Merck. Before use, the organic solvents and substrates were dried over molecular sieve 0.3 nm (beads 2 mm) from Merck.

### Reactions

*Equilibrium*

Fatty acid, glycerol, water, solvent (if present) and lipase from *Chromobacterium viscosum* were added in 10 ml stoppered glass bottles. The bottles were shaken by an end-over-end incubator (150 rpm) at 35 °C. At a certain incubation time, samples were

taken from the emulsion, and the aqueous and organic phase were separated by centrifugation. The organic phase was analyzed by HPLC and Karl Fischer titration.

The reactions with hexadecanoic acid (with solvent) and dodecanoic acid (without solvent), were incubated at 50 °C, because these fatty acids did not dissolve at a reaction temperature of 35 °C.

Experiments were carried out until equilibrium was achieved. To confirm the equilibrium, extra lipase was added. If this did not result in an increase of the product concentrations, the equilibrium was stated to be reached. Another way to obtain the equilibrium position is to carry out hydrolysis experiments, instead of esterification experiments.[5] In hydrolysis experiments, triacylglycerol was added instead of fatty acid. The initial number of moles were chosen in such a way that theoretically the same equilibrium position had to be reached as for the esterification experiments.

*Time courses*

The experiments to measure the time course of the reaction were done in a thermostated reaction vessel of 250 ml. The reaction mixture consisted of 200 mmole oleic acid, 400 mmole glycerol, 400 mmole water, and 250 mg lipase from *Chromobacterium viscosum*. In a number of experiments 200 mmole solvent was added. The emulsion was mixed thoroughly at 35 °C, and at regular time intervals, samples were taken out and after separation the organic phase was analyzed by HPLC. The initial reaction rate was determined by linear regression on the first 4 to 10 measurements, and expressed as mmole ester per g crude enzyme per hour.

**Analysis**

*HPLC*

The fatty acid, glycerol and ester concentrations were determined by HPLC, using two size exclusion columns (PLgel 30 cm, Polymer Laboratories), placed in serial order. The columns were eluted with tetrahydrofuran at a flow rate of 1.0 $ml.min^{-1}$, and the effluent was monitored with a refractive index detector. For the determination of calibration curves, solutions of pure fatty acids, glycerol, mono-, di-, and triacylglycerols were used. For monohexanoylglycerol, the slope of the calibration curve was estimated. It was assumed that this slope was proportional to the acyl group chain length. Concentrations were expressed as mole fractions, and the sum of mole fractions of fatty

acid, mono-, di-, and triacylglycerol, and solvent (if present) in the organic phase is 1. The solvents cannot be analyzed by this HPLC method and are assumed to be entirely present in the organic phase. Small errors will appear for the polar solvents, which are expected to be partly present in the aqueous phase.

*Karl Fischer titration*

Water concentrations were determined with a Mettler DL18 Karl Fischer Titrator (Mettler, Switzerland).

**Calculations**

For the estimation of activity coefficients the UNIFAC group contribution method was used.[2] In this study, the UNIFAC parameter table of Magnussen et al.[8] was used. This table is developed especially for the prediction of liquid-liquid equilibria, including the prediction in aqueous-organic two-phase systems at temperatures between 10 and 40 °C. The activity coefficients are calculated with reference to an ideal solution in the sense of Raoult's law.

The program TREP, Two-phase Reaction Equilibrium Prediction,[5,16] calculates the mole fractions of reactants in a two-phase system, in case both the phase equilibrium as well as the reaction equilibrium are achieved. At the phase equilibrium, the activity for each component in phase 1 equals the activity in phase 2 within a given accuracy (equation 1-2). The reaction equilibrium is assumed if the calculated ratio of activities equals the input equilibrium constants within a given accuracy (equations 3-5). The output of TREP consisted of the number of moles, the mole fractions, activity coefficients, and activities. In order to compare the measured and calculated mole fractions, the calculated mole fractions are corrected in the same way as the measured mole fractions. This means that glycerol and water are left out and the solvent is entirely present in the organic phase.

For the determination of the equilibrium constants, only that part of TREP is used where the phase equilibrium is calculated.

## RESULTS AND DISCUSSION

Subsequently the esterification of glycerol and several fatty acids is discussed. Then, the measured and calculated water and glycerol mole fractions are discussed. Finally, the time course of the esterification of oleic acid and glycerol in some solvents is discussed.

*Oleic acid*

For the esterification of glycerol and oleic acid, the equilibrium constants for monoester, diester and triester synthesis are determined in a reaction system without an

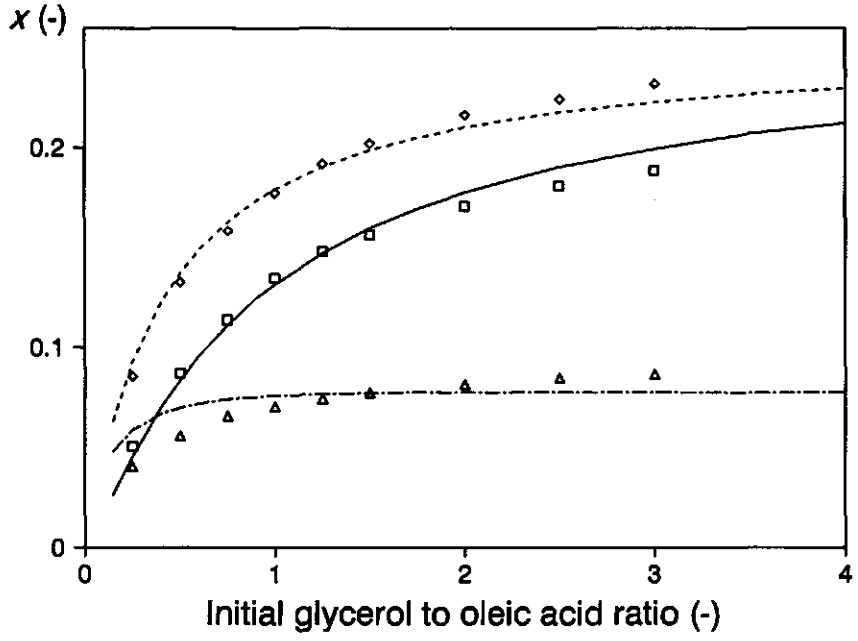



**Figure 1:** The measured and calculated ester mole fractions as a function of the initial glycerol to oleic acid ratio.

Experiments: 20 mmole oleic acid, equal molar amounts of glycerol and water, and 25 mg lipase were mixed and incubated for 215 h at 35 °C. Monooleyl- (□), dioleyl- (◇), and trioleylglycerol (△).

Calculations with TREP: $K_M$=1.1, $K_D$=0.8, and $K_T$=0.6; $T$=308 K; initial amounts are the same as in the experiments. Monooleyl- (——), dioleyl- (---), and trioleylglycerol (– · – ·).

organic solvent. Analogous to the esterification of decanoic acid,[5] experiments were carried out where the molar ratio of glycerol to oleic acid is varied, while the glycerol to water ratio is kept constant. The ester mole fractions at equilibrium are determined experimentally and the equilibrium constants are calculated according to equation (3)-(5). The average equilibrium constants are found to be 1.1, 0.8 and 0.6 for monoester, diester and triester synthesis, respectively. These constants are introduced into TREP to calculate the ester mole fractions at equilibrium. Measured and calculated ester mole fractions are shown in figure 1 and are found to be in good agreement.

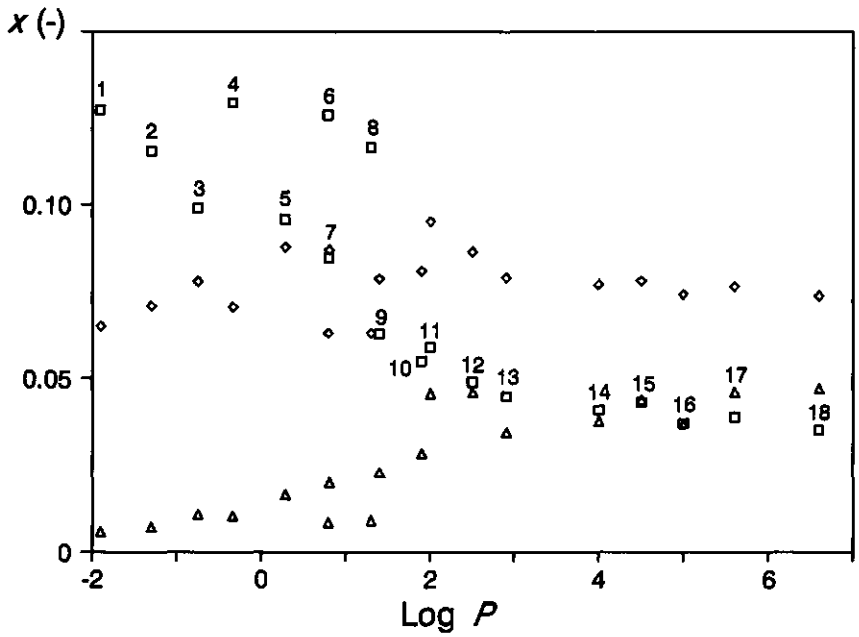



**Figure 2:** The measured monooleyl- (□), dioleyl- (◊), and trioleylglycerol (△) mole fraction in the organic phase as a function of log *P* of the solvent.
The reaction was carried out with 10 mmole oleic acid, 10 mmole solvent, 20 mmole glycerol, 20 mmole water, and 25 mg lipase. The samples were incubated for 185 h at 35 °C.
Solvents: Triglyme (1), diglyme (2), monoglyme (3), 4-hydroxy-4-methyl-2-pentanone (4), 2-butanone (5), 2-methyl-2-propanol (6), 2-pentanone (7), 2-methyl-2-butanol (8), *tert.* butylmethyl ether (9), isopropyl ether (10), chloroform (11), toluene (12), butyl ether (13), isoamyl ether (14), isooctane (15), hexyl ether (16), decane (17), dodecane (18).

The effect of solvents on the experimentally determined ester mole fractions, is shown in figure 2. Log $P$ is the logarithm of the partition coefficient of a given compound in the octanol-water two-phase system[12] and is used as parameter to indicate the polarity of the solvent. In polar solvents, with a low log $P$ value, the polar monoester is better solvated than the nonpolar triester. At equilibrium, this results in a high monoester mole fraction and a low triester mole fraction. In nonpolar solvents, which have a high log $P$ value, the mono- and triester mole fractions are more or less the same. The diester mole fractions are not very dependent on the polarity of the solvent. The relatively high monoester and the low triester mole fraction in tertiary alcohols, 2-methyl-2-propanol

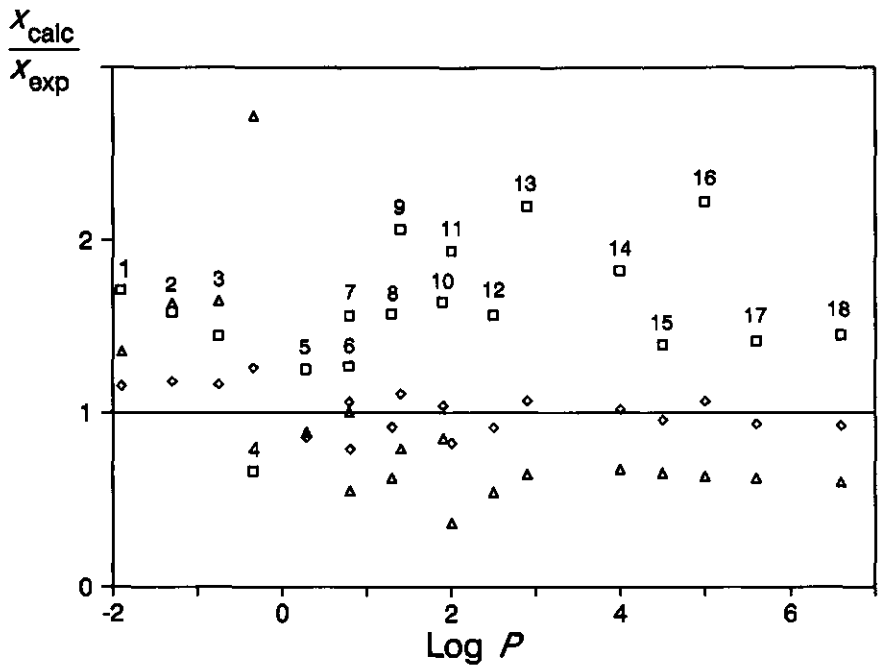



**Figure 3:** The calculated ester mole fractions divided by the measured ones as a function of log $P$ of the solvent.
Experiments: from figure 2.
Calculations with TREP: $K_M$=1.1, $K_D$=0.8, and $K_T$=0.6; $T$=308 K; initial amounts are the same as in the experiments. Monooleyl- (□), dioleyl- (◇), and trioleylglycerol (△). The solvents are numbered as in figure 2.

(number 6) and 2-methyl-2-butanol (number 8), are in agreement with previous results.[5] The oleic acid mole fractions, which are not shown in figure 2, are not very dependent on the polarity of the solvent. This was also found for the esterification of glycerol and decanoic acid.[5]

For the calculation of the ester mole fractions at equilibrium with solvents added, the average values for the equilibrium constants are introduced into TREP. Furthermore, the initial numbers of moles, the temperature and the required UNIFAC parameters are introduced. In the calculations the initial numbers of moles are the same as for the experiments of figure 2. The quotient of calculated and measured mole fractions is shown in figure 3. Systematic deviations appear to be present. The predicted values for the monoester mole fractions are generally to high, the maximum deviation is a factor 2.2. The predictions of the diester mole fractions are fairly good, deviations are less then a factor 1.3. The predicted values for the triester mole fractions are generally too low, deviations rise up till a factor 3. In case of 4-hydroxy-4-methyl-2-pentanone (number 4), the predicted monoester mole fraction is too low. In the calculations for this solvent, the solvent concentration in the aqueous phase is calculated to be very high and almost no solvent remains in the calculated organic phase. This means that the ester mole fractions are more or less the same as in a system without solvent. In all other polar solvents, the solvent is calculated to be mainly present in the organic phase.

The systematic deviations in mono-, di-, and triester mole fractions, were also found for esterification of decanoic acid,[5] and can be attributed to the parameter values used with UNIFAC. It can be concluded that the trends are well predicted.

*Octanoic acid*

The average equilibrium constants are also determined for the esterification of glycerol and octanoic acid. From experiments without an organic solvent, the average values for the equilibrium constants are found to be 1.4, 1.1 and 0.6 for monoester, diester and triester synthesis, respectively. These values are introduced into TREP to calculate the ester mole fractions at equilibrium without solvent. In figure 4, the measured and calculated ester mole fractions are shown.

Experimental and calculated data of the esterification of glycerol and octanoic acid in several solvents are not shown here. However, for the experimental results as well as for the calculations, the same trends are present as for the esterification with decanoic acid[5] and oleic acid (figures 2 and 3).

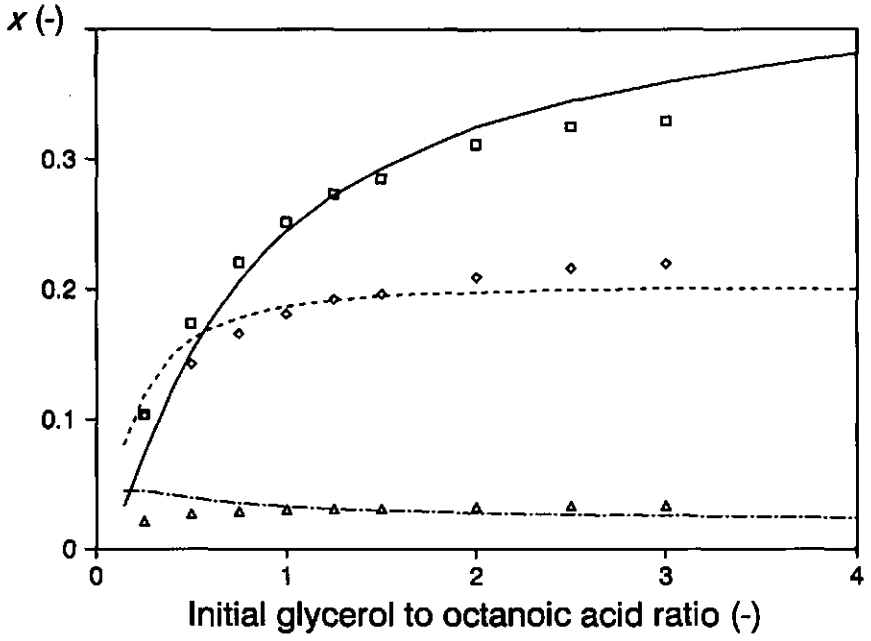



**Figure 4:** The measured and calculated ester mole fractions as a function of the glycerol to octanoic acid ratio.
Experiments: 20 mmole octanoic acid, equal molar amounts of glycerol and water, and 25 mg lipase were mixed and incubated for 215 h at 35 °C. Monooctyl- (□), dioctyl- (◇), and trioctylglycerol (△).
Calculations with TREP: $K_M$=1.4, $K_D$=1.1, and $K_T$=0.6; $T$=308 K; initial amounts are the same as in the experiments. Monooctyl- (——), dioctyl- (---), and trioctylglycerol (- · - ·).

*Chain length of fatty acids*

Table I shows the equilibrium constants of the reactions with several fatty acids. It can be seen that equilibrium constants for mono-, di- and triester synthesis, respectively, do vary although in a limited range. These three sets of equilibrium constants were used to calculate the ester mole fractions for the reaction between glycerol and saturated fatty acids with a chain length from 6 to 18 without use of solvent. The results are given in figure 5. The lines represent the smooth curves through the ester mole fractions, calculated with

**Table I:** The equilibrium constants for the esterification of glycerol and fatty acids.

| Fatty acid | $K_M$ | $K_D$ | $K_T$ |
|---|---|---|---|
| Octanoic acid | 1.4 | 1.1 | 0.6 |
| Decanoic acid | 1.1 | 0.5 | 0.4 |
| Oleic acid | 1.1 | 0.8 | 0.6 |
| Average | 1.3 | 0.8 | 0.6 |

the set of average equilibrium constants (see table I). From the calculations with the other sets of equilibrium constants, the highest and the lowest ester mole fractions are shown. The trends are in all cases the same and the ester mole fraction does not seem to change considerably with the changes of the equilibrium constants as given in table I. The set of average equilibrium constants is used to predict the equilibrium position of the esterification of glycerol and fatty acid with a different chain length, which will be discussed below.

In figure 6 the experimental results are given, represented by the symbols. The fatty acids used for the experiments in order of increasing chain length are hexanoic acid, octanoic acid, decanoic acid, dodecanoic acid and oleic acid. The open symbols are the results of experiments with saturated fatty acids, and the black symbols are the results of the experiment with oleic acid, which is unsaturated. Figure 6 clearly shows that the ester mole fractions at equilibrium are affected by the chain length of the fatty acid. With the short-chain fatty acids as organic phase, the solvability of the polar products is relatively good, resulting in high monoester mole fractions. However, with the long-chain oleic acid as organic phase, the nonpolar products are better solvated, resulting in relatively high di- and triester mole fractions. These trends are predicted well by the calculations with the set of average equilibrium constants. Highest deviations appear for the mono-, and diester of hexanoic acid ($C_6$). These deviations, expressed as the calculated mole fractions divided by the measured ones, are 1.22 for the monoester, and 0.46 for the diester. For the reaction with $C_{18}$ fatty acid, the ester mole fractions are calculated for a saturated fatty acid. However, the measured ester mole fractions are from experiments with oleic acid,

which is unsaturated. For calculations with unsaturated fatty acids as compared to the calculations with saturated fatty acid, the monoester mole fraction is decreased by 0.018, the diester mole fraction is equal, and the triester mole fraction is decreased by 0.025. These differences are low and for this reason only the calculations for saturated fatty acids are shown in figure 6.

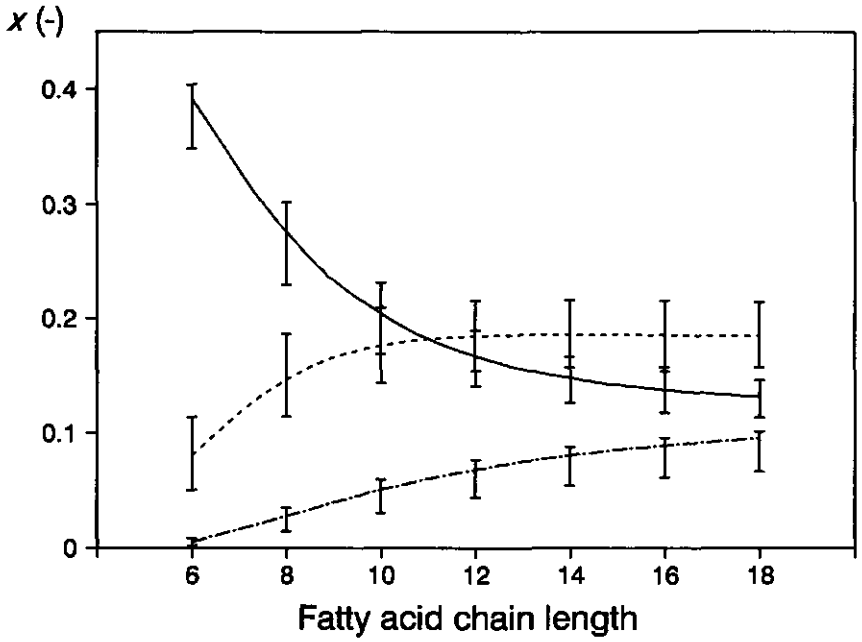



**Figure 5:** The calculated ester mole fractions as a function of the fatty acid chain length at different sets of equilibrium constants.
Calculations with TREP: Equilibrium constants: see table I; $T$=308 K; 20 mmole fatty acid (only saturated fatty acids), 20 mmole glycerol and 20 mmole water. Monoacyl- (——), diacyl- (- - -), and triacylglycerol (- · - ·). The curves represent smooth lines through the calculations with $K_M$=1.3, $K_D$=0.8, and $K_T$=0.6. Only the highest and lowest ester mole fractions from calculations with the other sets of equilibrium constants are shown.

