

Evaluation of Binary Solvent Mixtures for Efficient Monoacylglycerol Production by Continuous Enzymatic Glycerolysis

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This study was aimed at evaluating different binary solvent mixtures for efficient industrial monoacylglycerol (MAG) production by enzymatic glycerolysis. Of all investigated cases, the binary mixture of *tert*-butanol:*tert*-pentanol (TB:TP) 80:20 vol % was the most suitable organic medium for continuous enzymatic glycerolysis, ensuring high MAG formation in a short time, reasonable solvent price, and easy handling during distillation/condensation processing. A minimum solvent dosage of 44–54 wt % of the reaction mixture was necessary to achieve high MAG yields of 47–56 wt %, within 20 min. The melting and boiling points of the TB:TP mixture were estimated to be 7 and 85 °C, respectively, using thermodynamic models. These predictions were in good agreement with experimentally determined values. In spite of the high reaction efficiency in the binary TB:TP system, the mixture of glycerol and sunflower oil (containing 97.1% triacylglycerol) yielded surprisingly a liquid/liquid phase split behavior even at high temperatures (>80 °C). This in contrast to thermodynamic model calculations suggested full miscibility in all proportions. These findings suggest that enhanced reaction efficiency in organic solvent also depends upon aspects other than the system homogeneity such as reduced viscosity, reduced mass transfer limitations, and the accessibility of the substrate to the active site of the enzyme.

KEYWORDS: *Candida antarctica* lipase B; continuous reactor; glycerolysis; monoacylglycerols; tertiary alcohol media; vegetable oils

INTRODUCTION

Monoacylglycerols (MAG) are extensively used emulsifiers in the food, pharmaceutical, and cosmetic industries (1, 2). Besides bulk applications in the food and dairy industries, other applications of special MAGs with specific fatty acid profiles include medical, cosmetic, and hair care uses (1, 2). The commercial interest for producing special MAGs containing, for instance, *n*-3 polyunsaturated fatty acid (PUFA) is increasing due to growing demands for healthier food lipid ingredients and other high-added-value products.

Currently, MAGs are widely manufactured by chemical glycerolysis of fats and oils at high temperature of 200–250 °C (1–3). Chemical glycerolysis suffers from the drawbacks of the high temperature, which leads to increase in flavor and color impurities (1–3). Handling of PUFA oil raw material with currently used methods is difficult due to the high rate of oxi-

dization at high temperature (4). This has stimulated great interest in the gentler lipase-catalyzed glycerolysis process at ambient temperatures (1–3, 5–7).

Vegetable oils are considered to be easily accessible, relatively cheap neutral triacylglycerol (TAG) raw material for carrying nutritionally important PUFAs. Thus, enzyme-catalyzed glycerolysis of vegetable PUFA oils seems to have strong industrial potential for improving MAG processing, quality, and functionality and thereby expanding MAG applications in functional foods and pharmaceuticals, etc. (6).

In the glycerolysis reaction, the use of organic solvents is beneficial for creating a homogeneous reaction system of the immiscible reactants glycerol and oil and to facilitate continuous reactor processes (1, 2). Enzymatic glycerolysis in the organic solvents *tert*-butanol (TB) or *tert*-pentanol (TP) has previously been investigated and found to be very efficient for the production of MAG mixtures with PUFA profile (5–7). The two organic media enhance enzyme activity and accelerate the reaction rate dramatically compared to the solvent-free system (5–7).

However, from an industrial point of view, the use of binary solvent mixtures might be even more advantageous than that of pure solvents for glycerolysis. The use of pure TP (2-methyl-2-butanol/*tert*-amyl alcohol) has, for instance, the drawback of

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very high costs compared to pure TB (2-methyl-2-propanol/*tert*-butyl alcohol), especially at high purity of 99% (6). However, to avoid side reactions with solvent impurities, high solvent purity is necessary. Also, product purification is necessary to remove product impurities and achieve MAGs in highly pure form. MAGs in a pure form are advantageous to achieve emulsifying properties superior to those of MAG–diacylglycerol (DAG) mixtures (1, 2). In the case of using pure TB, solvent removal and product purification by traditional distillation/condensation techniques is rendered difficult, because its melting and boiling points are quite close (57 °C) (6). Also, TB's melting point (25 °C) is quite high. This involves the risk of solvent crystallization during condensation of the evaporated solvent. Solvent crystallization in the equipment makes continuous operations difficult and limits the possibility for easy reuse of the solvent (6).