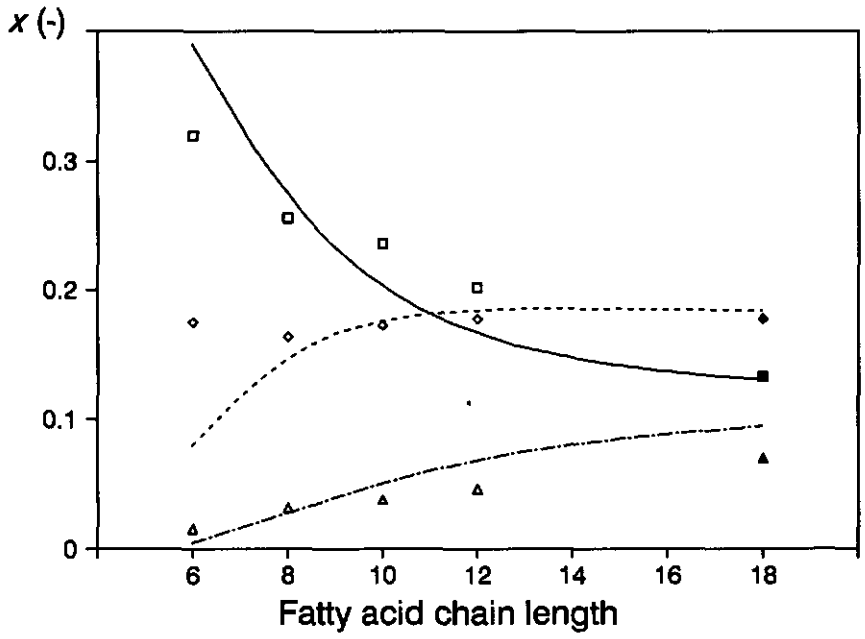



**Figure 6:** The measured and calculated ester mole fractions as a function of the fatty acid chain length.
*Experiments:* 20 mmole fatty acid, 20 mmole glycerol, 20 mmole water, and 25 mg lipase were mixed and incubated for 280 h at 35 °C. The experiment with dodecanoic acid ($C_{12}$) is carried out at 50 °C. Monoacyl- (□), diacyl- (◊), and triacylglycerol (△). Black symbols: reaction with unsaturated fatty acid.
*Calculations with TREP:* $K_M$=1.3, $K_D$=0.8, and $K_T$=0.6; $T$=308 K; *initial amounts are the same* as in the experiments (only saturated fatty acids). The smooth curves represent monoacyl- (——), diacyl- (---), and triacylglycerol (– · – ·).

Because the reaction temperature had to be above the melting point of the fatty acid, the experiment with dodecanoic acid ($C_{12}$) is carried out at 50 °C instead of 35 °C. The ester mole fractions at equilibrium are not expected to be very dependent on

temperature. This was shown by Blanco et al.,[1] who studied the esterification of N-acetyl-tryptophan and phenylethanol. They found no significant effect on the equilibrium position in the temperature range of 18 to 35 °C. Saturated fatty acids with a longer chain length than dodecanoic acid are not used. Because of the high melting points, temperatures above 55 °C are necessary for these reactions. Although oleic acid ($C_{18}$) has a longer chain length than dodecanoic acid, it could be used at 35 °C because it is unsaturated, which implicates a lower melting point.

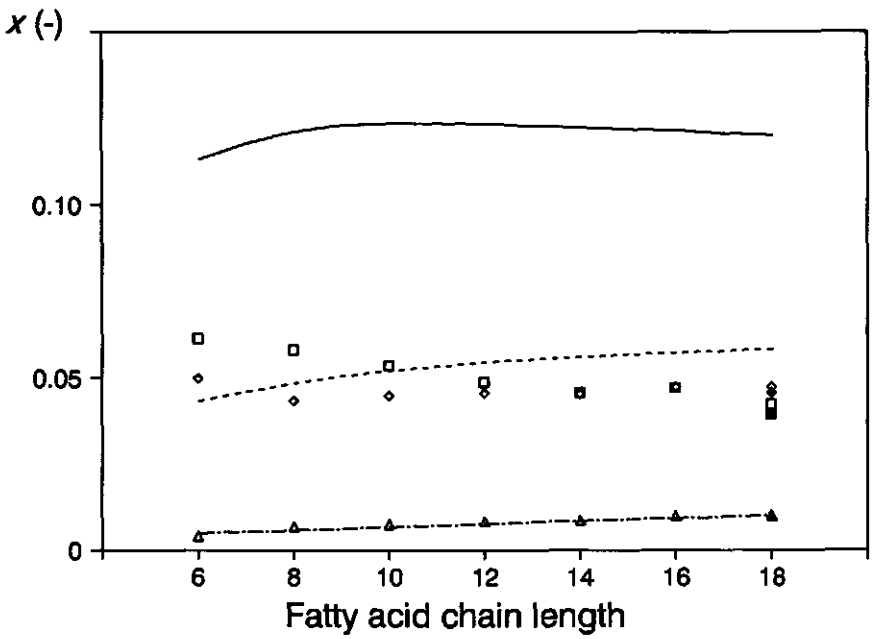



**Figure 7:** The measured and calculated ester mole fractions as a function of the fatty acid chain length in *tert.* butylmethyl ether.
Experiments: 10 mmole fatty acid, 20 mmole *tert.* butylmethyl ether, 20 mmole glycerol, 20 mmole water, and 25 mg lipase were mixed and incubated for 228 h at 35 °C. The experiment with octadecanoic acid ($C_{18}$) is carried out at 50 °C. Monoacyl- (□), diacyl- (◊), and triacylglycerol (△). Black symbols: reaction with unsaturated fatty acid.
Calculations with TREP: $K_M$=1.3, $K_D$=0.8, and $K_T$=0.6; $T$=308 K; initial amounts are the same as in the experiments (only saturated fatty acids). The smooth curves represent monoacyl- (——), diacyl- (---), and triacylglycerol (– · – ·).

In order to obtain experimental data for saturated long-chain fatty acids, experiments and calculations are performed in reaction systems with the solvents monoglyme, *tert.* butylmethyl ether, toluene and isooctane. The results of a typical one of this set of experiments (in *tert.* butylmethyl ether) are shown in figure 7. The measured results of figure 7 show that the fatty acid chain length has only little effect on the ester mole fractions at equilibrium with an organic solvent added. This is a completely different behaviour than for the case without solvent, as shown in figure 6. The lines in figure 7 represent the smooth curves through the calculated ester mole fractions for saturated fatty acids. The calculations predict also small changes only in the ester mole fraction on changing the fatty acid chain length. The calculated monoester mole fractions are systematically too high and deviations, expressed as the calculated mole fractions divided by the measured ones, are between 2 and 3. These deviations for the monoester mole fractions are in the same order of magnitude as the deviations that are found for the esterification of glycerol and oleic acid in several solvents (see figure 3). The fact that in solvents the ester mole fractions at equilibrium are not that dependent on the fatty acid chain length, is in agreement with the conclusions of Halling.[8] He argued that in dilute reaction systems, the effect of solvents on the equilibrium position is the same for all reactions of the same type. In figure 7, the initial solvent mole fraction in the organic phase is 0.67, which is found to be sufficient high to obtain the described effect on the equilibrium position.

In order to compare the equilibrium position of saturated and unsaturated fatty acid, experiments were carried out with saturated and unsaturated $C_{18}$ fatty acids. In *tert.* butylmethyl ether (see figure 7), monoglyme, toluene, and isooctane, the difference between saturated and unsaturated ester mole fractions was not more than 0.004 mole fraction. This indicates that one double bond in a fatty acid not significantly affects the equilibrium ester mole fractions. In literature,[13,14] a lower degree of esterification with saturated fatty acids is reported. However, it was already mentioned by the authors that it is most likely that the equilibrium was not achieved because the saturated fatty acids were in a solid state at the reaction temperature. In the calculations with TREP, differences between stearic acid and oleic acid ester mole fractions were higher as compared to the measurements. Calculations with $C_6$ to $C_{18}$ saturated and unsaturated fatty acids show that the differences between saturated and unsaturated ester mole

fractions did not exceed 0.010 for *tert.* butylmethyl ether, monoglyme, and isooctane. However, in the calculations for toluene these differences rose up to 0.023 mole fraction. This is probably due to deviations in the UNIFAC parameter table.

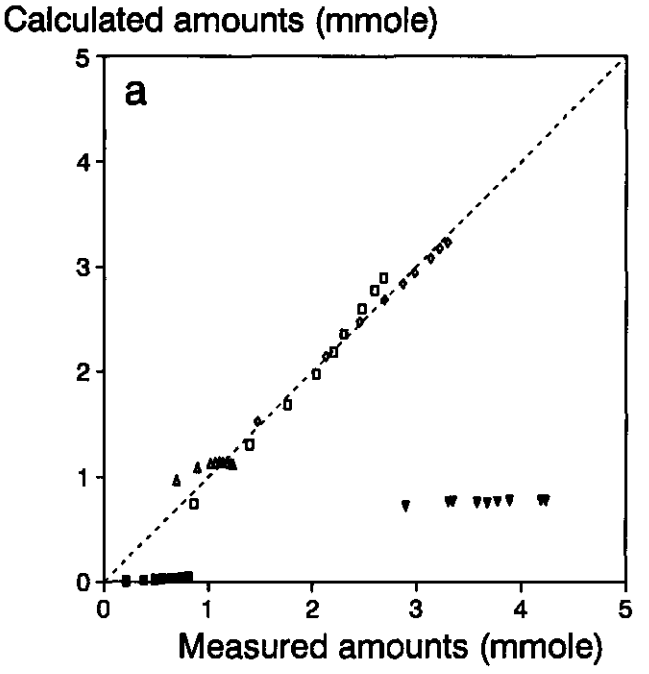
As shown in this section, one set of equilibrium constants can be used for the prediction of the equilibrium position of the esterification of glycerol and fatty acids with a chain length from 6 to 18. Good predictions are obtained in systems without as well as with solvents. The different behaviour between the reaction with and without solvent is confirmed by the calculations.

*Water and glycerol*

Throughout this paper, the sum of mole fractions of solvent, fatty acid, mono-, di-, and triester in the organic phase is 1. This means that water and glycerol are not included in the mole fraction. This is done because of the deviations that are found between the measured and calculated amounts of water and glycerol. The data are shown in figure 8. The mole fractions of the esterification of glycerol and oleic acid from figure 1, correspond with the amounts in mmole (figure 8a). As in figure 1, the deviations between calculated and measured amounts of ester are small. However, the calculated amounts of both water and glycerol are systematically too low. If these components were included in the mole fraction, this would lead to systematic deviations of all components.

In figure 8b, a comparison is made for the experiments and calculations for the esterification of glycerol and several fatty acids. For the calculations, the set of average equilibrium constants from table I is used. The deviations between calculated and measured amounts of ester show the same trends as those in figure 8a, while water and glycerol are different. The experimentally determined amounts of water and glycerol both decrease with increasing fatty acid chain length. The calculated amounts of glycerol and water show the same trends, but are systematically lower than the measured values. Only for the reaction with hexanoic acid (highest glycerol and water content), the calculated

**Figure 8:** Comparison between calculated and measured amounts of all reactants.
Data were obtained from experiments of figure 1 (part a, esterification with oleic acid) and figure 6 (part b, esterification with several fatty acids). In part b the chain length of the fatty acids is indicated for the amounts water.
Water (▼), glycerol (■), fatty acid (▽), monoacyl- (□), diacyl- (◊), and triacylglycerol (△).


Calculated amounts (mmole)
a
5
4
3
2
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1
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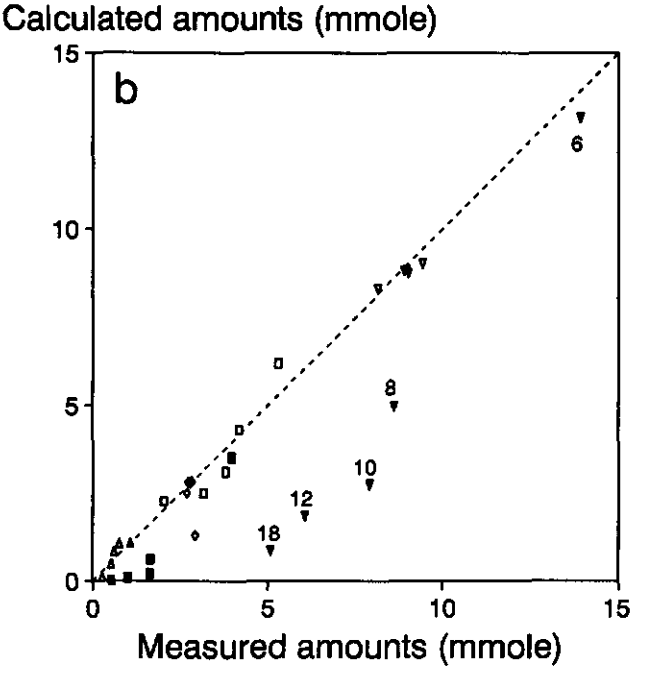
5
Measured amounts (mmole)


Calculated amounts (mmole)
b
15
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5
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6
8
10
12
18
Measured amounts (mmole)

and measured amounts of glycerol and water are in good agreement. The deviations between calculated and measured water and glycerol amounts, increase with increasing chain length of the fatty acid. By using fatty acids with a longer chain length, the polarity of the organic phase decreases. This could be an indication for a relation between the polarity of the medium and deviations in the calculated amounts of water and glycerol. However, the results of figure 3 were also plotted as calculated amounts versus measured amounts (data not shown). With these results no clear relation was found between the polarity of the medium and the deviations in the calculated amounts of water and glycerol.

It is clear that the calculated glycerol and water content of the organic phase is systematically lower than the measured values. In some cases, there seems to be a relation between the polarity of medium and the deviations. However, this correlation is not confirmed by the results from other experiments. A critical evaluation of the UNIFAC parameter table that is used in this study would be necessary to improve the glycerol and water predictions in the organic phase.

*Time courses*

In order to show the effect of solvents on the reaction rate, time courses for the esterification of glycerol and oleic acid are determined with and without addition of a solvent. The results for the experiment without solvent are shown in figure 9a. In figures 9b-9d, results are shown for the experiments in diglyme, toluene, and decane, respectively. In addition to an effect of the solvent on the equilibrium position, there is also a clear effect on the rate of mono-, di-, and triester production. Initial reaction rates are given in table II. In the experiments without solvent, the initial reaction rates for production of mono- and diester are almost equal. In the polar solvent diglyme, the monoester production rate is 2.5 times higher than the diester production rate, while in the solvents toluene and decane the diester rates are higher, 1.5 and 1.8 times the monoester reaction rate, respectively. The triester reaction rates are always much lower. In a previous study it was shown that high concentrations of a polar solvent are favorable for a high monoester concentration at equilibrium.[5] Therefore, reaction rates were also determined in a system with a high diglyme concentration. These are also given in table II and the monoester reaction rate is found to be 7.7 times higher than the diester reaction rate. The triester reaction rate is almost 0.

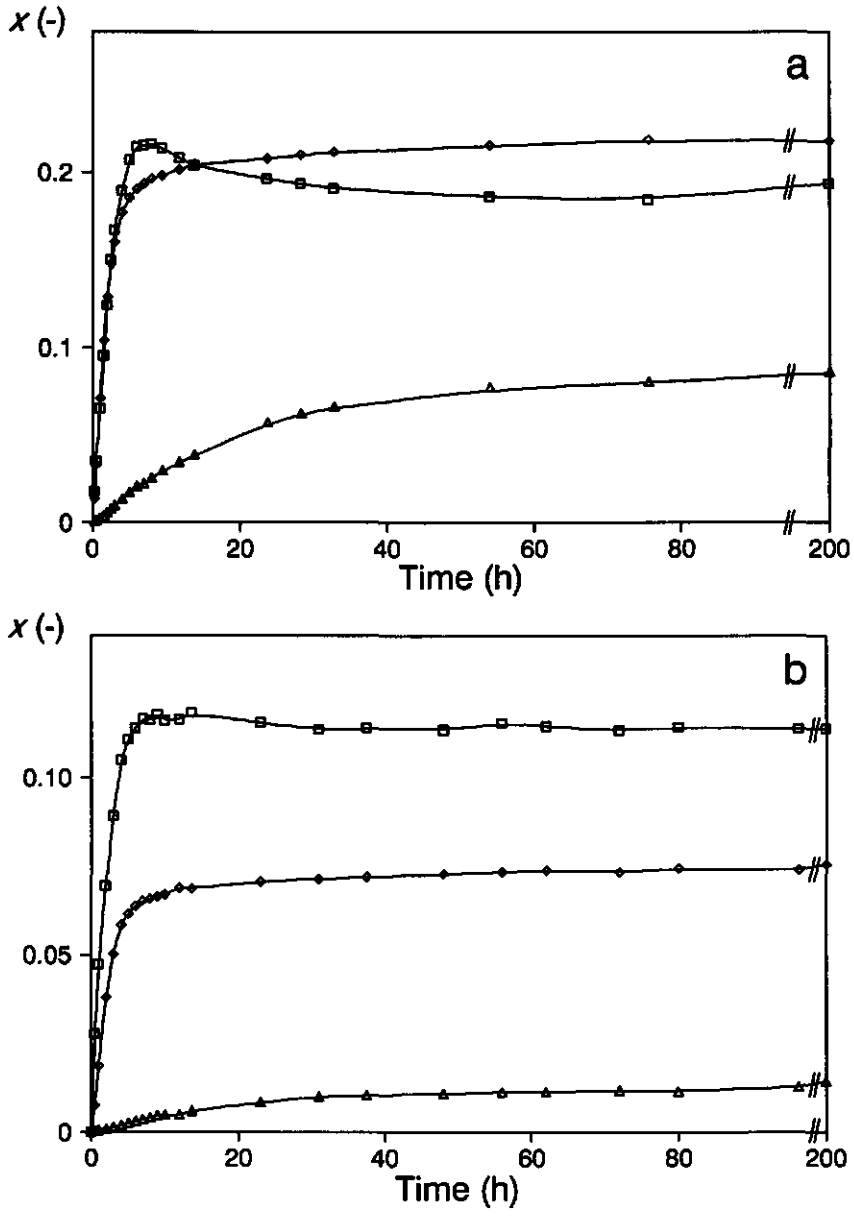
**Table II:** Initial reaction rates for the esterification of glycerol and oleic acid.

| Experiment | Initial reaction rate in mmole.g$^{-1}$.h$^{-1}$ | | | | $\tau_r$ in h |
|---|---|---|---|---|---|
| | monoester | diester | triester | oleic acid | |
| Without solvent | 48.5 | 50.6 | 2.0 | -151 | 1.9 |
| Diglyme[1)] | 74.4 | 29.7 | 0.7 | -136 | 2.0 |
| Toluene[1)] | 31.6 | 48.3 | 2.0 | -133 | 2.4 |
| Decane[1)] | 23.2 | 42.3 | 1.9 | -113 | 2.9 |
| Diglyme[2)] | 35.2 | 4.6 | 0.2 | -42 | 2.7 |

1) The initial solvent to fatty acid ratio is 1.
2) The initial solvent to fatty acid ratio is 5.

It is interesting that the monoester reaction rate in diglyme is enhanced by a factor 1.5 as compared to the reaction system without solvent. The diester reaction rate is found to be highest in the reaction system without solvent. The triester reaction rates do vary unsystematically for the reaction system without solvent and the reaction systems with toluene and decane. It is most likely that for formation of mono-, and diesters, esterification takes place at the two primary hydroxyl groups of glycerol. For triester production a secondary hydroxyl group has to be esterified and this can be expected to be relatively slow. The initial fatty acid removal rate is highest in the reaction system without solvent and is not varying much in the systems containing a solvent. In the reaction system with a high diglyme concentration, the fatty acid removal rate is lower than in the system with a low diglyme concentration.

Table II also shows the characteristic times of reaction ($\tau_r$). This value is defined as the time that is necessary to reach the average of the initial and the equilibrium fatty acid concentration. Table II shows that the characteristic times are more or less the same, indicating that differences in the initial reaction rates are not due to solvent effects on the enzyme. It is more likely that the reaction rates are proportional to the difference between the actual product concentration and the product concentration at equilibrium. This explains, for example, the high monoester reaction rate in diglyme, where the difference


x (-)
a
0.2
0.1
0
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20
40
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Time (h)
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b
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Time (h)

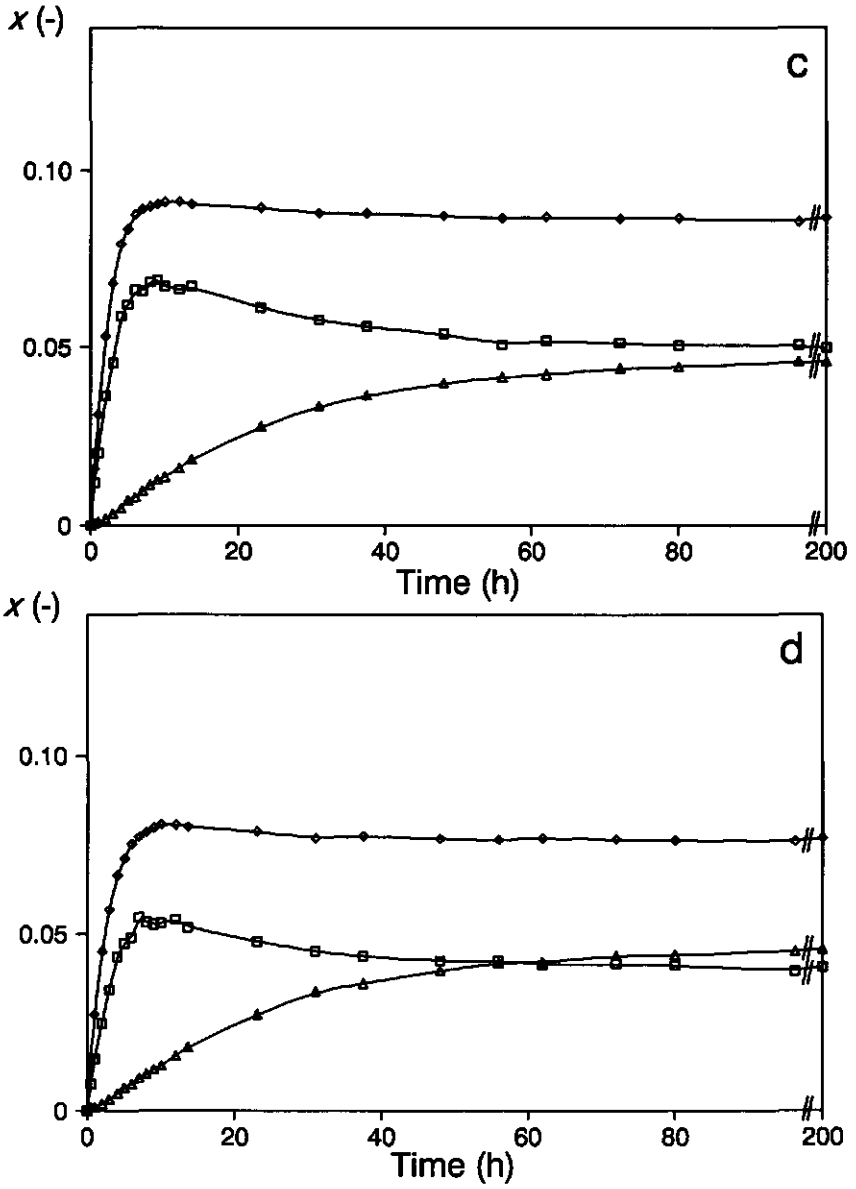


**Figure 9:** Time course of the esterification of glycerol and oleic acid, without solvent (a), in diglyme (b, log $P$ = -1.3), toluene (c, log $P$ = 2.5) and decane (d, log $P$ = 5.6).
Experiments: see 'Materials and Methods'
Monooleyl- (□), dioleyl- (◊), and trioleylglycerol (△).

between initial and equilibrium monoester mole fractions is 0.12, and the low monoester reaction rate in decane, where this difference is only 0.04. For a proper description of the kinetics of the esterification of glycerol and fatty acid, further studies are necessary.

For the esterification of dodecanol and decanoic acid,[15] and for the esterification of N-acetyl-tryptophan and phenylethanol,[1] an increase of the initial reaction rate by decreasing the polarity of the solvent was reported. In agreement with our findings, the equilibrium position of these reactions is highest in nonpolar solvents, resulting in a difference between the actual and equilibrium product concentrations being the highest for the nonpolar solvents.

## CONCLUSIONS

The usefulness of the program TREP, which is developed for the prediction of ester mole fractions at the reaction equilibrium in nondilute reaction systems, was already shown for the lipase-catalyzed esterification of glycerol and decanoic acid. Now, this work is extended for the esterification of glycerol and fatty acids of different chain length. The fatty acids octanoic acid and oleic acid (cis-9-octadecenoic acid) are extensively studied. The equilibrium constants are found to be more or less the same as for esterification with decanoic acid. Also the effect of solvents on the measured ester mole fractions is in agreement with esterification with decanoic acid; for the production of monoesters, a polar solvent is favorable and for production of triesters a nonpolar solvent has to be chosen. With the program TREP, these effects were predicted within margins.

The equilibrium position of the esterification of glycerol and other fatty acids, such as hexanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid and octadecanoic acid can be predicted with an average set of equilibrium constants. Determination of all separate equilibrium constants was not necessary. The ester mole fractions at equilibrium are found to be dependent on the fatty acid chain length if no solvent is present in the reaction system. However, the equilibrium ester mole fractions are about independent on the chain length of the fatty acid, with a solvent added at a sufficient high concentration.

The calculated amounts of ester and fatty acid are in reasonable agreement with the measured values. The trends are predicted, however, systematic deviations arise between measured and calculated amounts of water and glycerol. Especially in nonpolar media, deviations in water and glycerol amounts are high. This is probably due to deficiencies in the UNIFAC calculation method or parameter table.

Besides the equilibrium position, also the reaction rates are affected by the solvent that is added. Some preliminary experiments show that it is possible to enhance the monoester reaction rate by a factor 1.5, if a polar solvent is added to the reaction system. This is a result of the shift in equilibrium position.

## NOMENCLATURE

| | | |
|---|---|---|
| $a$ | activity | (-) |
| $K$ | equilibrium constant | (-) |
| $P$ | partition coefficient in n-octanol-water | $(M_{org}.M_w^{-1})$ |
| $\tau_r$ | characteristic time of reaction | (h) |
| $x$ | mole fraction | (-) |
| $\gamma$ | activity coefficient | (-) |

**Subscripts:**

| | |
|---|---|
| calc | calculated |
| D | diester |
| exp | experimental |
| F | fatty acid |
| G | glycerol |
| i | component |
| M | monoester |
| T | triester |

**Superscripts:**

| | |
|---|---|
| I | phase 1 |
| II | phase 2 |

## ACKNOWLEDGEMENTS

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# chapter 6

# SOLVENT EFFECTS ON THE EQUILIBRIUM POSITION OF LIPASE-CATALYZED ESTERIFICATION OF DECANOIC ACID AND VARIOUS ALCOHOLS


## SUMMARY

The lipase-catalyzed esterification of decanoic acid and several alcohols (1-dodecanol, 1-butanol, 1,3-propanediol, and sorbitol) has been studied in aqueous-organic two-phase systems. The ester mole fractions at equilibrium are dependent on the polarity of the organic solvent that is added. For the synthesis of dodecyl decanoate and butyl decanoate, the ester mole fractions increase with decreasing polarity of the solvent. The mole fraction of propanediol monoester is not very dependent on the solvent polarity, and the diester mole fraction increases with decreasing polarity of the solvent. The mole fractions of sorbitol esters are very dependent on solvent polarity. Almost no esters were present at equilibrium in systems with nonpolar solvents, while reasonable high ester mole fractions can be obtained in systems with polar solvents.

For the prediction of the mole fractions of all reaction components, the computer program TREP (Two-phase Reaction Equilibrium Prediction) is used. This program is based on mass balances and the UNIFAC group contribution method and is developed



This chapter is submitted for publication by the authors A.E.M. Janssen, N. Wessels Boer and K. Van 't Riet.

for nondilute reaction systems. Equilibrium constants are estimated from experiments without an organic solvent and are clearly affected by the type of alcohol that is chosen as a substrate. The mole fractions at equilibrium are calculated for the reactions with dodecanol, butanol and propanediol. The calculated ester mole fractions were in some cases too high and in other cases too low, but did not deviate more than a factor of 1.5 from the measured ones. For the water mole fraction, the trends are calculated. However, it appears that the calculated water mole fractions deviate systematically in the downwards direction. This could be expected, since the UNIFAC parameter table that is used, is known to give deviations in the prediction of the solubility of water. No calculations are presented for the reaction with sorbitol, because the UNIFAC parameter table that we have used was not suitable for carbohydrate solutions.

## INTRODUCTION

Numerous examples are known now of esterification of alcohols and fatty acids that are catalyzed enzymatically. In many cases an organic solvent was added to the reaction system to increase the solubility of substrates and/or products. Usually these reaction systems consist of two phases, an organic phase which contains the organic solvent and the nonpolar substrate(s) and product(s), and an aqueous phase, which contains water and the polar substrate(s) and product(s). Two-phase reaction systems can roughly be divided in emulsion-type systems and trapped aqueous phase systems.[2] In an emulsion-type system the volume of the organic and the aqueous phase is in the same order of magnitude. This results in a water-in-oil or an oil-in-water emulsion. In trapped aqueous phase systems, the aqueous phase is very small and not always clearly visible, since this phase can be restricted to the pores of the suspended enzyme particles. Although the amount of water can be very low, the water activity is still close to 1. Usually if the water activity of trapped aqueous phase systems drops below 1, the system converts to a one-liquid-phase system.

The importance of the water activity in esterification reactions, where water is one of the products, is frequently emphasized. Many attempts are made to control the water activity during the reaction. Recently, progress is made by addition of solid salt hydrates

to the reaction mixtures.[4] Another possibility is to control the water activity of the air above the reaction mixture. This could be realized by recycling the air through a saturated salt solution or by recycling the air through a condensor.[13]

Besides the water activity, also the thermodynamic activities of other reaction components are of importance. These activities are affected by the organic solvent that is added to the reaction system. Recently Valivety et al.[11] studied the equilibrium position of the reaction between dodecanol and decanoic acid. They showed a great difference in the degree of esterification in different solvents, while the water activity was kept close to 1 in all experiments. Furthermore they argued, in agreement with the studies of Halling,[3] that for prediction of solvent effects on the equilibrium position, only the reacting groups are of importance. This means that for an esterification reaction, the hydroxyl, carbonyl, water, and ester group should be taken into account. As a result, the equilibrium position in a certain solvent is equal for all esterification reactions.

In our previous work,[7] we studied the effect of organic solvents on the equilibrium position of lipase-catalyzed esterification of glycerol and decanoic acid in aqueous-organic two-phase systems. Here, the mono-, di-, and triester concentrations were found to be very dependent on the type of solvent used. The production of monoesters was favorable with addition of polar organic solvents, while the addition of nonpolar organic solvents resulted in relatively high triester concentrations. These phenomena were predicted with the program TREP (Two-phase Reaction Equilibrium Prediction), which is based on mass balances and the UNIFAC group contribution method. For the esterification of glycerol and decanoic acid TREP was found to calculate the ester mole fraction at the reaction equilibrium under all conditions within some margins.[7,12]

In this chapter, the esterification of decanoic acid and several alcohols, namely 1-dodecanol, 1-butanol, 1,3-propanediol, and sorbitol is studied. The reaction systems with 1-dodecanol and 1-butanol are trapped aqueous phase systems with a water activity close to 1. The reaction systems with 1,3-propanediol and sorbitol are emulsion type systems with a water activity below 1. The latter reaction systems are comparable to the reaction system for the esterification of glycerol and decanoic acid.[7]

The aim of this study is to investigate whether the program TREP also can be used for prediction of the equilibrium ester mole fractions for esterification reactions using another alcohol than glycerol in different types of reaction systems. Experimentally determined ester concentrations are compared with calculated concentrations. Attention

is paid to the experimental and calculated water concentration in the organic phase in addition to the ester concentrations. The possibilities and limitations of the program TREP for the prediction of equilibrium concentrations are discussed.

## THEORY

In two-phase systems, all components partition between both phases. At phase equilibrium, the thermodynamic activities of component $i$ ($a_i$) in phase 1 and 2 are equal.

$$a_i^{I} = a_i^{II} \tag{1}$$

The activity can be expressed as $a_i = x_i \cdot \gamma_i$, where $x_i$ is the mole fraction and $\gamma_i$ is the activity coefficient of component $i$. For nondilute solutions, the activity coefficient is dependent on the mole fractions of all components in the system. The UNIFAC group contribution method can be used for calculation of activity coefficients.[1]

For an esterification reaction according to the following scheme:

$$A + B \rightleftharpoons C + H_2O$$

The reaction equilibrium can be described by

$$K = \frac{a_C \cdot a_{H_2O}}{a_A \cdot a_B} \tag{2}$$

where $K$ is the equilibrium constant.

The program TREP, Two-phase Reaction Equilibrium Prediction, is developed to calculate the mole fractions of all reactants at phase and reaction equilibrium. In this program, activity coefficients are calculated by using the UNIFAC group contribution method. TREP can be used for every reaction for which the equilibrium constant, temperature, initial amounts of all components, and the required UNIFAC parameters are known.

In previous work,[7] calculations were made for the prediction of the equilibrium position of the esterification of glycerol and decanoic acid. Here, we extend this work for esterification of decanoic acid and several alcohols. For reactions with 1-dodecanol or 1-butanol, monoester and water are the products and a singly equilibrium constant, analog to equation (3), can be defined. For reaction with 1,3-propanediol, besides monoester and water, diester is formed. Subsequently two equilibrium constants are defined, a $K_M$ for monoester synthesis and a $K_D$ for diester synthesis. For the reaction with the polyol sorbitol, where water, and only mono-, di-, and triester are formed, three equilibrium constants can be defined: $K_M$, $K_D$, and $K_T$ for monoester, diester and triester synthesis, respectively.

The reaction system consists of two-phases, however, there is a clear difference between the reaction system of the monoalcohols and those of propanediol and sorbitol. In the systems with the monoalcohols, both substrates, the fatty acid and the alcohol, are present in the organic phase. The aqueous phase is relatively small and consists mainly of water, which results in a water activity of the system close to 1. In the reaction systems with propanediol and sorbitol, the fatty acid is mainly present in the organic phase and the alcohol is mainly present in the aqueous phase. The water activity of these systems is mainly dependent on the alcohol concentration in the aqueous phase and will be below 1.

## MATERIALS AND METHODS

### Materials

Lipase from *Chromobacterium viscosum* was obtained from Biocatalysts Ltd. (UK). The enzyme had a specific activity of 29 U/mg solid (determined by Biocatalysts with olive oil as a substrate). The substrates 1-butanol (99.5%), 1-dodecanol (98%), 1,3-propanediol (98%), sorbitol and decanoic acid (98%) were obtained from Merck (Germany). Hexane was HPLC grade from Rathburn. Toluene and chloroform were of 99% purity from Janssen Chimica (Belgium). Isopropyl ether (98%), pentyl ether (95%), isoamyl ether (95%), and hexyl ether (97%) were from Merck). All other solvents had a purity of at least 99% and were also obtained from Merck. The organic solvents and substrates were dried over a molecular sieve of 0.3 nm (beads 2 mm) from Merck.

**Reactions**

Decanoic acid, alcohol, water, solvent (if present), and lipase from *Chromobacterium viscosum* were mixed in 10 ml stoppered glass bottles. The bottles were shaken by an end-over-end incubator (150 rpm) at 35 °C. After a certain incubation time, samples were taken from the emulsion, and the aqueous and organic phase were separated by centrifugation. The organic phase is used for analysis.

Samples of the reactions with 1-dodecanol and 1-butanol were analyzed by fatty acid and Karl Fischer titration. Analysis by HPLC was not possible, since the peaks of dodecanol and decanoic acid and the peaks of decanoic acid and butyl decanoate can not be separated by the HPLC method used (see Analysis). Samples of the reactions with 1,3-propanediol were analyzed by HPLC and by Karl Fischer titration. Samples of the reactions with sorbitol were analyzed by HPLC.

Experiments were carried out until equilibrium was achieved. To confirm the equilibrium, extra lipase was added. If this did not result in an increase of the product concentration, the equilibrium was stated.

**Analysis**

*HPLC*

The organic phase was determined by HPLC, using two size exclusion columns (PLgel 30 cm, Polymer Laboratories), placed in serial order. The columns were eluted with tetrahydrofuran at a flow rate of 1.0 $ml.min^{-1}$, and the effluent was monitored with a refractive index detector. With this method, decanoic acid, 1,3-propanediol and the esters formed can be detected. The mole fractions were calculated by using the slope of the calibration curves of the pure components. The esters of 1,3-propanediol and sorbitol were not commercially available. Therefore, calibration of these components was estimated from experiments. For this purpose mass balances were used and the assumption was made that the slope of the calibration curves of the involved esters were equal. Concentrations were expressed as mole fractions, and the sum of mole fractions of fatty acid, alcohol, ester(s), water and solvent (if present) in the organic phase is 1. This is in contrast with chapter 4 and 5. The solvents cannot be analyzed by the HPLC method and are assumed to be entirely present in the organic phase.

*Karl Fischer titration*

The water concentration was determined with a Mettler DL18 Karl Fischer Titrator (Mettler, Switzerland).

*Fatty acid titration*

The fatty acid concentration was determined by titration of the organic phase in 25 ml ethanol, containing 0.05% phenolphthalein, with 0.1 N NaOH. The ester concentration was calculated from the difference between the initial and the equilibrium fatty acid concentration.

**Calculations**

For the estimation of activity coefficients the UNIFAC group contribution method was used.[1] In this study, the UNIFAC parameter table of Magnussen et al.[8] is used. This table is especially developed for the prediction of liquid-liquid equilibria at temperatures between 10 and 40 °C. However, this parameter table is known to give deviations in the prediction of the solubility of water in alkanes. Activity coefficients were calculated with reference to an ideal solution in the sense of Raoult's law.

For the determination of the equilibrium constants, a computer program was used that calculated the activities of all components at phase equilibrium.[7] Besides temperature and the required UNIFAC parameters, the input variables are the measured amounts of esters, decanoic acid in the organic phase, and the total amount of water, dodecanol, butanol, and/or propanediol at equilibrium. The output of this computer program consisted of the mole fractions, activity coefficients, and activities of all components in both phases at the phase equilibrium. The activities were used for the calculation of the equilibrium constant(s) according to equation (2). If the concentrations of all components are known in the organic phase, the belonging activity coefficients and activities also can be calculated directly for this phase. However, if this method is followed, the water activity is calculated to exceed 1. Therefore, it is preferred to use the computer program where the organic as well as the aqueous phase is taken into account.

The program TREP, Two-phase Reaction Equilibrium Prediction, calculates the mole fractions of reactants in a two-phase system, in case both the phase equilibrium as well as the reaction equilibrium are achieved.[7] The input consists of the equilibrium constant(s), temperature, initial amounts of the substrates, water and solvent, and the required UNIFAC parameters. In the calculations, the phase equilibrium is assumed

when the activity for each component in phase 1 equals the activity of that component in phase 2 within a given accuracy (usually 0.001). The reaction equilibrium is assumed when the calculated ratio of activities equals the input equilibrium constants within a given accuracy. This accuracy is usually 1% of the value of the equilibrium constant.

In order to compare the experimental and calculated mole fractions, the calculated mole fractions are corrected in such a way that the solvent is entirely present in the organic phase.

## RESULTS AND DISCUSSION

### Experimental Data

The solvents used in this study and the corresponding log *P* values are listed in table I. Log *P*, which is the partition coefficient of a given compound in the octanol-water two-phase system, is used as parameter to indicate the polarity of the solvent. It is not our aim to find the best solvent parameter to correlate ester concentrations at equilibrium. Log *P* is just chosen because it is a well-known parameter, that is tabulated or can be calculated for every solvent. Recently, good results were obtained with the solubility of water in the solvent as parameter.[3,11] However, this parameter cannot be used for polar solvents, which are miscible with water.

*1-Dodecanol and 1-butanol*

The results of the experiments for the esterification of monoalcohols and decanoic acid are shown in figure 1. Water miscible solvents were not used in these experiments. Figure 1 shows that at log *P* values above 2, the ester concentration is not very dependent on the polarity of the solvent. At log *P* values below 2, the ester concentration decreases by increasing the polarity of the reaction medium. This increase can be explained in terms of solvability, the nonpolar product of the reaction is better solvated in nonpolar solvents. In case of the tertiary alcohol, 2-methyl-2-butanol (number 13), the ester concentration is clearly lower than in the other solvents. The behaviour with tertiary alcohols also showed deviation in previous work on the esterification of glycerol and decanoic acid.[7] In that study, the monoester mole fraction in tertiary alcohols was higher as compared to the other solvents, while the diester and triester mole fractions were lower than in the other

solvents. In agreement with these findings, the mole fractions of dodecyl and butyl decanoate, which are nonpolar products, are also lower in the tertiary alcohol when compared to other solvents.

**Table I:** Log *P* of the organic solvents used in this study.

| | Solvent | log *P*[a] |
|---|---|---|
| 1. | Triglyme[b] | -1.9 |
| 2. | Diglyme[b] | -1.3 |
| 3. | N,N-Dimethylformamide | -1.0 |
| 4. | Monoglyme[b] | -0.75 |
| 5. | 4-Hydroxy-4-methyl-2-pentanone | -0.34 |
| 6. | Acetonitrile | -0.33 |
| 7. | Acetone | -0.23 |
| 8. | 2-Butanone | 0.28 |
| 9. | Dichloromethane | 0.60 |
| 10. | 2-Methyl-2-propanol | 0.79 |
| 11. | 2-Pentanone | 0.80 |
| 12. | Ethyl ether | 0.85 |
| 13. | 2-Methyl-2-butanol | 1.3 |
| 14. | *tert.* Butylmethyl ether | 1.4 |
| 15. | Isopropyl ether | 1.9 |
| 16. | Chloroform | 2.0 |
| 17. | Toluene | 2.5 |
| 18. | Butyl ether | 2.9 |
| 19. | Hexane | 3.5 |
| 20. | Isoamyl ether | 4.0 |
| 21. | Isooctane | 4.5 |
| 22. | Hexyl ether | 5.0 |
| 23. | Decane | 5.6 |
| 24. | Dodecane | 6.6 |

a) Log *P* is the logarithm of the partition coefficient of a given compound in the octanol-water two-phase system, calculated according to Rekker.[10]

b) Triglyme is triethylene glycol dimethyl ether, diglyme is diethylene glycol dimethyl ether an monoglyme is ethylene glycol dimethyl ether.

Furthermore, the water mole fraction in the organic phase is shown. In both figures the water mole fraction in the organic phase decreases by increasing the log *P* value of the added solvent. In case of the tertiary alcohol, 2-methyl-2-butanol (number 13), the water mole fraction is twice as high as in *tert.* butylmethyl ether (number 14), which has almost the same log *P* value. The water mole fractions in the experiments with butanol are slightly higher than in the experiments with dodecanol. Butanol is a more polar substrate than dodecanol. If high substrate concentrations are used, the water mole fraction is not only determined by the type of solvent, but also by the polarity of substrates and products.