The present study aims to evaluate the behavior of industrial continuous enzymatic glycerolysis in mixed binary solvent systems and follows up on earlier investigations related to glycerolysis in organic media (5–7). Detailed investigations are carried out with emphasis on continuous operations, high MAG formation in a short time, low solvent price, a relatively broad melting–boiling profile of the solvent medium, and easy handling compared to selected pure solvent systems.

MATERIALS AND METHODS

Materials. Aarhus United, Aarhus, Denmark, provided sunflower oil, a triacylglycerol oil with a purity of 97.1%. The oil consisted mainly of unsaturated long-chain fatty acids: C16:0, 6.7 wt %; C18:1, 26.3 wt %; C18:2, 61.2 wt %. A more detailed description of the oil is found elsewhere (5, 6). DANISCO A/S kindly provided DIMODANHR, a commercially available distilled MAG with a purity of $\geq 90\%$. Novozymes A/S, Bagsværd, Denmark, supplied Novozym435, a commercially available *Candida antarctica* lipase B (CALB) immobilized by physical adsorption onto a macroporous hydrophobic polymethyl methacrylate (PMMA) matrix. Glycerol with a purity of 99.5 wt % was purchased from VWR International Ltd., Albertslund, Denmark. All solvents used were of analytical grades with a purity of 96–99.7%. The solvents were purchased from Sigma-Aldrich, Brøndby, Denmark.

Thermodynamic Analysis. UNIFAC (8) was used to estimate the displacement in physical properties of selected binary solvent mixtures compared to the pure compounds. Melting and boiling points for the mixtures and pure components were calculated using isofugacity expressions for solid/liquid equilibrium and liquid/vapor equilibrium and are presented in phase diagrams. Further description of UNIFAC and UNIFAC calculations are given elsewhere (8).

Melting Point (MP) Measured by Differential Scanning Calorimetry (DSC). The transformation from solid to liquid of selected binary mixtures was studied by DSC (DSC 821e/HSS7, Mettler Toledo). Fifteen microliter samples were sealed in a ceramic sensor plate with a thermocouple that measured the difference between the heat flow to the sample and a reference. The samples were analyzed by initially cooling from 20 to -25 °C at 10 °C/min and held at -25 °C for 5 min. Subsequently, the samples were heated from -25 to 50 °C at a heating rate of 5 °C/min while onset, endset, offset and major peak maximum temperatures were measured. The melting point of the completely melted sample is determined as average values of triple determination of the endset values.

Continuous Glycerolysis in Packed Enzyme Column. A reaction mixture of sunflower oil, glycerol, and solvent was mixed in a feeding container/flask by magnetic stirring and preheated to 50 °C. The mixture, maintained at the set temperature and continuously stirred, was pumped (up-flow) through a column reactor (i.d. = 15 mm, o.d. = 38 mm, and length = 200 mm) with a Fluid Metering Inc. FMI Lab Pump QG150 (flow range = 0–16 mL/min) purchased from Micro Lab AARHUS A/S, Højbjerg, Denmark. The reactor consisted of a jacketed steel column dry packed with 8.0 g of enzyme, corresponding to 227 kg of enzyme/m³ column. A weight-based reaction time was

used defined as

$$\tau = \frac{W}{\nu_0 \rho} \quad (1)$$

where τ is the weight reaction time (min), W is the weight of catalyst pellets on a dry matter basis (g), ρ is the bulk density of the dry enzyme (g/cm³), and ν_0 is the volumetric flow rate of reactant mixture (cm³/min = flow rate/cross section of column). The column temperature was maintained at 40 °C by a water bath circulation. The column was initially stabilized, and bubbles were removed by running reaction mixture through the column equivalent to at least 3 times the reactor volume. The first product from the enzyme bed, equal to at least 4 times the weight time, was discarded to ensure the product outlet was representative. Samples were thereafter withdrawn and stored at -20 °C prior to analysis by gas chromatography (GC), which is described elsewhere (6). Results from GC analysis are expressed as weight percentages of the product mixture of glycerol, mono-, di-, and triacylglycerols, free fatty acids, and fatty acid esters.