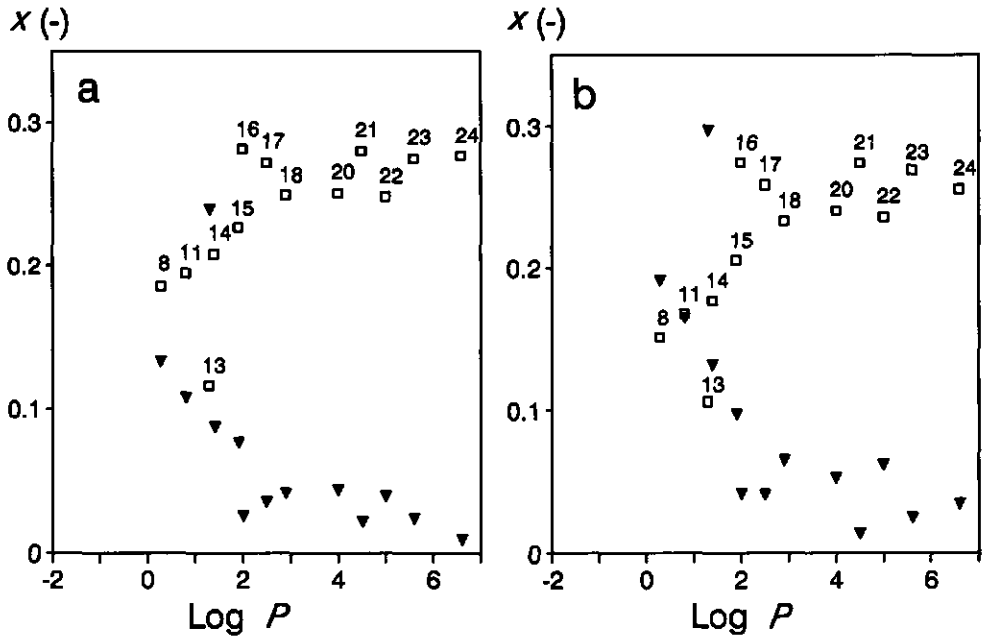


**Figure 1:** Experimental results of the equilibrium mole fraction in the organic phase of monoester (□), and water (▼) for esterification of decanoic acid with 1-dodecanol (a) and 1-butanol (b).
The reaction was carried out with 10 mmole decanoic acid, 10 mmole alcohol, 10 mmole water, 20 mmole solvent, and 25 mg lipase. The samples were incubated for 210 h at 35 °C. The solvents are numbered as in table I.

Valivety et al.[11] studied the synthesis of dodecyl dodecanoate and reported also that nonpolar solvents are preferred for esterification. They have used a 'practical equilibrium constant', which is the ester concentration divided by the product of the alcohol and fatty acid concentration. In their study, the 'practical equilibrium constant' increased from 1.8 in diethylketone till 1600 in isooctane. The 'practical equilibrium constant' is calculated from our data converted to mole.kg$^{-1}$ as units. The value is found to increase only from 1.3 in 2-methyl-2-butanol to 43 in isooctane. The fact that our 'practical equilibrium constant' in isooctane is much lower than the one of Valivety et al.,[11] is probably due to the high substrate concentrations that we have used. In our system, substrate concentrations are in the order of 1.1 - 1.7 M, while Valivety et al.[11] have used lower substrate concentrations (0.25 M). At low substrate concentrations and subsequently high solvent concentrations, the effect of the solvent on the solvability of the product seems to be more pronounced. In accordance, Valivety et al.[11] argued that the 'practical equilibrium constant' only can be used for reasonably dilute solutions where the activity coefficients are approximately constant. In our reaction system, with high substrate concentrations, this requirement is not fulfilled.

*1,3-Propanediol*

In figure 2, the results of the esterification of decanoic acid and 1,3-propanediol are shown. The monoester mole fraction is not very dependent on the polarity of the solvent, while the diester mole fraction increases by increasing the log *P* value of the solvent. These phenomena also can be explained in terms of the solvability of the products.

The water mole fraction in the organic phase (figure 2) is about 0.05 in nonpolar solvents and raises up to 0.45 in the polar solvents. The propanediol mole fraction, which is not shown if figure 2, increases from 0.02 in nonpolar solvents to 0.15 in the polar solvents. The mole fractions of water and propanediol increase with increasing polarity of the organic phase. Deviations arise for the tertiary alcohols (numbers 10 and 13) and for 4-hydroxy-4-methyl-2-pentanone (number 5). These solvents are miscible with water (numbers 5 and 10) or the solubility of water is high (number 13).[7] In figure 1, also a higher water mole fraction was measured in the tertiary alcohol 2-methyl-2-butanol.

Figure 2 is different from previous results of the esterification of decanoic acid and propanediol.[6] In that paper, the monoester mole fraction clearly decreases with increasing log *P*. However, in those experiments, water and propanediol concentrations were not determined. That means that in the organic phase, the sum of mole fractions of

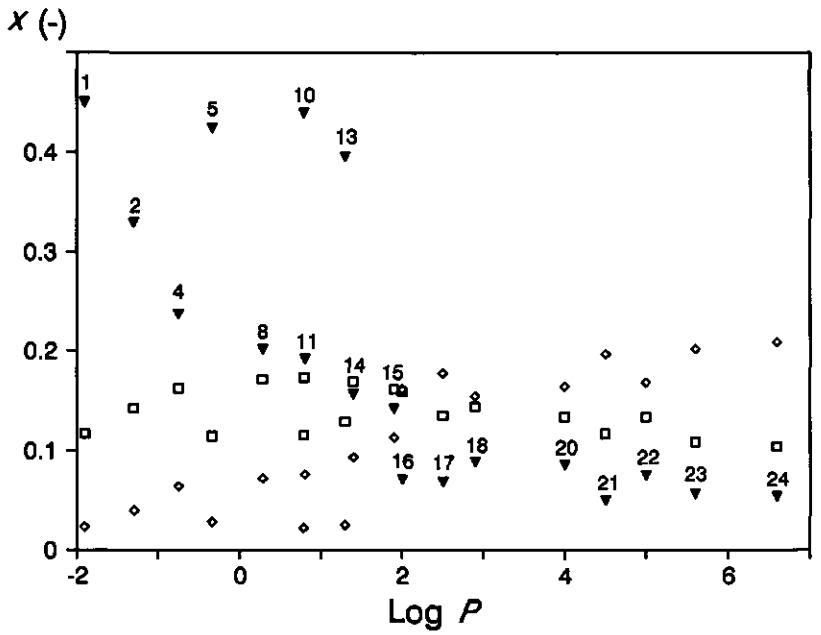


**Figure 2:** Experimental results of the equilibrium mole fraction in the organic phase of monoester (□), diester (◊), and water (▼) for esterification of 1,3-propanediol and decanoic acid.
The reaction was carried out with 10 mmole decanoic acid, 20 mmole 1,3-propanediol, 20 mmole water, 10 mmole solvent, and 25 mg lipase. The samples were incubated for 192 h at 35 °C. The solvents are numbered as in table I.

solvent, fatty acid, mono- and diester was 1. In this study, we also measured propanediol and water concentrations in the organic phase and in the results of figure 2, the sum of mole fraction of solvent, fatty acid, monoester, diester, propanediol and water is 1. In the experiments of figure 2 with the numbers 1, 5, 10, and 13, the water mole fraction in the organic phase exceeds 0.4. Consequently, the mono- and diester mole fractions are low as compared to the experiments with other solvents. If the number of moles of mono- and diester were plotted versus log *P*, a decrease of monoester and an increase of diester was observed by decreasing the polarity of the solvent.

*Sorbitol*

The results of the esterification of sorbitol and decanoic acid are plotted in figure 3. In these experiments, the water and sorbitol concentrations in the organic phase were not determined and therefore, the sum of mole fractions of solvent, fatty acid, mono-, di- and triester is 1. It was observed before that sorbitol ester concentrations at equilibrium are low.[6] From figure 3 it is clear that nonpolar solvents with a log *P* value above 2, are unfavorable for the production of polar sorbitol esters. The low solvability of the polar sorbitol esters results in ester mole fractions below 0.002. In polar solvents, the solvability of the sorbitol esters is much better and ester mole fractions up to 0.04 are measured. The relative monoester mole fraction is highest in solvents with log $P < 0$ and in tertiary alcohols.

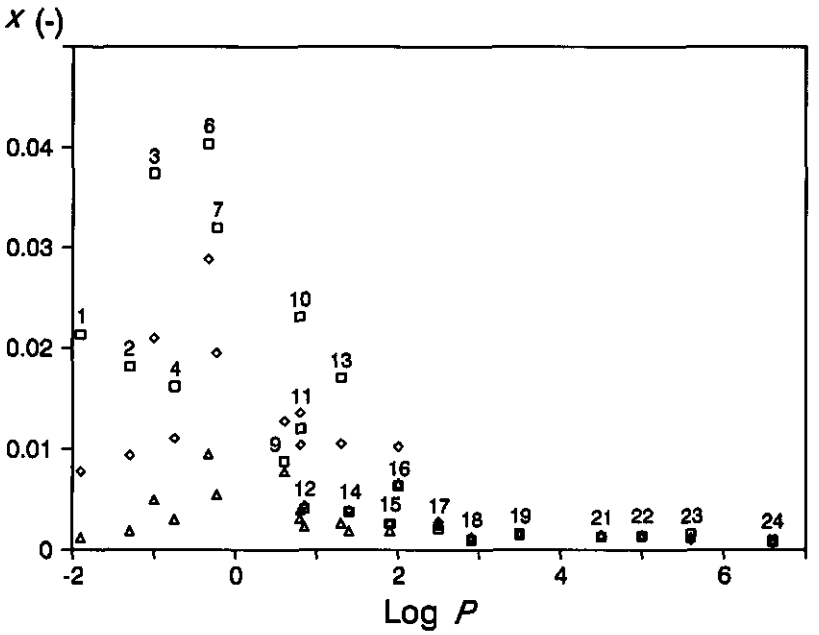


**Figure 3:** Experimental results of the equilibrium mole fraction in the organic phase of monoester (□), diester (◇), and triester (△) for esterification of sorbitol and decanoic acid. The reaction was carried out with 10 mmole decanoic acid, 20 mmole sorbitol, 71 mmole water, 10 mmole solvent, and 50 mg lipase. The samples were incubated for 1000 h at 35 °C. The solvents are numbered as in table I. In this graph, the sum of the mole fractions of solvent, fatty acid, mono-, di-, and triester is 1.

**Determination of equilibrium constants**

In order to calculate the ester mole fraction at equilibrium with the computer program TREP, the equilibrium constant(s) (equation (2)) had to be known. Analog to the esterification of glycerol and decanoic acid,[7] the mole fractions or total amounts at equilibrium were determined experimentally in a reaction system without an organic solvent. The activity coefficients and activities were calculated by that part of the program TREP that calculates the phase equilibrium.

*1-Dodecanol and 1-butanol*

Experiments were performed, where the molar ratio of alcohol to fatty acid is varied, and the initial amounts of water and fatty acid were the same in all experiments. The average equilibrium constants for dodecyl decanoate and butyl decanoate synthesis are 50 and 35, respectively. In figure 4 the experimental and calculated results are shown.

In figures 4a and 4b, the same trends are found for both reactions. The experimental results show that the maximum ester mole fraction is obtained at an initial alcohol to decanoic acid ratio near to 1. At ratios below one, there is an excess of fatty acid at equilibrium and the alcohol mole fraction is low as compared to the fatty acid mole fraction. By increasing the initial alcohol to fatty acid ratio, the equilibrium alcohol mole fraction increases and the equilibrium fatty acid mole fraction decreases. The water mole fraction in the organic phase increases with increasing the medium polarity, i.e. with increasing the alcohol mole fraction. As can be expected the polarity of the reaction system with butanol is higher than with dodecanol and subsequently the water mole fraction is higher in the former one.

The calculated mole fractions, represented by the lines of figures 4a and 4b, are in good agreement with the experimental mole fractions. At alcohol to fatty acid ratios below 1, deviations appear between experimental and calculated water mole fractions. A higher water mole fraction in the system with butanol as compared to the system with dodecanol, as was shown for the experiments, is also found in the calculations.

*1,3-Propanediol*

The equilibrium constants for propanediol mono- and diester synthesis are determined in experiments where the molar ratio of propanediol to decanoic acid is varied, and the initial propanediol to water ratio is the same in all experiments. The experimental results are shown in figure 5a, and an increase in the monoester mole

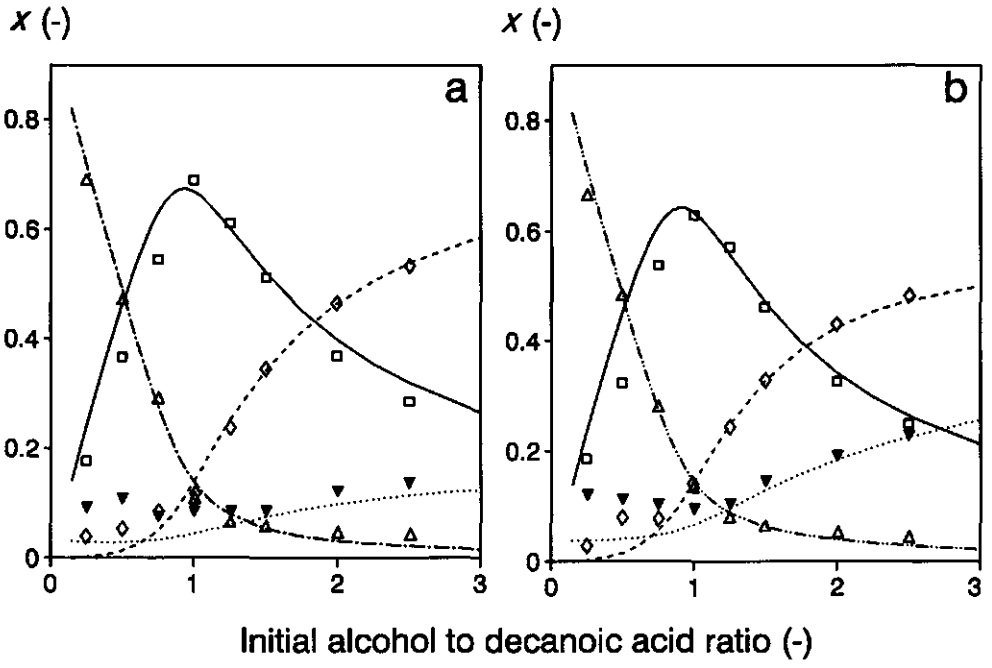


**Figure 4:** Measured and calculated mole fractions as a function of the initial 1-dodecanol (a) and 1-butanol (b) to decanoic acid ratio.
Experiments: 20 mmole decanoic acid, 5 mmole water, a variable number of mmoles of alcohol, and 25 mg lipase were mixed and incubated for 140 h at 35 °C.
Calculations with TREP: $K_M$=50 (1-dodecanol) and $K_M$=35 (1-butanol); $T$=308 K; initial numbers of mmoles are the same as in the experiments.
Measured and calculated mole fractions of monoester (□, ——), alcohol (◇, ---), decanoic acid (△, - · - ·), and water (▼, · · · · ·).

fraction can be seen with increasing the initial propanediol to decanoic acid ratio. The diester mole fraction shows an optimum, while the water mole fraction is more or less constant.

Figure 5a also shows the calculated results for which the average equilibrium constants for monoester and diester synthesis are 10 and 9, respectively. The calculations

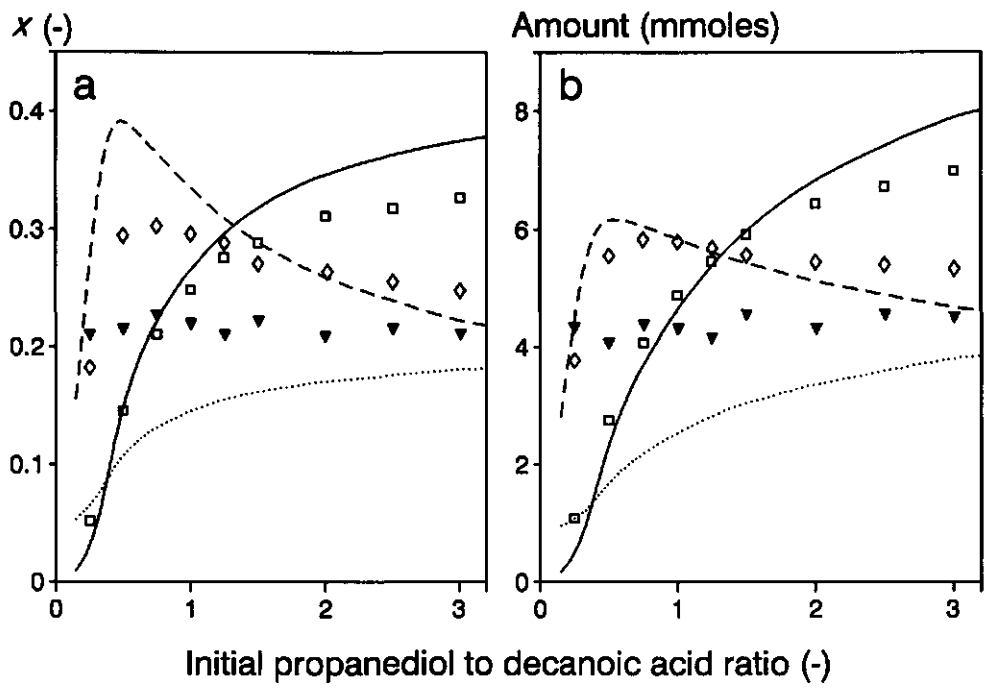


**Figure 5:** Measured and calculated mole fractions (a) and amounts (b) as a function of the initial propanediol to decanoic acid ratio.
Experiments: 20 mmole decanoic acid, variable numbers of mmoles of propanediol and water, with equal molar amounts, respectively, and 25 mg lipase were mixed and incubated for 168 h at 35 °C.
Calculations with TREP: $K_M$=10 and $K_D$=9; $T$=308 K; initial amounts are the same as in the experiments.
Measured and calculated mole fractions of monoester (□, ——), diester (◊, ---), and water (▼, · · · · ·).

for the monoester mole fraction show small deviations from the experimental results. The ratio of calculated and experimental monoester mole fraction did not exceed 1.15. The data of the fatty acid and propanediol mole fraction in the organic phase are not shown in figure 5a. For the fatty acid, the ratio between calculated and experimental mole fractions is between 1.05 and 1.16, which indicate that calculations and experiments are

in good agreement. For propanediol this ratio is between 0.63 and 1.02, especially at low propanediol to decanoic acid ratios, the experimental propanediol mole fraction is higher than the calculated mole fraction. Highest deviations appear for the diester and water mole fractions. The calculations for the diester mole fraction show a more pronounced optimum than found in the experiments. The calculated water mole fraction increases by increasing the propanediol to decanoic acid ratio, while the experimental water mole fraction is constant with only a random scatter. Furthermore, the calculated water mole fraction is lower than the experimental values.

Instead of the mole fraction in mole/mole, the amounts of all components can be plotted, as is shown in figure 5b. The deviations, expressed as the calculated amounts divided by the experimental amount, for decanoic acid, mono- and diester are between 0.84 and 1.14, indicating a good prediction of these amounts. Highest deviations appear for the amount of water and values are between 0.25 and 0.85. These high deviations for water will affect the mole fractions of the other components, since the mole fraction here is expressed as the amount of moles of a component divided by the sum of the amounts of moles of all components in one phase.

A better agreement between calculated and experimental results is obtained if these are expressed in number of moles. However, it is preferred to express the results as mole fractions (in mole/mole), because the mole fractions can be related to activities by the activity coefficients. If deviations between calculated and experimental mole fractions are mainly due to deviation of one component, this will be mentioned.

*Sorbitol*

The water activities of sorbitol/water solutions, calculated with the UNIFAC group contribution method and by using the formulas of Norrish,[11] were compared. Deviations increased with increasing sorbitol mole fraction. At a sorbitol mole fraction of 0.22, the differences of the activity between both calculation methods was 0.1. For glycerol/water mixtures these differences are less than 0.012.[7]

Experiments were carried out where the sorbitol to decanoic acid ratio was varied and the initial sorbitol to water ratio was the same in all experiments. By application of the UNIFAC method, the average equilibrium constants for monoester, diester and triester synthesis were found to be 1.5, 0.05 and 0.07, respectively. These average equilibrium constants were used to calculate the ester mole fractions at equilibrium for the above mentioned experiments without solvent and deviations appeared to be fairly

high. For example, deviations in the monoester mole fraction, expressed as the calculated divided by the experimental mole fraction, increased from 0.4 at low sorbitol to fatty acid ratios to 1.4 at sorbitol to fatty acid ratio of 2.5. Preliminary calculations for experiments with solvents showed that in some cases the calculated ester mole fractions were a factor of 20 too high.

These results show that the UNIFAC parameter table that is used in this study is not suitable for prediction of activity coefficients in the presence of high carbohydrate concentrations. Group-interaction parameters should be determined for the carbohydrates especially.

**Equilibrium constants**

The calculated equilibrium constants for esterification of decanoic acid and several alcohols are listed in table II. Also the activity and mole fraction of decanoic acid are shown in this table, for experiments that were carried out with an alcohol to fatty acid ratio of 1. It appears that for these alcohols, a higher equilibrium constant, results in a

**Table II:** Equilibrium constants and fatty acid mole fraction at equilibrium for esterification of decanoic acid and several alcohols.

| Reaction | $K_M$ | $K_D$ | $K_T$ | $a_{\text{decanoic acid}}$[a)] | $x_{\text{decanoic acid}}$[b)] |
|---|---|---|---|---|---|
| 1-Dodecanol | 50 | | | 0.07 | 0.11 |
| 1-Butanol | 35 | | | 0.09 | 0.14 |
| 1,3-Propanediol | 10 | 9 | | 0.13 | 0.18 |
| Glycerol[c)] | 1.1 | 0.5 | 0.4 | 0.48 | 0.34 |
| Sorbitol[d)] | | | | | 0.90 |

a) The decanoic acid activity at equilibrium from calculations without solvent at an alcohol to decanoic acid ratio of 1.

b) The decanoic acid mole fraction at equilibrium from experiments without solvent at an alcohol to decanoic acid ratio of 1.

c) Data from Janssen et al.[7]

d) The equilibrium constants are not shown in this table, since the reliability of these values is low (see text).

lower decanoic acid activity. This also leads to a lower decanoic acid mole fraction and thus to a higher degree of esterification.

From previous work on the esterification of glycerol and fatty acids, the equilibrium constants for monoester, diester, and triester synthesis were found to be more or less the same for reactions with different types of fatty acids (chapter 5). However, in the present study it is shown that the equilibrium constants are clearly affected by the type of alcohol that is chosen as a substrate. Although there are only four alcohols studied, there seems to be a relationship between the polarity of the alcohol and the value of the equilibrium constant. The equilibrium constant for monoester synthesis ($K_M$) increases with decreasing polarity of the alcohol. However, the lower values of the $K_D$ and $K_T$ as compared to the value of $K_M$ cannot be explained by the relationship between polarity of the substrate and the value of the equilibrium constant. Further investigations are necessary to gain a better understanding of factors that affect the values of equilibrium constants.

There is another aspect that could be of importance for the equilibrium position. For glycerol or propanediol monoester synthesis, one of the substrates (alcohol) is present in the aqueous phase, while the other substrate (fatty acid) is present in the organic phase. The produced esters are present in the organic phase and the glycerol or propanediol part of the ester will change the properties of the organic phase. For the synthesis of dodecyl decanoate, butyl decanoate, propanediol diester, and glycerol di-, and triester, both substrates as well as the produced esters are present in the organic phase. These reasonings throw another light on the argument of Halling,[3] that the effect of changing solvent on the equilibrium position should be the same for all esterification reactions. These arguments are indeed valid if both substrates as well as the ester, are present in the same phase. However, for esterification of glycerol and fatty acid, the produced monoester will change the properties of the organic phase. This indicates that the arguments of Halling will not be valid for these type of esterification reactions.

**Calculations of solvent effects with TREP**

The average values of the equilibrium constants are introduced into TREP. Furthermore the initial amounts, as were used in the experiments, the temperature and the required UNIFAC parameters are introduced. Now, the mole fractions of all

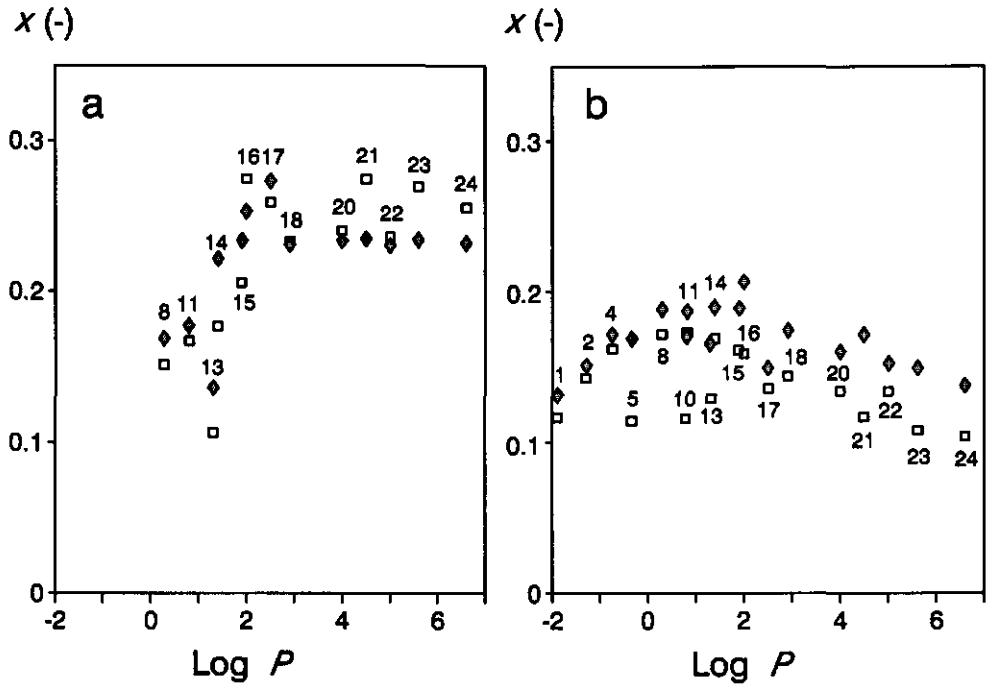


**Figure 6:** Calculated and measured ester mole fractions as a function of log $P$ of the added solvent, for butyl decanoate (a) and propanediol monoester (b) synthesis.
Experiments: from figure 1b and 2. The solvents are numbered as in table I.
Calculations with TREP: $K_M$=35 (1-butanol); $K_M$=10 and $K_D$=9 (1,3-propanediol); $T$=308 K; initial amounts are the same as in the experiments.
Calculated (◆) and experimental (□) ester mole fraction.

components at equilibrium can be calculated. Below the correlations between calculated and experimental mole fractions are discussed. This is done separately for the esters and for water.

*Esters*

In figure 6a, the calculated and experimental ester mole fractions are shown for esterification of 1-butanol and decanoic acid. The calculated ester mole fractions, showed the same trends as the experimental ester mole fractions. The calculated mole fractions

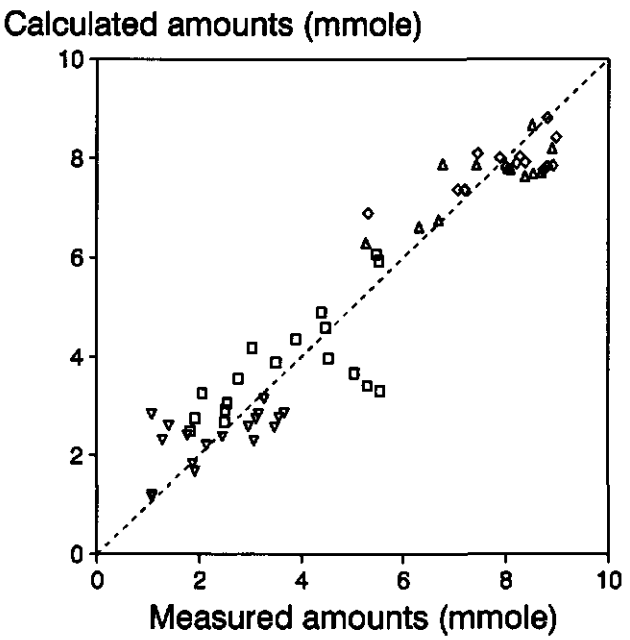


**Figure 7:** Calculated as a function of measured amounts of dodecyl decanoate (◇), butyl decanoate (△), propanediol monoester (□), and propanediol diester (▽).
Data are obtained from figures 1,2 and 6.

are too low as compared to the experimental mole fractions, in solvent with a log *P* value below 3 and too high in solvent with a log *P* value above 3. The deviations, expressed as the calculated divided by the experimental ester mole fractions are between 0.86 and 1.28. The lower measured ester mole fraction in the tertiary alcohol 2-methyl-2-butanol as compared to the other solvents is also found in the calculations. The same trends as in figure 6a, are observed for the esterification of 1-dodecanol and decanoic acid (data not shown). The deviations between the calculated and experimental ester mole fractions are between 0.86 and 1.44.

For the esterification of propanediol and decanoic acid, the calculated and experimental results for the monoester are shown in figure 6b. The calculated mole fraction deviates always in the upwards direction, which is in agreement with the calculations for the esterification of glycerol and decanoic acid.[7] The deviation is less than a factor of 1.5 of the measured mole fraction. For the diesters, the calculated mole fractions are much too high in solvents with a log *P* value below 0 and deviations rise up to a factor of 5 (data not shown). In solvents having a log *P* value between 0 and 2, the calculated diester mole fractions are in good agreement with the measurements. For solvents with a log *P* value above 2, the calculated diester mole fractions are too low with maximum deviations of 0.70.

In figure 7, the calculated amounts of ester are plotted versus the measured amounts of ester for reaction systems where a solvent is added. In this graph dodecyl decanoate, butyl decanoate, and 1,3-propanediol mono-, and diester are shown. Figure 7 shows that the calculated amounts of ester are sometimes higher and sometimes lower than the measured amounts. Although there are some deviations in the absolute values, the trends in the amounts of ester are predicted with the program TREP.

*Water*

In figure 8a, the calculated and experimental water mole fractions are shown for the esterification of 1-butanol and decanoic acid. The calculated trends are in good agreement with the experimental results. However, in most cases the experimental water mole fraction is higher than the calculated mole fraction and deviations are between 0.30 and 1.33. The same results are obtained with 1-dodecanol as a substrate. For dodecanol, the deviations between calculated and experimental water mole fractions are between 0.30 and 1.50.

For the esterification of 1,3-propanediol and decanoic acid, the calculated and measured water mole fractions are shown in figure 8b. For solvents with a log *P* value above 2, the trends are calculated and deviations, which are in most cases in the downwards direction, are between 0.53 and 1.10. Higher deviations are observed in the polar solvents with a log *P* value below 2. In these solvents, the ratio of calculated and experimental mole fractions is between 0.25 and 1.10.

In figure 9, the calculated amounts of water are plotted versus the measured amounts. It appears that, the calculated amounts of water are in almost all cases lower than the measured amounts. This systematic deviation is probably due to the UNIFAC parameter table that is used in this study. In their paper, Magnussen et al.[8] already mentioned that deviations could arise between the calculated and experimental solubility of water in alkanes. Especially for the polar solvents, the parameters in this table should be improved to obtain a better agreement between calculated and experimental water mole fractions.

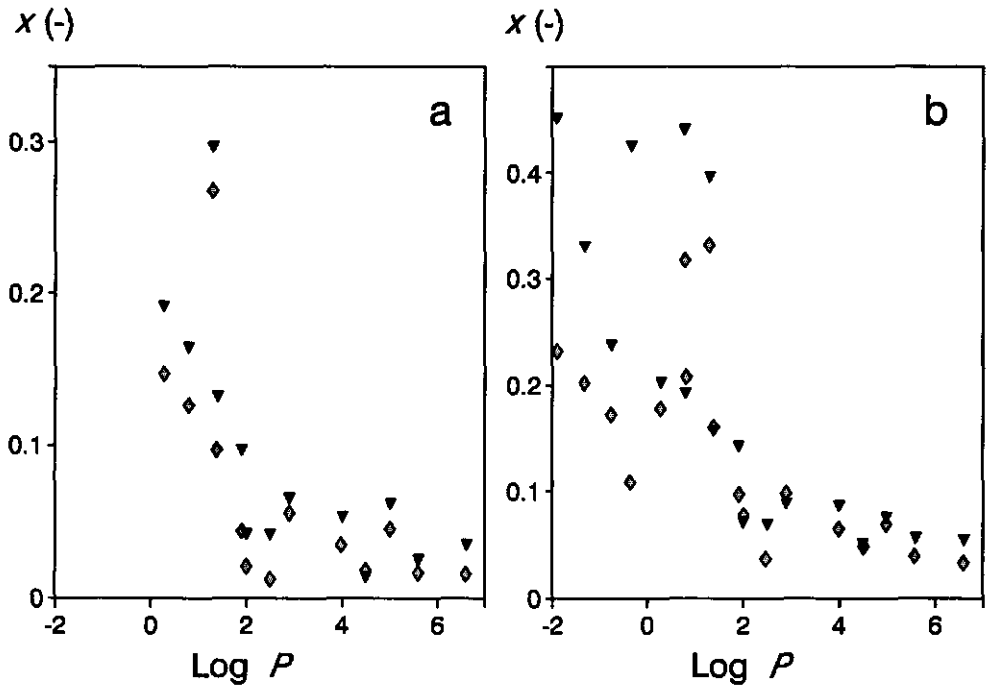


**Figure 8:** Calculated and measured water mole fractions as a function of log $P$ of the added solvent, for esterification with butanol (a) and propanediol (b).
Experiments: from figure 1b and 2. The solvents are numbered as in table I.
Calculations with TREP: $K_M$=35 (1-butanol); $K_M$=10 and $K_D$=9 (1,3-propanediol); $T$=308 K; initial amounts are the same as in the experiments.
Calculated (◆) and experimental (▼) water mole fraction.

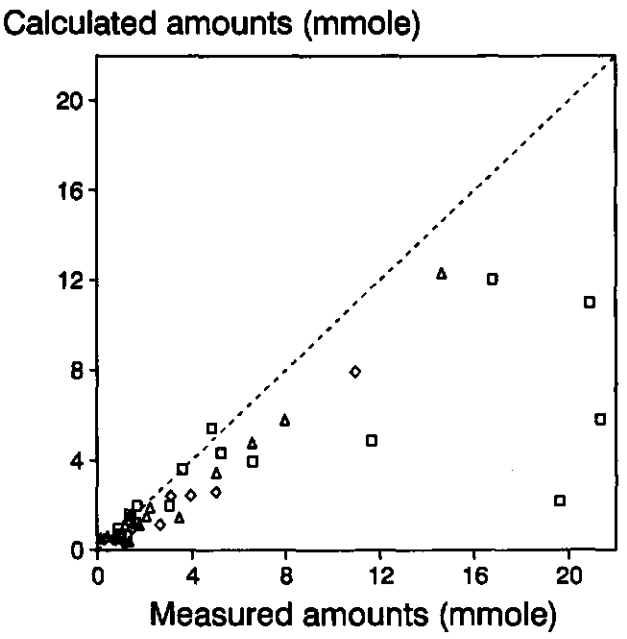


**Figure 9:** Calculated as a function of measured amounts of water for dodecyl decanoate (◇), butyl decanoate (△), and propanediol ester (□) synthesis.
Data are obtained from figures 1,2 and 8.

## CONCLUSIONS

The program TREP, which is based on mass balances the UNIFAC group contribution method, is developed for the calculation of the equilibrium mole fractions of all components of a reaction in a non-dilute two-phase system. The usefulness of TREP was already shown for the esterification of glycerol and decanoic acid. Here, this work is extended by the esterification of decanoic acid and various alcohols. The equilibrium constants are found to be affected by the alcohol that is chosen as the substrate. For polar substrates, such as glycerol and 1,3 propanediol, the equilibrium constants are lower than for nonpolar substrates, such as 1-dodecanol and 1-butanol.

For the reactions with 1-dodecanol, 1-butanol and 1,3-propanediol in organic solvents, good agreement is obtained between calculated and experimental ester, alcohol and fatty acid mole fractions. Systematic deviations appear for the calculated water mole fractions, which are in most cases lower than the experimentally determined water mole fractions.

For the prediction of the sorbitol ester mole fractions, deviations between calculated and experimental mole fractions rise up till a factor 20. This could be caused by the UNIFAC parameter table that was used in this study, which is not suitable for reaction systems containing carbohydrates.

## NOMENCLATURE

| | | |
|---|---|---|
| $a$ | activity | (-) |
| $K$ | equilibrium constant | (-) |
| $P$ | partition coefficient in n-octanol-water | $(M_{org}.M_w^{-1})$ |
| $x$ | mole fraction | (-) |
| $\gamma$ | activity coefficient | (-) |

**Subscipts:**

| | |
|---|---|
| calc | calculation |
| exp | experimental |
| i,A,B,C | components |

**Superscripts:**

| | |
|---|---|
| I | phase 1 |
| II | phase 2 |

## ACKNOWLEDGEMENTS

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# chapter 7

# GENERAL DISCUSSION

Chapter 1 of this thesis gives an overview models in literature for the prediction of the equilibrium position in dilute two-phase reaction systems. In contrast to chapter 1, chapters 4, 5, and 6 are focussed on the equilibrium position in nondilute two-phase systems. In this chapter, the models from chapter 1 are compared with the model that is described in chapters 4, 5, and 6 of this thesis.

Lipase catalyzed esterification of carbohydrates and fatty acids is discussed in chapters 2 and 3. Especially the reaction system that is described in chapter 3 is interesting for commercial applications, since it only consists of the substrates, water and lipase and modification of the substrates is not necessary. However, the reaction rate and equilibrium concentration are low. The possibility to increase the reaction rate and the equilibrium concentration by increasing the temperature is discussed in this chapter.

Sucrose esters are of commercial interest, since these esters have good emulsifying properties and sucrose is a cheap substrate. Chapters 2 and 3 indicate that sucrose esters are difficult to obtain enzymatically. Experimental results and theoretical considerations are given in this chapter of the enzymatic synthesis of sucrose esters in an aqueous-organic two-phase system.

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Part of this chapter and part of chapter 1 will be submitted for publication by the authors A.E.M. Janssen, A Van der Padt and K. Van 't Riet.

In chapter 2, the molecular structure of the sorbitol monoester is described. The structure of sorbitol di-, tri-, and tetraesters are determined and a short description is given of the purification from the reaction mixture and elucidation of the structure of these compounds.

## SOLVENT EFFECTS ON EQUILIBRIUM

In chapter 1, the equilibrium position in an aqueous-organic two-phase system is described by using the partition coefficients of substrates and products and the volume ratio of organic and aqueous phase. For a reaction

$$A + B \rightleftharpoons C + H_2O$$

Martinek et al.[9] proposed to use an apparent equilibrium constant ($K_{bi}$), which can be described as

$$K_{bi} = K_w \frac{(1+\alpha \cdot P_C)(1+\alpha \cdot P_{H_2O})}{(1+\alpha \cdot P_A)(1+\alpha \cdot P_B)} \qquad (1)$$

where the partition coefficient $P_i$ is defined as the concentration in the organic phase, divided by the concentration in the aqueous phase when the distribution has achieved equilibrium. The volume ratio $\alpha$ is the volume of the organic phase divided by the volume of the aqueous phase. The apparent equilibrium constant $K_{bi}$ and the equilibrium constant for an ideal dilute aqueous solution $K_w$, are defined as

$$K_{bi} = \frac{[C]_{bi} \cdot [H_2O]_{bi}}{[A]_{bi} \cdot [B]_{bi}} \quad \text{and} \quad K_w = \frac{[C]_w \cdot [H_2O]_w}{[A]_w \cdot [B]_w} \qquad (2)$$

where $[i]_w$ is the concentration of component $i$ in the aqueous phase and $[i]_{bi}$ is the biphasic concentration of component $i$.

Eggers et al.[5] argued that for a dilute system, which also was a requirement for the equations of Martinek et al.,[9] the water activity of the two-phase system is constant. They have used an equilibrium constant in which the amount of water is expressed as mole fraction, while the amounts of components *A*, *B*, and *C* are expressed as concentrations. This leads to a definition of the equilibrium constant in which neither the water concentration nor the partition coefficient of water play any role. The apparent equilibrium constant for the biphasic system can be rewritten to the following expression

$$K_{bi}^{*} = K_{w}^{*}\frac{(1+\alpha\cdot P_C)(1+\alpha)}{(1+\alpha\cdot P_A)(1+\alpha\cdot P_B)} \tag{3}$$

where $K_{bi}^{*}$ and $K_{w}^{*}$ are defined as

$$K_{bi}^{*} = \frac{[C]_{bi}}{[A]_{bi}\cdot[B]_{bi}} \quad \text{and} \quad K_{w}^{*} = \frac{[C]_{w}}{[A]_{w}\cdot[B]_{w}} \tag{4}$$

As defined in chapter 1, the superscript * is used to mark the equilibrium constants according to Eggers et al.[5] Both $K_{bi}$ and $K_{bi}^{*}$ as well as $K_{w}$ and $K_{w}^{*}$ have different values and are related by

$$K_{bi}^{*} = \frac{K_{bi}}{[H_2O]_{bi}} \quad \text{and} \quad K_{w}^{*} = \frac{K_{w}}{[H_2O]_{w}} \tag{5}$$

The differences between equation (1) and (3) are discussed in chapter 1. Now these models for dilute systems will be compared with the model for nondilute reaction systems as is described in chapter 4, 5, and 6 of this thesis. In this model the reaction equilibrium is described by

$$K = \frac{a_C\cdot a_{H_2O}}{a_A\cdot a_B} = \frac{x_C\cdot x_{H_2O}}{x_A\cdot x_B}\cdot\frac{\gamma_C\cdot\gamma_{H_2O}}{\gamma_A\cdot\gamma_B} \tag{6}$$

where $K$ is the equilibrium constant and $a_i$, $x_i$ and $\gamma_i$ are the thermodynamic activity, the mole fraction and the activity coefficient of component *i*, respectively. The mole fractions at equilibrium are calculated using the computer program TREP (Two-phase Reaction

Equilibrium Prediction) which is based on the UNIFAC group contribution method. The program TREP is developed for calculation of the equilibrium position in nondilute two-phase systems.

In order to compare the program TREP to the models of Martinek et al.[9] and Eggers et al.,[5] the mole fractions at equilibrium are calculated with TREP as function of the volume ratio $\alpha$ in a dilute reaction system. As in chapter 1, the esterification of 1-propanol and butanoic acid in a two-phase system of water and hexane is used as example. In each calculation with TREP, the biphasic volume and the initial biphasic concentrations of the substrates $A$ and $B$ are the same. The output of TREP contains the mole fractions of all components and for comparison with the models of Martinek et al.[9] and Eggers et al.,[5] the mole fractions have to be converted to biphasic and aqueous phase concentrations. These concentrations are used to calculate $K_{bi}$, $K_w$, $K_{bi}^*$, and $K_w^*$ according to equation (2) and (4). The results of these calculations are shown in figure 1 (solid lines). The dotted lines in figure 1 are taken from chapter 1 and are shown for comparison. Figure 1 shows that for dilute systems equations (1) and (3) and TREP predict about the same data. Only interactions between water and solvent are considered for determination of the partition coefficients that are used in equations (1) and (3). Interactions between all components in the reaction system are taken into account in the program TREP. However, in dilute systems, the interaction between the reactants can be neglected. This means that for a dilute system such as shown in figure 1, it is logical that the results obtained with TREP are similar to the results of Martinek et al.[9] (figure 1a) and Eggers et al.[5] (figure 1b).