Screening of Different Binary Mixture for Continuous Glycerolysis. Glycerol was mixed with sunflower oil, equal to a molar ratio of 5. Four different binary solvent mixtures were tested: TB:TP 80:20 and 90:10 vol %, TB:cyclohexane (cH) 80:20 vol % and TB:*n*-hexane (nH) 80:20 vol %, each mixture in an amount of 20 mL/10 g of oil. The purities of the solvents were as follows: TP, 96%; cH, 99%, nH, 97%; and TB, 99%. The flow rate was 0.6 mL/min, corresponding to laminar flow and a weight-based reaction time of 30 min. Results are expressed as average values of triple determination of samples withdrawn after 4, 7, and 24 h.

Effect of Solvent Dosage in Continuous Glycerolysis. Glycerol was mixed with sunflower oil, equal to a molar ratio of 3 and a binary mixture of TB:TP 80:20 vol % in four different dosages (28, 44, 54, and 61 wt % of reaction mixture). The purities of the TP and TB were 99 and 99.7%, respectively. The flow rates were 0.47 and 0.93 mL/min, corresponding to laminar flow and reaction–weight times of 20 and 40 min. Results are expressed as average values of double determination of samples withdrawn after 4 and 6 times the weight time.

Statistical Analysis. The Excel Analysis Toolpack: Analysis of Variances (ANOVA): Single Factor was used to test for significant differences between samples grouped by one variable (solvent combination or solvent dosage). The significance of the results was established at $P \leq 0.05$.

Phase Split Behavior of Reactant Mixtures. Reaction mixtures, with a total volume of 100 mL, were mixed of 50 wt % TB:TP 80:20 and 50 wt % glycerol plus oil in molar ratio of 3.7 or 5, respectively. The reaction mixtures were preheated to a selected temperature during magnetic stirring (40, 60, 75, 80, or 100 °C). When the selected temperature was reached, the magnetic stirrer was stopped, and the mixtures were left for 30 min and 24 h. Subsequently, the occurrence of a phase split was observed, indicating whether the reactants were miscible or not. The two different phases obtained from the reactant mixture of glycerol to oil molar ratio of 3.7 heated to 40 and 75 °C were withdrawn and collected in separate flasks. From each collected phase, 1 mL was used for GC analysis of the compound distribution, and the remaining part was used for analysis of the solvent distribution by rotation evaporation under vacuum at 70 °C. Results are based on double determinations.

Solvent Removal by Rotation Evaporation under Vacuum. A weighted sample amount (10–160 g) was heated in a water bath at a set temperature during rotation. The solvent was removed from the mixture by evaporation under vacuum at the selected temperature (60 or 73 °C). Subsequently, the distillate was condensed by cooling water and collected in a vessel (Büchi, Glasapparatefabrik Flawil, Switzerland). The solvent/byproduct collected was weighed, and the evaporated solvent was then calculated as weight percent of the initial solvent amount in the sample. Results are expressed as average values of double determinations.

RESULTS AND DISCUSSION

Predicted MP and BP of Different Binary Solvent Mixtures. MPs and BPs of binary TB:TP; TB:nH, and TB:cH mixtures can be evaluated through Txy (vapor/liquid equilibrium) and Tx (solid/liquid equilibrium) phase diagrams. Such phase diagrams were calculated using isofugacity relationships expressed in terms of UNIFAC activity coefficients as well as melting points, heats of melting, and vapor pressure relationships of the pure components. The resulting Tx diagrams are shown in **Figure 1a**, and the resulting Txy diagrams are shown in **Figure 1b**. For the solid/liquid systems, it is assumed that the two components are immiscible in the solid phase, so only pure solid phases are formed. This assumption leads to eutectic diagrams, with one branch corresponding to solidification of a single component at equilibrium with the (liquid) solvent mixture. The two branches coincide at the eutectic point/temperature. In general, two curves are shown per diagram: a lower curve resulting from assuming the liquid mixture to be an ideal solution and an upper resulting from taking into account liquid phase nonideality using UNIFAC activity coefficients and appropriate standard states. Perhaps not unexpectedly, the greatest differences between ideal mixture calculations and nonideal mixture calculations are found in the diagrams for alcohol/alkane systems. In the alcohol/alcohol (TB/TP) diagram the two curves are superimposed.

The binary solvent mixtures show in general broader/wider ranges between melting and boiling points than the pure TB system. The range between melting and boiling point of 57 °C for the pure TB was, for instance, enhanced to ranges of 78, 62, or 70 °C, when 20 vol% TP, normal hexane, or cyclohexane was added. Adding nH to TB lowers the melting and boiling points, with the MP depression effect dominating. Adding TP to TB lowers the MP and raises the BP. Adding cH to TB lowers the MP and does not alter the bubble point temperature significantly from the boiling temperature of TB.