As was discussed by Eggers et al.,[5] for comparison of two-phase reaction systems, the biphasic product concentration is a more useful parameter than $K_{bi}/K_w$ or $K_{bi}^*/K_w^*$. The biphasic product concentration $[C]_{bi}$ can be calculated from mass balances if the initial biphasic substrates concentrations and either $K_{bi}$ and $K_w$ or $K_{bi}^*$ and $K_w^*$ are known. Figure 2 shows the biphasic product concentrations, calculated with the program TREP and with the equations of Martinek et al.,[9] and Eggers et al.[5] It should be noted that for the calculation of $[C]_{bi}$ according to Martinek et al.[9] and Eggers et al.,[5] values of $K_w$ and $K_w^*$ are used that are estimated from calculations with TREP. Figure 2 shows that the equations of Martinek et al.,[9] and Eggers et al.[5] are the same. These values of $[C]_{bi}$ are slightly higher than the values of $[C]_{bi}$ calculated with TREP. This is caused by the partition coefficient of $C$, which is found to be very dependent on the concentration of $C$. This partition coefficient is calculated to be 460 in a hexane-water two-phase system at a

biphasic concentration of 0.1 kmole.m$^{-3}$. However, at a biphasic concentration of 0.02 kmole.m$^{-3}$, the partition coefficient is decreased to 350. If this lower value of $P_C$ is used in the equations of Martinek et al.[9] and Eggers et al.,[5] the values of $[C]_{bi}$ are again similar to the values of $[C]_{bi}$ calculated with TREP. The equations of Martinek et al.,[9] Eggers et al.,[5] and the program TREP are all based on mass balances. This means that if the partition coefficients are exactly known, the equations of Martinek et al.,[9] Eggers et al.,[5] and the program TREP lead to the same biphasic product concentration at equilibrium.

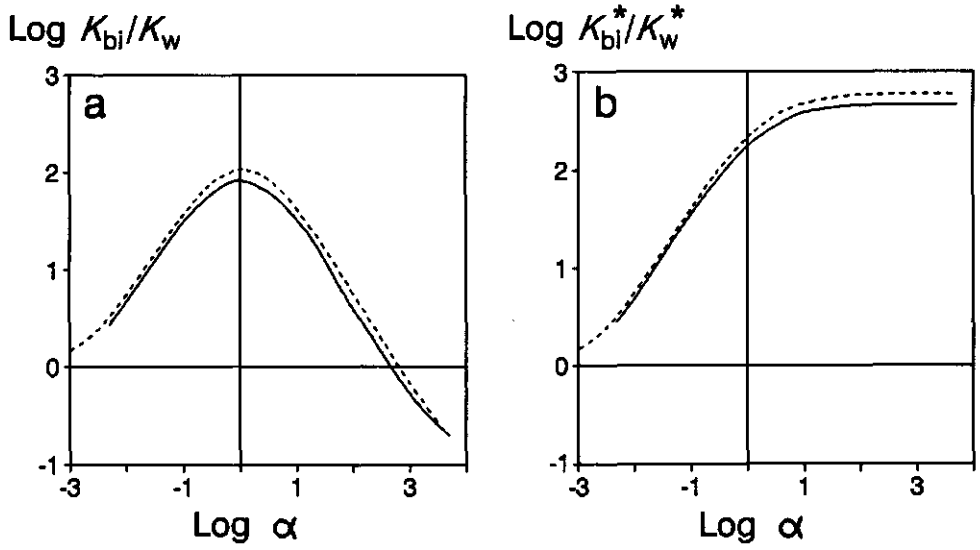


**Figure 1:** Prediction of equilibrium data in hexane as a function of log α with the equations for dilute systems (- - -) and with the program TREP (——).
The dotted lines are the same as in figure 2 of chapter 1, part a is calculated with equation (1) and part b is calculated with equation (3).
The solid lines represent the calculations with TREP: $K$=10; $T$=298 K; initial amounts: 0.2 mole butanoic acid, 0.2 mole 1-propanol and variable amounts of water and hexane ($V_{org}$ + $V_w$ = 2.10$^{-3}$ m$^3$). Part a is calculated with equation (2) and part b is calculated with equation (4).

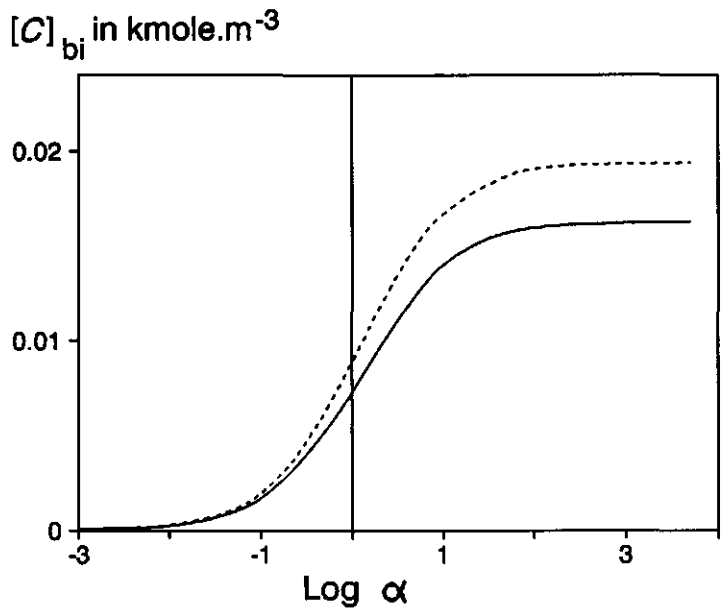


**Figure 2:** Prediction of the biphasic product concentration in hexane as a function of log $\alpha$ with the equations of Martinek et al.[9] or Eggers et al.[5] (- - -), and with the program TREP (——).
Calculations of $[C]_{bi}$ with equations of Martinek et al.[9] and Eggers et al.[5]: see figure 3 of chapter 1.
Calculations of $[C]_{bi}$ with TREP: see figure 1 of this chapter.

For the calculations with TREP in figure 1 (solid lines), no values are shown below a volume ratio of 0.005, because at these low volume ratios, hexane was calculated to be completely dissolved in the aqueous phase. As a result the system consisted of one instead of two phases, which means that $K_{bi}$ and $K_w$ are identical. For a nonpolar solvent, such as hexane, this only happens at extreme volume ratios, however, for more polar solvents the system can be converted to a one-phase system at less extreme volume ratios. This is shown in figure 3. Here, the logarithm of $K_{bi}^*/K_w^*$ is plotted versus the logarithm of $\alpha$ for several solvents. The lines in figure 3a, are calculated with equation (3) and the lines of

figure 3b are calculated by using TREP and equation (4). The calculations with TREP show that a chloroform-water two-phase system is altered in a one-phase system at volume ratios below 0.005 and above 500. A reaction system of the polar solvent 2-methyl-2-butanol, and water only consists of two phases at volume ratios between 0.05 and 5. With equations (1) and (3) no distinction is made between one- and two-phase systems. Therefore, care has to be taken that the reaction system always consists of two phases, when using these equations.

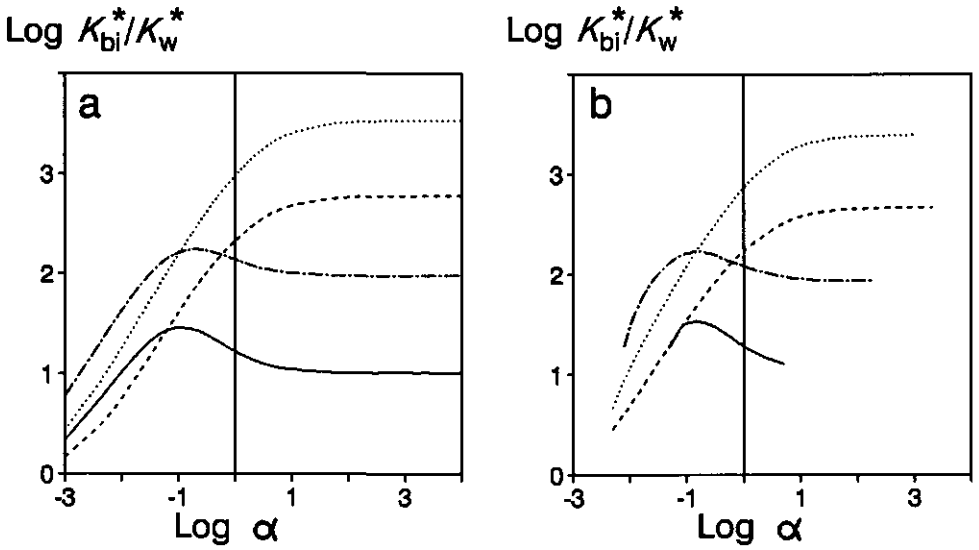


**Figure 3:** Prediction of equilibrium data in several solvents as a function of log α with equation (3) for dilute systems (part a) and with the program TREP (part b).
Partition coefficients are from table I of chapter 1:
Benzene: $P_A$ = 0.28; $P_B$ = 1.8; $P_C$ = 1700. Hexane: $P_A$ = 0.35; $P_B$ = 2.2; $P_C$ = 460.
Chloroform: $P_A$ = 15; $P_B$ = 3.5; $P_C$ = 5000. 2-Methyl-2-butanol: $P_A$ = 17; $P_B$ = 6.9; $P_C$ = 1200.
Calculations with TREP: $K$=10; $T$=298 K; initial amounts: 0.2 mole butanoic acid, 0.2 mole 1-propanol and variable amounts of water and solvent ($V_{org} + V_w = 2.10^{-3}$ m³).
Solvents: Benzene (· · ·), hexane (– – –), chloroform (– · – ·), and 2-methyl-2-butanol (——).

Furthermore, figure 3 shows the different effects of several solvents as a function of the volume ratio. At low volume ratios, the highest $K_{bi}^*/K_w^*$ is obtained in chloroform, due to the high partition coefficient of the product. At high volume ratios, $K_{bi}^*/K_w^*$ is found to be highest for benzene. At high volume ratios, low partition coefficients for the substrates *A* and *B*, and a high partition coefficient for the product *C* is favorable. Figure 3 also shows that for some solvents an optimum for $\alpha$ exists that gives a maximum for $K_{bi}^*/K_w^*$. This optimum $\alpha$ also results in a maximum in the plot of $[C]_{bi}/[C]_w$ as function of $\log \alpha$. Analogous to Martinek et al.,[9] a maximum in the plot of $K_{bi}^*/K_w^*$ or $[C]_{bi}/[C]_w$ as a function of $\alpha$ arises if

$$P_C + 1 > P_A + P_B \quad \text{and} \quad 1/P_C + 1 > 1/P_A + 1/P_B \tag{7}$$

These prerequisites for a maximum are fulfilled for chloroform and 2-methyl-2-butanol.

Application of equation (3) is based on the prerequisite that the partition coefficients and the equilibrium constant in the aqueous phase ($K_w^*$) have a constant value at every volume ratio. The calculations with TREP are used to check whether or not the partition coefficients and $K_w^*$ are the same at every value of $\alpha$. Therefore, the equilibrium concentrations of *A*, *B*, and *C* obtained with TREP, are used to calculate the partition coefficients and $K_w^*$. The partition coefficients and $K_w^*$, that are obtained from the calculations with TREP of figure 1 are presented by the solid lines of figure 4. The partition coefficients of *A* and *B* vary less than 3 % of the average value, however, that of *C* does decrease with increasing values of $\alpha$. Accordingly, the $K_w^*$ value is not constant anymore at high values of $\alpha$.

The equilibrium position is also calculated with TREP at initial amounts of substrates that are 10 and 20 times higher than for the calculations of figure 1. The volume ratio is affected by the high amounts of substrates and products and therefore volumes and concentrations are corrected for this. The results are shown in figure 4 and it is clear that the partition coefficients and $K_w^*$ are dependent on the composition of the reaction system at high reactant concentrations. Figure 4 wrongly suggests that the partition coefficients are a direct function of $\alpha$. Partition coefficients are only a function of the composition of the reaction medium, which changes with $\alpha$. A plot of the partition coefficient versus $\alpha$ is just chosen, because it shows clearly the effect of increasing the substrate and product concentrations.

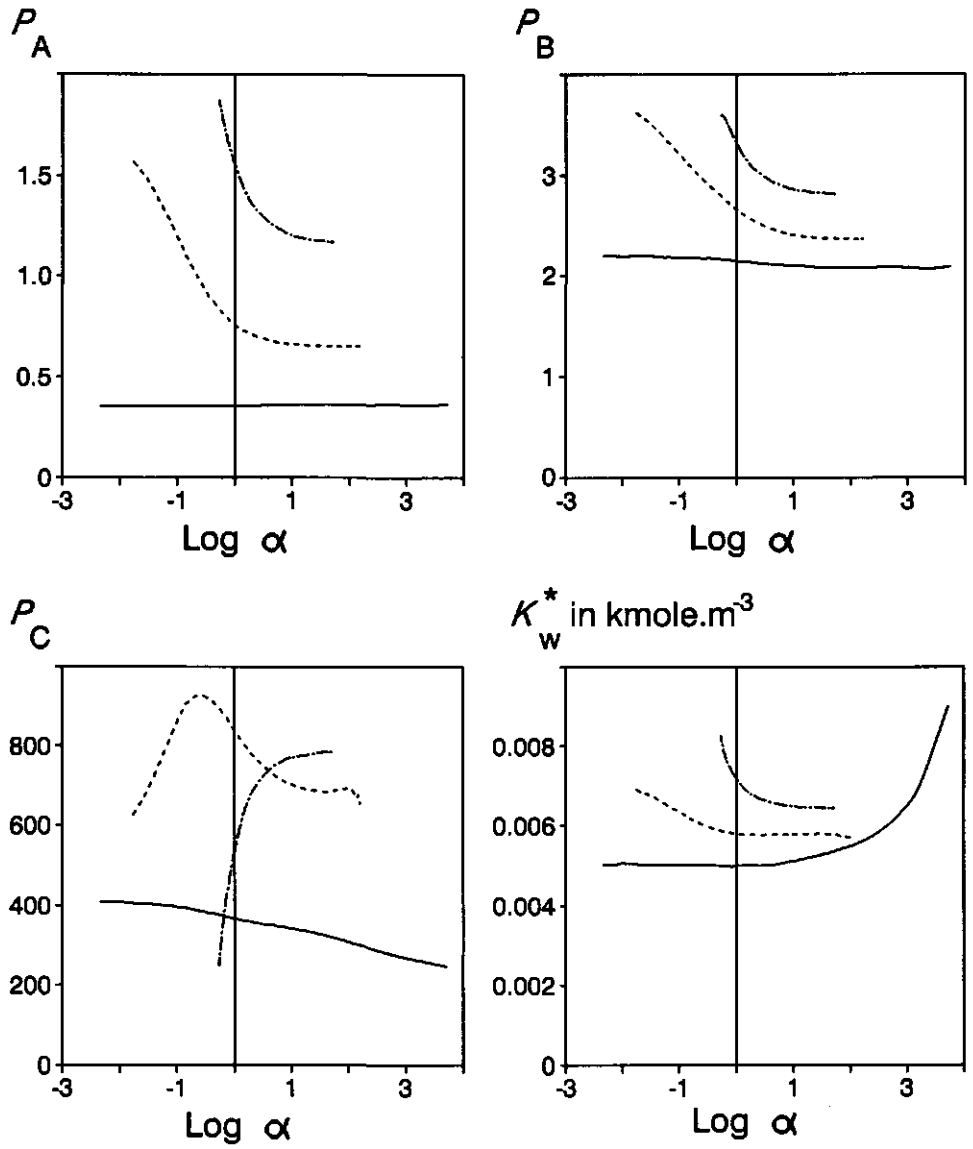


**Figure 4:** Partition coefficients of components *A*, *B*, and *C* and $K_w^*$ in a hexane-water two-phase reaction system plotted versus log α .
Calculations with TREP: $K$=10; $T$=298 K; initial amounts: 0.2 (——), 2 (- - -), and 10 (- · - ·) mole butanoic acid and 1-propanol, and variable amounts of water and hexane ($V_{org} + V_w$ = $2.10^{-3}$ $m^3$). Corrections are made for changes in the total volume and the volume ratios because of high substrate and product concentrations.

The partition coefficient of water is also dependent on the composition of the reaction medium at high initial substrate concentrations. The partition coefficient of water is not included in the equations for the calculation of $K_{bi}^*$. However, a requirement for this procedure is that the water activity of the reaction system is one. Calculations with TREP showed that the water activity was reduced slightly if high substrate concentrations were used.

In principle, the equations of Martinek et al.,[9] and Eggers et al.[5] can be used at high reactant concentrations, if the partition coefficients are known at every volume ratio. However, experimental determination of each partition coefficient as a function of the concentrations of all reactants is a time-consuming job. For this reason, the equations of Martinek et al.,[9] and Eggers et al.[5] are not very useful for nondilute reaction systems.

In this section, $K_{bi}/K_w$ or $K_{bi}^*/K_w^*$ and $[C]_{bi}$ are used as a measure of the product yield. However, often only one of the two phases will be processed down-stream. This means that besides the biphasic product concentration, also the product concentration in the aqueous and organic phase, respectively, is of importance. Analogous to $[C]_{bi}$ these concentrations can be calculated with the equations of Martinek et al.,[9] Eggers et al.,[5] and with TREP. The results with TREP are shown in figure 5. The plot of the biphasic concentration shows the increase in $[C]_{bi}$, and only a slight decrease in $[A]_{bi}$ and $[B]_{bi}$. In the aqueous phase, only the concentrations of $A$ and $B$ are shown, since the concentration of $C$ is very low and therefore not visible in this plot. At values of $\alpha$ below 0.01, the aqueous phase is relatively large and the organic phase is only small. Here, the concentrations in the aqueous phase are almost equal to the biphasic concentrations. At values of $\alpha$ above 100, the volume of the aqueous phase can be neglected, and the concentrations in the organic phase are almost equal to the biphasic concentrations. It is remarkable that the product concentration in the organic phase is almost independent of $\alpha$. As opposed to the product concentration in the organic phase, the concentration of residual substrates in the organic phase is dependent on $\alpha$. If substrate $A$ is most easy to remove during down-stream processing, a high volume ratio is preferred. Whereas, in case substrate $B$ is easy to remove, a low volume ratio is preferred. From figure 5 it can be concluded that besides the product yield $[B]_{bi}$, also other aspects, such as the product and substrate concentrations in the organic phase, should be taken into account.

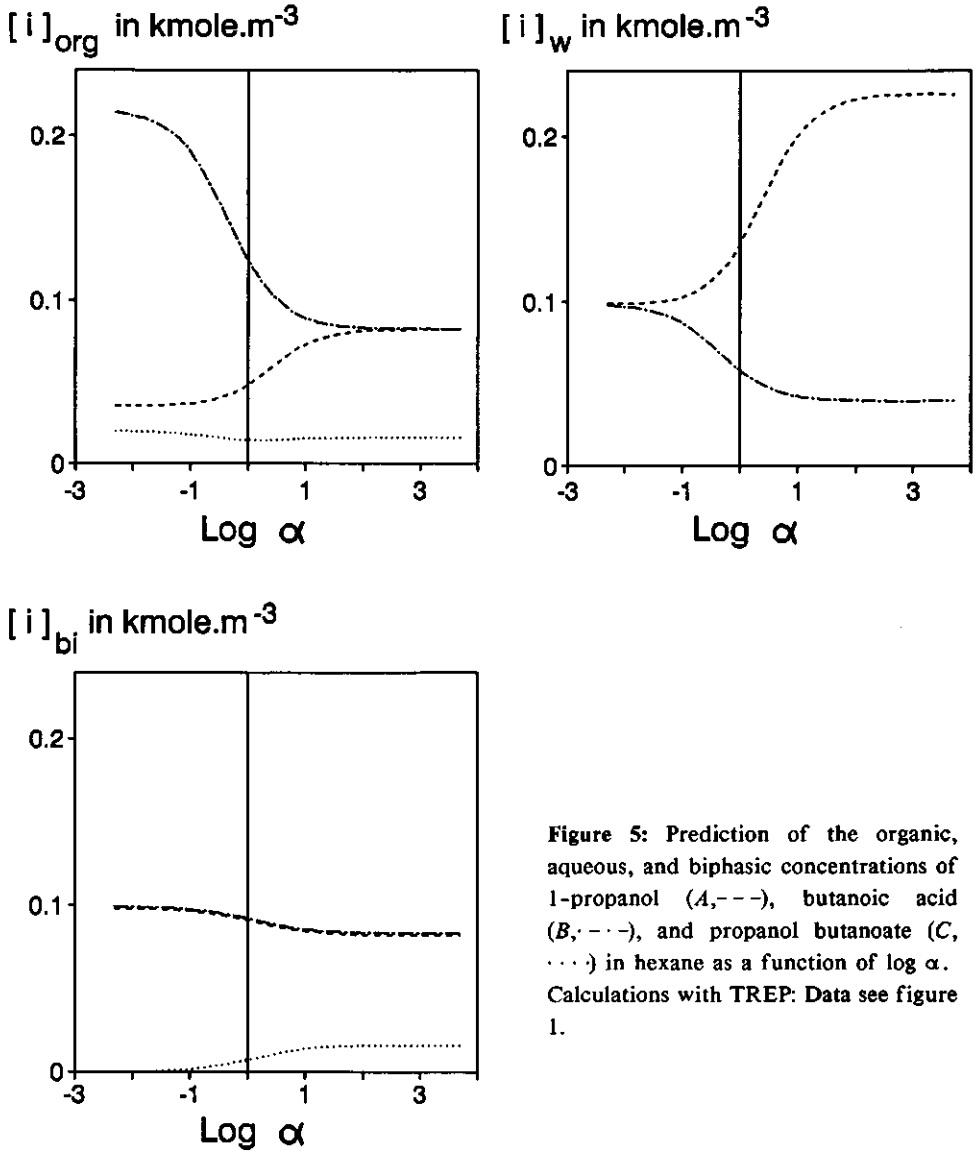


**Figure 5:** Prediction of the organic, aqueous, and biphasic concentrations of 1-propanol (*A*,– – –), butanoic acid (*B*,· – · –), and propanol butanoate (*C*, · · · ·) in hexane as a function of log α. Calculations with TREP: Data see figure 1.

From the calculations that are made in this section it can be concluded that the equations as derived by Martinek et al.,[9] and Eggers et al.[5] are only useful for dilute two-phase reaction systems. It is important that the reaction system really consists of two phases. At extreme high or extreme low volume ratios, a one-phase system could arise. Furthermore it is convenient if the partition coefficients and $K_w$ are constant at different volume ratios. If these values are not constant, the equations still can be used if the partition coefficients are known at every volume ratio. This means that for nondilute reaction systems each partition coefficient has to be determined as a function of the concentrations of the reactants. Experimentally this is hardly feasible. However, calculation methods, such as the UNIFAC group contribution method, are available, where interactions between all components of a reaction mixture are taken into account. The program TREP, which is developed in this thesis, is based on the UNIFAC group contribution method and mass balances. TREP is shown to be very useful for prediction of the equilibrium position in two-phase reaction systems. TREP can be used for dilute as well as nondilute two-phase reaction systems. It is shown that models that are developed for dilute reaction systems, fail if the partition coefficients are not constant anymore.

## EFFECT OF TEMPERATURE ON ESTERIFICATION OF POLYOLS AND FATTY ACIDS

For the esterification of carbohydrates and fatty acid in an aqueous-organic two-phase system as presented in chapter 3, the carbohydrate concentration in the aqueous phase is limited by its solubility. A method to increase the carbohydrate concentration in the aqueous phase, is to increase the temperature of the reaction medium.

The solubility of carbohydrates in water is dependent on the temperature. The increase in solubility of four carbohydrates is shown in figure 6. The solubility of sorbitol increases from a mole fraction of 0.22 at 35 °C up to 0.53 at 90 °C. The relative increase in the glucose and fructose mole fractions versus temperature are more or less the same as for sorbitol. However, it is worth to be noticed that the sucrose mole fraction only increases from a mole fraction of 0.11 at 35 °C to 0.18 at 90 °C.

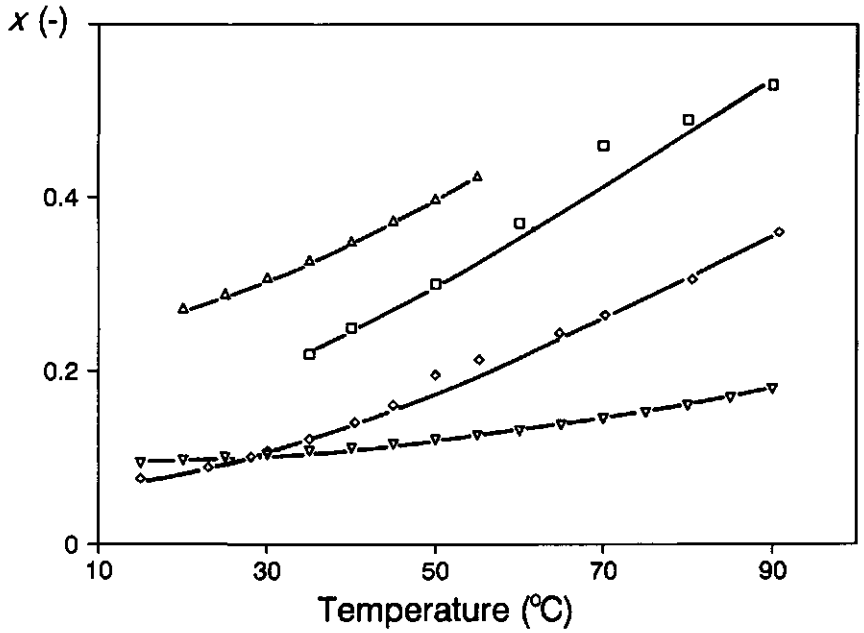


**Figure 6:** The solubility of sorbitol (□), fructose (△), glucose (◇), and sucrose (▽) as a function of temperature.
Data of fructose and sucrose are obtained from reference 13, data of glucose are obtained from reference 11 and data of sorbitol are determined experimentally.

In this section, some preliminary results on the effect of temperature on the equilibrium position and the reaction rate of lipase-catalyzed ester synthesis are presented.

## Effect of temperature on equilibrium

*Introduction*

The equilibrium position of a reaction is dependent on the standard Gibbs free energy of all reactants. The change in standard Gibbs free energy between products and substrates ($\Delta G^0(T)$) and the equilibrium constant ($K(T)$) are related by

$$\Delta G^{\circ}(T) = -R \cdot T \cdot \ln K(T) \tag{8}$$

where $R$ is the gas constant and $T$ is the temperature. The standard Gibbs free energy change of reaction $\Delta G^{0}(T)$ also can be written as

$$\Delta G^{\circ}(T) = \Delta H^{\circ}(T) - T \cdot \Delta S^{\circ} \tag{9}$$

where $\Delta H^{0}(T)$ is the standard enthalpy change of reaction and $\Delta S^{0}$ is the standard entropy change of reaction. Often $\Delta H^{0}(T)$ is not very dependent on temperature. If $\Delta H^{0}(T)$ can be assumed to be constant over a certain temperature range, the dependence of the equilibrium constant on temperature ($T$) can be described by

$$\frac{\partial \ln K(T)}{\partial T} = -\frac{1}{R} \cdot \frac{\partial\left(\frac{\Delta G^{\circ}(T)}{T}\right)}{\partial T} = \frac{\Delta H^{\circ}}{R \cdot T^{2}} \tag{10}$$

The aim of this study is to describe the consequences of an increase in temperature on the equilibrium position of an esterification reaction. From equation (10) it can be concluded that $K$ is temperature independent if $\Delta H^{0} = 0$. If $\Delta H^{0}$ has a constant value, a linear relationship will exist between $\ln K$ and $1/T$. In the latter case, determination of a number of equilibrium constants at different temperatures is a method to find a temperature-average value of $\Delta H^{0}$.

Temperature effects were studied for the esterification of glycerol and decanoic acid. In order to have temperature as the only variable, the initial mole fractions of all components were the same in each experiment. The same set of experiments was carried out for the esterification of sorbitol and decanoic acid. However, for the latter reaction, also a set of experiments was carried out in which the sorbitol mole fraction in the aqueous phase at each temperature was equal to the solubility of sorbitol. This set of experiments shows the maximum ester mole fractions that can be achieved at a certain temperature in this reaction system.

*Experimental*

Lipase of *Chromobacterium viscosum* (Biocatalysts, UK) is used for the experiments. Only for the experiments at 90 °C, the thermostable lipase of *Candida antarctica* was used. This lipase (Novo SP.4.35L) was a gift of Novo Nordisk (Denmark).

Decanoic acid, glycerol or sorbitol, water and lipase were mixed in 10 ml stoppered glass bottles and shaken by an end-over-end incubater in a thermostated cupboard. This type of cupboard cannot resist temperatures above 70 °C. Therefore, the experiments at 80 and 90 °C were carried out in a water bath. The shaking was carried out by hand at intervals of approximately 1 hour (during day time). Samples of the organic phase were analyzed by HPLC. This method is described in chapter 3.

Experiments were carried out until equilibrium was achieved. The equilibrium was confirmed by addition of extra lipase. If addition of lipase did not result in an increase of the ester concentration, the reaction equilibrium was assumed to be achieved.

*Results*

The results of the effect of temperature on the esterification of glycerol and decanoic acid, at a constant initial glycerol mole fraction, are shown in figure 7. The ester mole fractions are almost the same at different temperatures.

The results of the effect of temperature on the esterification of sorbitol and decanoic acid, at a constant initial sorbitol mole fraction, are shown in figure 8a. Here, an increase in the ester mole fraction at increasing temperatures can be seen. The ester mole fractions at 80 °C deviate from the trend shown by the other ester mole fractions. The reason might be that the experiments at 80 and 90 °C were carried out without continuous shaking. For the experiment at 90 °C a heat-stable lipase (*Candida antarctic*) is used, while for the experiment at 80 °C the same lipase as in the experiments at lower temperatures (*Chromobacterium viscosum*) is used. In the experiments without continuous shaking, concentration gradients can exist due to limited diffusion velocity and for this reason reaction rates can be low. It is also possible that inactivation of lipase did occur before the reaction equilibrium was reached. However, it is not clear why addition of extra lipase did not result in an increase of the ester concentrations.

In figure 8b, the results of the esterification of sorbitol and decanoic acid at increasing initial sorbitol mole fractions and increasing temperatures are shown. If the temperature is increased from 35 °C to 90 °C, the total ester mole fraction, which is the

sum of the mono-, di-, tri-, and tetraester mole fractions, increases from 0.07 to 0.32, which is more than a factor 4. The ratio between mono-, di-, tri-, and tetraester mole fractions is hardly affected by temperature and initial sorbitol mole fraction.

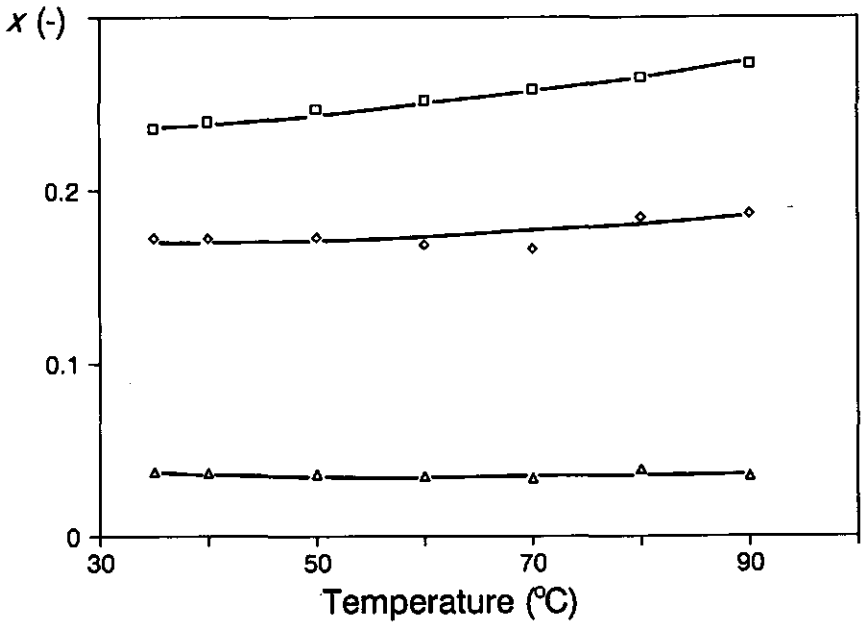


**Figure 7:** Glycerol mono- (□), di- (◊), and triester (△) mole fractions at equilibrium as a function of the temperature.
Experimental: 20 mmole decanoic acid, 20 mmole glycerol, 20 mmole water, and 25 mg lipase (*Chromobacterium viscosum)* were mixed and incubated until the reaction equilibrium is reached. For the reaction at 90 °C lipase from *Candida antarctica* was used.

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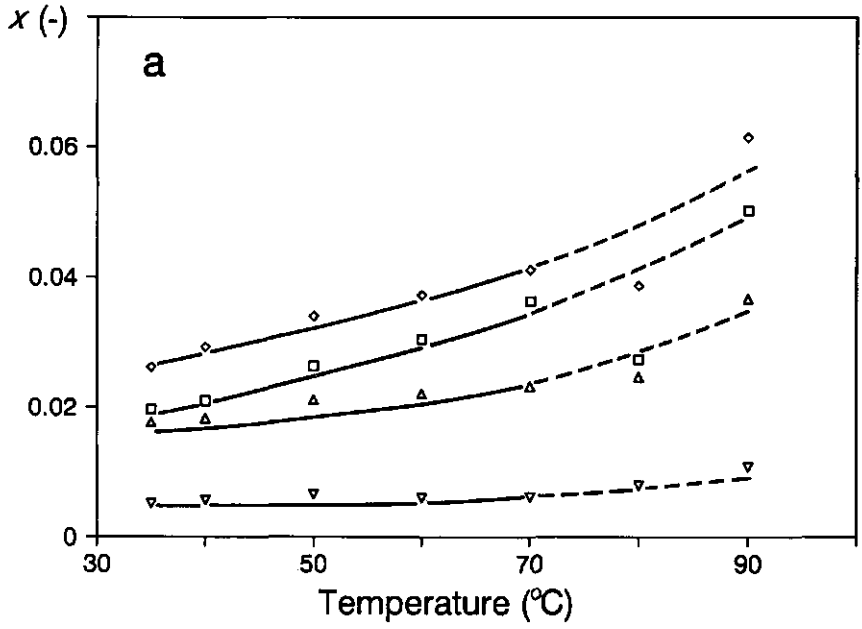
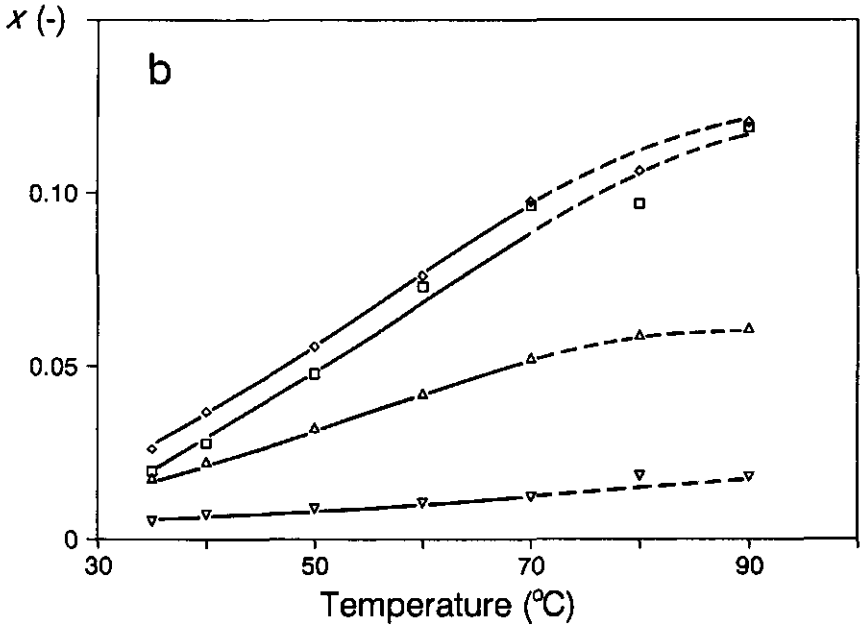
**Figure 8:** Sorbitol mono- (□), di- (◊), tri- (△), and tetra (▽) mole fractions at equilibrium as a function of the temperature at constant initial mole fraction (a) and at increasing mole fraction sorbitol (b).
Experimental: 20 mmole decanoic acid, 20 mmole sorbitol, 71 mmole water (a) or a variable amount of water (b), and 25 mg lipase (*Chromobacterium viscosum)* were mixed and incubated until the reaction equilibrium is reached. For the reaction at 90 °C lipase from *Candida antarctica* was used.


X (-)
a
0.06
0.04
0.02
0
30
50
70
90
Temperature (°C)


X (-)
b
0.10
0.05
0
30
50
70
90
Temperature (°C)

In chapter 4, the determination of equilibrium constants is described. The activity coefficients can be calculated with the UNIFAC group contribution method and the experimental mole fractions at equilibrium. With the equilibrium mole fractions and the activity coefficients, the equilibrium constants can be calculated. However, the UNIFAC parameter table that is used in this thesis,[8] is only suitable at temperatures between 10 and 40 °C. Therefore, no equilibrium constants can be calculated at different temperatures and consequently $\Delta H^0$ can not be determined.

The dependence of water activity on temperature can be obtained from sorption isotherms. A sorption isotherm gives the relationship between water activity and water content at constant temperature. Sorption isotherms of sorbitol at 25, 60 and 80 °C[14] show that at higher water contents, where sorbitol is in solution, the water activity is not very dependent on temperature. This means that in the reaction systems the water activities are assumed to be constant at different temperatures, for constant sorbitol mole fractions. The ester mole fractions are also not very dependent on the temperature at a constant initial sorbitol mole fraction. From these facts, it is expected that the ester activities as well as the substrate activities are not very dependent on the temperature. For this reason the equilibrium constant (equation (6)) is not expected to be dependent on the temperature. In that case the value of $\Delta H^0$ is low for esterification of sorbitol and decanoic acid. Also for the esterification of glycerol and decanoic acid a low value of $\Delta H^0$ is expected.

**Effect of temperature on reaction rate**

*Introduction*

The enzyme activity increases by increasing the temperature, resulting in a higher reaction rate. However, enzyme stability is also influenced by temperature, high temperatures cause a decrease in stability. In this section, the effect of temperature on the initial reaction rate of the esterification of sorbitol and decanoic acid is studied. It is assumed that enzyme inactivation can be neglected for the time period needed for measurement of the initial reaction rate. As for the equilibrium experiments, a set of

experiments was carried out, where the initial mole fractions of all components were the same. In another set of experiments the sorbitol mole fraction in the aqueous phase at each temperature was equal to the solubility of sorbitol.

*Experimental*

Lipase of *Chromobacterium viscosum* (Biocatalysts, UK) and *Candida antarctica* (Novo Nordisk, Denmark) were used for the experiments.
Experiments were carried out in a thermostated reaction vessel of 250 ml. The reaction mixture consisted of 0.2 mole decanoic acid, 0.71 mole water, variable amounts of sorbitol and 500 mg lipase. The emulsion was mixed thoroughly and at regular time intervals, samples were taken out. After separation, the organic phase was analyzed by HPLC. The initial reaction rate is determined from a plot of the amount of esterified fatty acid (in mole) versus time. Linear regression is carried out from t = 0 till the time that the amount of esterified fatty acid is 20% of the amount at equilibrium. At a temperature of 35 °C this time is about 50 h, while at 90 °C this time is only about 5 h. The initial reaction rate is expressed as mmole ester per g crude enzyme per hour.

*Results*

In figure 9, the initial reaction rates are plotted versus the temperature. This figure shows a tremendous increase in reaction rate with temperature. If the same sorbitol mole fraction is used ($x_s$ = 0.22), the reaction rate is increased by a factor 27, by increasing the temperature from 35 °C to 70 °C. If, besides the temperature, also the sorbitol mole fraction is increased, the reaction rate increases by a factor 70 over the same temperature range. At high temperatures, the heat-stable lipase of *Candida antarctica* is used. This lipase shows a lower reaction rate per gram enzyme than lipase of *Chromobacterium viscosum* at 70 °C. However, at 90 °C the reaction rate is increased to 13.6 mmole/g.h. These reaction rates are in the same order of magnitude as presented by Björkling et al.[2] From their data of the reaction between alkylglucosides and dodecanoic acid, catalyzed by *Candida antarctica* lipase at 70 °C, reaction rates of 7 - 16 mmole/g.h can be calculated. Until now, these were the highest reaction rates for esterification of carbohydrates presented in literature.

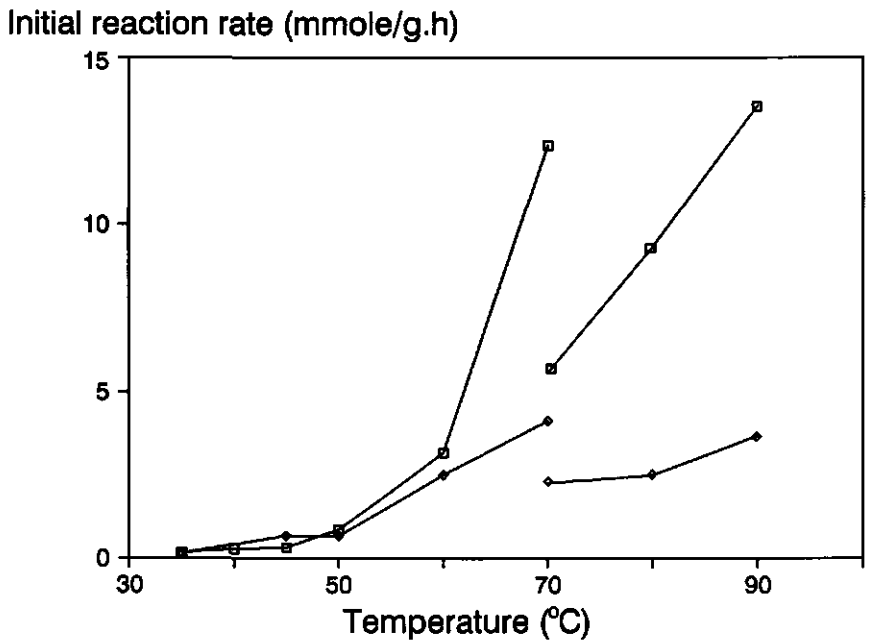



**Figure 9:** Initial reaction rates as a function of the temperature.
Open symbols: catalysis with lipase of *Chromobacterium viscosum*; closed symbols: catalysis with lipase of *Candida antarctica*. Experiments with saturated sorbitol solutions (□), and with a sorbitol mole fraction of 0.22 (◇).

The esterification of decanoic acid and other carbohydrates at 90 °C, catalyzed by *Candida antarctica* is also studied. At 90 °C and an initial fructose mole fraction in the aqueous phase of 0.65, the initial reaction rate is 12.2 mmole/g.h. The reaction with glucose, at an initial mole fraction of 0.32 is much lower, 0.4 mmole/g.h. At this high temperature, browning took place after some time. Esterification of decanoic acid and sucrose is discussed in the next section of this chapter.

The initial reaction rates at temperatures between 35 and 60 °C seem to be independent of the sorbitol mole fraction, indicating zero-order kinetics with respect to the sorbitol concentration. However, at 70 °C the initial reaction rate is clearly dependent

on the sorbitol mole fraction. Also the reaction rates with *Candida antarctica* lipase are clearly dependent on the sorbitol mole fraction. Further studies are necessary to gain a better understanding of the kinetics of this reaction.

The stability of lipase at high reaction temperatures is not studied. However, from literature it is known that enzymes are stabilized by water-polyol mixtures.[7,10] Back et al.[1] discussed the increased thermal stability of proteins in the presence of sugars and polyols. They have studied 4 proteins and the denaturation temperature increases by 11-18 °C in a 50 % (w/w) sorbitol solution. For example, for lysozyme the denaturation temperature increases from 71 °C in an aqueous solution to 88 °C in a 50 % (w/w) sorbitol solution. In our study, sorbitol concentrations are between 74 % (w/w) and 92 % (w/w). In chapter 3, lipase of *Candida rugosa* is found to be stable in a 74 % (w/w) sorbitol solution at 35 °C. However, high concentrations of glycerol (> 88 % (w/w) are found to inactivate lipase from *Candida rugosa* very rapidly.[12] For long-term processes at high temperatures, the stability of lipase is important. Further studies on lipase stability at high sorbitol concentrations and high temperatures are necessary.

Blanco et al.[3] also studied the role of temperature and their results are in agreement with our findings. They studied the effect of temperature on the α-chymotrypsin-catalyzed esterification of *N*-acetyl-tryptophan and phenylethanol, and reported that temperature has only little effect on the equilibrium position, but really affects the initial reaction rate. For a temperature increase of 10 °C, the reaction rate increased by a factor 2. It can be concluded that increasing the temperature is attractive for enzymatic carbohydrate ester synthesis in aqueous-organic two-phase systems. Because higher carbohydrates mole fractions can be used, the ester mole fraction at equilibrium increases. Furthermore, by an increase in the temperature, the reaction rate increases, however, also the rate of inactivation increases. For long-term processes at high temperatures it is important that heat-stable lipases will be used.

## ENZYMATIC SYNTHESIS OF SUCROSE ESTERS

There is a general interest in sucrose esters, since these esters have good emulsifying properties and sucrose is a cheap substrate. In the aqueous-organic two-phase system as discussed in chapter 3, no esterification was detected with sucrose as a substrate. The

reason for this could be the formation of a very tight enzyme-product complex,[6] which means that the product prefers the active site of the enzyme rather than the organic or aqueous phase. Very tight enzyme-product complexes can be prevented by making the organic phase more attractive for sucrose esters for example by addition of polar solvents. In this section attempts to prepare sucrose esters enzymatically by addition of polar solvent are described. Also the results of experiments at elevated temperatures will be discussed. It has been reported that lipase from *Chromobacterium viscosum* catalyses the transesterification between an activated fatty acid and an acylated sucrose in dry acetone.[4] This means that sucrose can be recognized as a substrate by this lipase and for this reason, this lipase is used to study the esterification of sucrose and decanoic acid.

Experiments were carried out in which polar solvents were added, such as triglyme, dimethylformamide, acetone, acetonitrile, and 2-methyl-2-propanol. After 2 weeks of incubation, a small peak was observed in the HPLC chromatogram, which is indicative of sucrose monoester. The mole fraction was about 0.003 and only increased slightly after longer incubation times and addition of more lipase.

Although the solubility of sucrose is not increasing with temperature as much as the carbohydrates that are mentioned before, also experiments were carried out at 80 °C. After one week of incubation, the HPLC chromatogram showed three small peaks, which could be sucrose mono-, di-, and triesters. The mole fractions were between 0.001 and 0.002 and did not increase significantly after longer incubation times and addition of more lipase. An explanation for the low sucrose ester concentration is discussed in chapter 6: Equilibrium ester mole fractions are found to decrease with increasing polarity of the the alcohol. If this trend is extrapolated to sucrose esters, these ester mole fractions will be very low.

It can be concluded that sucrose esters are probably synthesized in an aqueous-organic two-phase system. The sucrose esters should be purified, in order to confirm their structures by spectrometric techniques. However, concentrations of sucrose esters are very low and for this reason the reaction system is commercially of no interest.

## STRUCTURE OF SORBITOL ESTERS

In chapter 2, the purification of the monoester of sorbitol and oleic acid was described. The purified ester was found to be a mixture of 1-monoacyl- and 6-monoacylsorbitol. This was an indication that lipase of *Chromobacterium viscosum* exhibited a preference towards primary hydroxylgroups. Furthermore, in the $^{13}$C-NMR spectrum, no signals were visible derived from C-C double bonds, which was an indication that the *Chromobacterium viscosum* lipase was more reactive towards saturated fatty acids. Until now, this subject is not thoroughly studied, however, preliminary experiments in a reaction system with 2-pyrrolidone (chapter 2), containing a mixture of oleic acid (cis-9-octandecenoic acid) and palmitic acid (hexadecanoic acid), did not show higher ester concentrations at high palmitic acid concentrations. Also in chapter 6, where the esterification of glycerol and several fatty acids, catalyzed by *Chromobacterium viscosum* lipase was studied, no indications were found that this lipase has a preference to saturated fatty acids.

In this section, the elucidation of the structure of di-, tri-, and tetraesters of sorbitol and decanoic acid is described. The esters were synthesized in a reaction system at 70 °C, as described in the second section of this chapter ('Effect of temperature on esterification of polyols and fatty acids'). The reaction was catalyzed by *Chromobacterium viscosum* lipase. The reaction products were purified with a silicagel column, which was eluted with a chloroform/methanol mixture. The methanol concentration of this mixture was initially 1 % (v/v) and was gradually increased till 10 % (v/v). The effluent was fractionated and monitored by thin layer chromatography. Fractions containing di-, tri-, and tetraesters were separately pooled and analyzed by $^{1}$H-NMR, infrared and mass spectroscopy. The structures of the esters were elucidated by interpretation of the $^{13}$C-NMR spectra. The structure of the diester was found to be 1,6-didecanoylsorbitol. This is in agreement with previous results on the preference of *Chromobacterium viscosum* lipase for primary hydroxyl groups. The triester formed appeared to be mainly 1,5,6-tridecanoylsorbitol, whereas the the tetraester was found to be 1,2,5,6-tetradecanoylsorbitol. Computer programs are available now, that calculate the most stable structure of a molecule. For sorbitol triester, the 1,5,6-ester was found to be the most stable isomer, which is the same structure as that of the enzymatically formed triester. It is remarkable that the most stable tetraester structure was calculated to be the 1,4,5,6-ester. This is different from the structure of the purified tetraester. This could be an indication that lipase of

*Chromobacterium viscosum* distinguishes between the four secondary hydroxyl groups rather than just synthesize the most stable structure of a tetraester. It also indicates that intramolecular acyl group migration did not take place, because this would result in the most stable structure of the tetraester.

## CONCLUSIONS

In an aqueous-organic two-phase reaction system, lipase-catalyzed synthesis of carbohydrate esters is possible. Modification of the carbohydrates or fatty acids is not necessary in the process that is described, which is an advantage as compared to the reaction systems that are described in literature. A low water activity is required to obtain high ester concentrations at the reaction equilibrium. Therefore, a two-phase membrane reactor is a suitable type of reactor for carbohydrate ester synthesis, since the water activity can be kept low during the reaction. In general, the reaction rate and the equilibrium concentrations of enzymatic carbohydrate esterification are low. Both can be increased considerably by an increase of the reaction temperature.

Organic solvents are often used in enzymatic synthesis and the effect of organic solvents on the activity and stability of the enzyme is frequently studied. In this thesis, the effect of organic solvents on the equilibrium position of various reactions is studied. The choice of solvent and its concentration is found to be extremely important for the product concentrations at equilibrium. For the prediction of these equilibrium data, a model is developed. The models that were available in literature, were based on partition coefficients and mass balances. These models were developed for dilute two-phase systems. However, for industrial applications, high product concentrations are desired, which implicate the use of nondilute reaction systems. Therefore, the program TREP is developed, which is based on the UNIFAC group contribution method. The esterification of glycerol and decanoic acid in several organic solvents is used to show that TREP calculates the ester mole fractions at equilibrium under all conditions for all mixtures of solvents within some margins. TREP is shown to be useful for equilibrium predictions of several other types of reactions. Most of these reactions are performed in an emulsion-type two-phase system, where the volume of both phases are in the same order of magnitude. However, some reactions are performed in a trapped aqueous phase

system, where the aqueous phase is very small and the water activity is close to 1. Although the type of reaction and the reaction system are clearly different, TREP is useful for prediction of the equilibrium position. With TREP a better insight is gained in factors that affect the equilibrium position of a reaction. For example, reactions conditions could be predicted for the esterification of glycerol and fatty acid acid at which the monoester is the only product.

## NOMENCLATURE

| | | |
|---|---|---|
| $a$ | activity | (-) or (kmole.m$^{-3}$) |
| $\Delta G^0(T)$ | standard free energy change of reaction | (J.mol$^{-1}$) |
| $\Delta H^0(T)$ | standard enthalpy change of reaction | (J.mol$^{-1}$) |
| $[i]$ | concentration of component i | (kmole.m$^{-3}$) |
| $K$ | equilibrium constant | (-) |
| $K_{bi}$ | biphasic equilibrium constant | (-) |
| $K_w$ | equilibrium constant for dilute aqueous phase | (-) |
| $K_{bi}^*$ | biphasic equilibrium constant | (m$^3$.kmole$^{-1}$) |
| $K_w^*$ | equilibrium constant for dilute aqueous solution | (m$^3$.kmole$^{-1}$) |
| $P$ | partition coefficient | ($M_{org}.M_w^{-1}$) |
| $R$ | gas constant | (J.mol$^{-1}$.K$^{-1}$) |
| $\Delta S^0$ | standard entropy change of reaction | (J.mol$^{-1}$.K$^{-1}$) |
| $T$ | temperature | (K) |
| $V$ | volume | (m$^3$) |
| $x$ | mole fraction | (-) |
| $\alpha$ | volume ratio | (-) |
| $\gamma$ | activity coefficient | (-) |

**Subscripts:**

| | |
|---|---|
| bi | biphasic |
| i,A,B,C | components |
| org | organic phase |
| w | aqueous phase |

**Superscripts:**

* using water mole fraction instead of water concentration

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# SUMMARY

The last decade increasingly attention is paid to lipases as catalysts for synthesis of components, such as fatty acid-based surfactants, flavors, edible oil equivalents, monomers and polymers, and amides. In this thesis, the lipase-catalyzed esterification of polyols and fatty acids is described. These esters consist of a nonpolar part (fatty acid) and a polar part (polyol). Therefore, polyol esters have surface-active properties and are used as emulsifier in food, pharmaceutics and cosmetics. One of the aims of this thesis is to develop a reaction system for the esterification of polyols (carbohydrates) and fatty acids, without any modification of the substrates. Also, high reaction rates are desired.

Enzymatic esterification is often performed in the presence of organic solvents. Besides activity and stability of the enzymes, the solvents will affect the equilibrium position of reactions. In literature, models were described for the prediction of the equilibrium position in dilute two-phase systems. However, for industrial applications, high product concentrations are desired, which implicate the use of nondilute reaction systems. Another aim of this thesis is to gain a better insight in factors that affect the equilibrium position of a reaction and to predict the product concentrations at equilibrium in non-dilute two-phase systems.

In chapter 2 and 3, the lipase-catalyzed esterification of sorbitol and fatty acid is studied in two different two-phase reaction systems. In chapter 2, 2-pyrrolidone is used as a cosolvent for sorbitol. In this study, the lipase from *Chromobacterium viscosum* is used and the initial esterification rate is high as compared to literature data. The water activity is found to be important for the ester concentrations at equilibrium. High concentrations of the cosolvent 2-pyrrolidone should be avoided, because these will inactivate the lipase. In the reaction system that is described in chapter 3, water is used to dissolve sorbitol. *Candida rugosa* lipase is used in this study and initial esterification rates are slightly higher than in chapter 2. The water activity is dependent on the sorbitol mole fraction in the aqueous phase and lowering of the water activity is limited by the solubility of sorbitol. A two-phase membrane reactor is a suitable type of reactor, since the water activity of the

aqueous phase can be kept constant during the experiment and lipase possesses a good stability. In both reaction systems, besides sorbitol also glucose and fructose can be used as a substrate, while disaccharides, such as sucrose, are not reactive at all.

In chapter 4, the lipase-catalyzed esterification of glycerol and decanoic acid has been studied in aqueous-organic two-phase systems. The addition of an organic solvent is found to influence the ester mole fractions at equilibrium. For the synthesis of polar products (monoesters), a polar solvent (low log P) is favorable, while for the synthesis of nonpolar products (triesters), it is better to choose a nonpolar solvent (high log P). The computer program 'Two-phase Reaction Equilibrium Prediction' (TREP) has been developed for the prediction of the ester concentrations in nondilute two-phase systems, in case both the reaction equilibrium as well as the phase equilibrium are achieved. This program is based on mass balances and the UNIFAC group contribution method. Deviations in the prediction with TREP are generally less then a factor of 2 and are due to inaccuracies of the UNIFAC group contribution method.

The lipase-catalyzed acylglycerol synthesis with fatty acids of different chain length is studied in chapter 5. For predictions with TREP, one set of equilibrium constants is used for monoester, diester, and triester synthesis. It is shown that with this set the equilibrium position of the reaction between glycerol and all saturated fatty acids with a chain length from 6 to 18 and oleic acid can be calculated within some margins. For fatty acids with different chain length, the ester mole fractions at equilibrium are clearly different. With the short-chain hexanoic acid, the monoester mole fraction is highest, while for the long-chain oleic acid, the diester mole fraction is the highest one. Besides the equilibrium position, also the reaction rates are affected by the solvent that is added. In polar solvents, the monoester production rate is enhanced. This is caused by the shift in the equilibrium mole fractions.

In chapter 6, the effect of solvents on the esterification of decanoic acid and several alcohols, such as 1-dodecanol, 1-butanol, 1,3-propanediol, and sorbitol is studied. In agreement with the previous results, the ester mole fractions at the reaction equilibrium are dependent on the solvability of the ester in the organic phase. This effect is most striking for the polar sorbitol esters. Almost no esters are present at equilibrium in systems with nonpolar solvents, while reasonable high ester mole fractions can be obtained in systems with polar solvents. In contrast with the results of chapter 5, the equilibrium constants are clearly affected by the type of alcohol that is chosen as a

substrate. Calculations with TREP showed that the calculated ester mole fractions did not deviate more than a factor of 1.5 from the measured ones. However, it appears that the calculated water mole fractions deviate systematically in the downwards direction.

Chapter 7 shows a comparison between models in literature for the prediction of the equilibrium position in dilute two-phase reaction systems and calculations with TREP. It is shown that the models from literature are limited to reaction systems in which partition coefficients are constant. The program TREP can be used for nondilute as well as dilute reaction systems.

Furthermore, this chapter shows that the ester mole fractions at equilibrium can be increased with increasing temperature. This is due to the increase of the solubility of sorbitol with increasing temperature. Most pronounced is the effect of temperature on the reaction rate, which is increased enormously. However, for long-term processes at high temperatures it is important that heat-stable lipases will be used.

# SAMENVATTING

De laatste jaren wordt in toenemende mate aandacht besteed aan het gebruik van het enzym lipase als katalysator voor de synthese van allerlei stoffen zoals emulgatoren, geur- en smaakstoffen, eetbare oliën, mono- en polymeren en amiden. In dit proefschrift wordt de door lipase gekatalyseerde verestering van polyolen en vetzuren beschreven. De gevormde esters bestaan uit een apolair gedeelte (vetzuur) en een polair gedeelte (polyol). Hierdoor zijn deze esters oppervlakte-actief, wat ze geschikt maakt voor het gebruik als emulgator in de levensmiddelen-, farmaceutische en cosmetische industrie. Het doel van het in dit proefschrift beschreven onderzoek is het ontwikkelen van een reactiesysteem voor de verestering van polyolen (suikers) en vetzuren. Vereisten hierbij zijn de toepasbaarheid voor ongemodificeerde substraten en de hoge reactiesnelheden.

De enzymatische verestering wordt vaak uitgevoerd in aanwezigheid van organische oplosmiddelen. Het oplosmiddel beïnvloedt niet alleen de activiteit en stabiliteit van het enzym, maar ook de thermodynamische evenwichtsligging van de reactie. In de literatuur zijn modellen beschreven die de evenwichtsligging in ideale tweefasenreactiesystemen voorspellen. Voor industriële toepassingen is het echter gewenst om met hoge product concentraties te werken. Dit houdt in dat de reactiesystemen niet ideaal zijn. Het tweede doel van dit onderzoek is daarom inzicht te verkrijgen in factoren die de evenwichtsligging van een reactie beïnvloeden en te voorspellen wat de productconcentraties zijn wanneer het thermodynamisch evenwicht is bereikt in een niet-ideal tweefasensysteem.

In hoofdstuk 2 en 3 van dit proefschrift is de lipase gekatalyseerde verestering van sorbitol en vetzuur beschreven voor twee verschillende reactiesystemen. In hoofdstuk 2 is 2-pyrrolidon gebruikt als oplosmiddel voor sorbitol. De reactie wordt gekatalyseerd door lipase van *Chromobacterium viscosum* en de initiële reactiesnelheid is hoog in vergelijking met literatuurwaarden. De esterconcentraties bij evenwicht zijn afhankelijk van de wateractiviteit van het systeem. Hoge 2-pyrrolidonconcentraties moeten vermeden worden, aangezien het lipase hierdoor geïnactiveerd wordt. In het reactiesysteem dat beschreven is in hoofdstuk 3, is water gebruikt als oplosmiddel voor sorbitol. In dit

reactiesysteem is het lipase van *Candida rugosa* gebruikt en de initiële reactiesnelheid is hoger dan in het reactiesysteem van hoofdstuk 2. Ook hier is de wateractiviteit van belang en deze is afhankelijk van de molfractie sorbitol in the waterfase. De wateractiviteit kan slechts verlaagd worden totdat de maximale oplosbaarheid van sorbitol in water bereikt is. De tweefasenmembraanreactor is een geschikte reactor voor dit type reacties, omdat de wateractiviteit in de waterfase laag gehouden kan worden tijdens de reactie. Bovendien is de stabiliteit van het lipase goed in dit reactorsysteem. In beide reactiesystemen kunnen naast sorbitol ook glucose en fructose gebruikt worden als substraat. Met saccharose als substraat wordt geen verestering waargenomen.

In hoofdstuk 4 is de verestering van glycerol en decaanzuur in een tweefasensysteem met water en een organisch oplosmiddel beschreven. De esterconcentraties bij het thermodynamisch evenwicht, worden beïnvloed door de keuze van het oplosmiddel. Het blijkt dat polaire oplosmiddelen (lage log *P* waarden) heel geschikt zijn wanneer polaire producten (mono-esters) gewenst zijn, terwijl voor de synthese van apolaire producten (tri-esters) juist beter een apolair oplosmiddel (hoge log *P* waarden) gebruikt kan worden. Het programma TREP (Two-phase Reaction Equilibrium Prediction) voorspelt de concentraties van alle componenten in niet-ideale tweefasensystemen wanneer zowel het reactie- als het fasenevenwicht bereikt is. Dit programma is gebaseerd op massabalansen en de UNIFAC-groepsbijdragen-methode. De voorspelde concentraties wijken in het algemeen minder dan een factor 2 af van de gemeten concentraties. Deze afwijkingen zijn te wijten aan de beperkingen van de UNIFAC-groepsbijdragen-methode.

De verestering van glycerol en vetzuren met een verschillende ketenlengte is beschreven in hoofdstuk 5. Voor berekeningen met TREP kunnen voor de evenwichtsconstanten voor mono-, di- en tri-ester synthese, respectievelijk, gemiddelde waarden gebruikt worden. Met deze gemiddelde waarden kunnen de concentraties berekend worden voor de reacties tussen glycerol en oliezuur en elk verzadigd vetzuur met een ketenlengte tussen 6 en 18. Bij verestering met hexaanzuur (C6), is de molfractie monoester het hoogste, terwijl bij verestering met oliezuur (C18), de molfractie van de diester het hoogste is. Naast de evenwichtsligging beïnvloedt het oplosmiddel ook de reactiesnelheid. In polaire oplosmiddelen is de reactiesnelheid van de monoester 1.5 keer hoger dan in een reactiesysteem zonder oplosmiddel. Dit wordt veroorzaakt door het verschil in de evenwichtsconcentraties.

In hoofdstuk 6 is het effect van oplosmiddelen op de verestering van decaanzuur en verschillende alcoholen, zoals 1-dodecanol, 1-butanol, 1,3-propaandiol en sorbitol, bestudeerd. In overeenstemming met eerdere resultaten, zijn ook hier de esterconcentraties bij evenwicht afhankelijk van de mate waarin de ester oplost in de organische fase. Dit effect is het meest duidelijk voor de sorbitolesters. In apolaire oplosmiddelen lossen deze polaire esters nauwelijks op, terwijl in polaire oplosmiddelen redelijke esterconcentraties worden gemeten. In tegenstelling tot de resultaten in hoofdstuk 5, worden de evenwichtsconstanten wel degelijk beïnvloed door het alcohol dat gebruikt wordt als substraat. Berekeningen met TREP tonen aan dat de voorspelde esterconcentraties niet meer dan een factor 1.5 afwijken van de gemeten concentraties. De berekende water concentraties zijn echter bijna altijd te laag.

In hoofdstuk 7 zijn de modellen voor de voorspelling van de evenwichtsligging in ideale tweefasensystemen, die in de literatuur beschreven zijn, vergeleken met de berekeningen die gemaakt zijn met TREP. De modellen voor ideale reactiesystemen blijken alleen bruikbaar te zijn, wanneer de verdelingscoëfficiënten constant zijn, terwijl TREP voor zowel ideale als niet-ideale reactiesystemen gebruikt kan worden.
Bovendien is in dit hoofdstuk beschreven dat de sorbitolesterconcentraties bij evenwicht verhoogd kunnen worden door bij hogere temperatuur te werken. Dit komt doordat de oplosbaarheid van sorbitol toeneemt bij toenemende temperatuur. De temperatuur heeft ook grote invloed op de reactiesnelheid. Bij continue processen bij hoge temperaturen is echter de stabiliteit van het lipase van groter belang en kunnen beter hitte-bestendige lipasen gebruikt worden.

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