Partial substitution of TB is desirable to avoid the risk of crystallization during purification processing after glycerolysis reaction. However, the substitution should be minimized to maintain the high reaction efficiency and low costs in the pure TB system (6, 7). A MP below approximately 15 °C and a temperature range between the melting and boiling points of at least 65 °C was deemed to be sufficient to avoid practical problems with crystallization. Thus, replacement of 10–20 vol % of the TB with either TP, nH, cH seems appropriate to be to obtain sustainable practical operations.

Comparison of Predicted and Experimentally Determined MP of Binary Solvent Mixtures. To validate the UNIFAC predictions, MPs were determined experimentally using DSC. **Table 1** compares experimental and predicted values with literature values. Also, the price index for the different solvent combinations is given. Measured MPs for pure and binary solvent mixtures agreed well with predicted as well as literature values. The changed melting/boiling profile for TB:TP 90:10 vol % compared to that of pure TB was only minor, still allowing the risk of crystallization during downstream processing. Thus, with respect to practical operation at industrial facilities, only binary mixtures with no more than 80 vol % TB seem to be suitable.

Highly flammable and harmful risks are related to all four different solvents tested (12–15). *n*-Hexane has the advantage of being accepted as a food-grade extraction medium by the international Food and Nutrition Board, supported by the U.S. Food and Drug Administration (FDA) (16). However, the low BP of nH at 68.7 °C (9) has the disadvantage of pronounced

flammable risk and easy release of harmful volatiles at industrial facilities maintained at typical temperatures of 20–25 °C. Cyclohexane has better acceptance in the industry than *n*-hexane, reflected by a doubling of the maximum exposure limit of cH compared to nH (12–15). Further details about the exposure limits are found under Safety. This is due to cyclohexane's higher boiling point at 80.7 °C (9), which leads to reduced vapor solvent at room temperature and, thus, improved health and safety environment at practical handling. Even though food legalization aspects slightly favor the use of nH for partial substitution of TB, it is difficult to recommend one solvent over the others on the basis of hazard considerations only.

Comparison of industrial obtained prices shows that TB:cH 80:20 vol % is the most economical choice, whereas TB:nH 80:20 vol % is the most costly mixture. The price difference of 44% between the cheapest and most expensive solvent combination is of considerable commercial importance. However, it was not found to be reliable to exclude any of the binary mixtures without further cost–benefit analysis of other aspects such as space–time requirements and product yield in the different solvent mixtures.

Glycerolysis Reaction in Binary Solvent Mixtures. Continuous glycerolysis reaction efficiency was tested in selected binary solvent mixtures and compared to the efficiency in the pure TP. Results are shown in **Figure 2**. In general, high MAG contents of 45–56 wt % were achieved in all of the tested binary solvents and were in a range close to an expected equilibrium MAG content of approximately 55 wt % at glycerol to oil molar ratios of 5 (5). Of the four different solvent mixtures tested, glycerolysis conducted in 80:20 vol % TB:TP resulted in the significantly highest MAG yield and was similar to the yield achieved in pure TP. Hexane (normal as well as cyclo) is much less polar than TP (17). It is very plausible that a reduced polarity in the two TB:hexane systems has a negative impact on the behavior of the reactant mixture, resulting in the lower MAG formation. This might be due to a coarser and more unstable emulsion of glycerol and oil in the less polar medium and, hence, to diminished substrate access to the active site of the enzyme.

The significant variations in MAG content between the 80:20 and 90:10 vol % ratio of the TB:TP system cannot be explained by polarity variations. TP is only slightly less polar than TB due to one more carbon atom placed in the alcohol chain (17). Anyhow, the TB:TP 80:20 vol % seems to be very promising for an efficient glycerolysis reaction with reaction efficiency similar to the efficiency in the pure TP. Accordingly, on the basis of the melting/boiling profile, costs, and reaction efficiency, the TB:TP 80:20 vol % solvent system is regarded as a competitive alternative to the pure TP and TB system. The TB:TP 80:20 vol % mixture has the advantage of improved physical properties compared to the pure TB system and reduced costs compared to pure TP system.

Effect of Solvent Dosage and Reaction Time in the Column Reactor. To evaluate the impact of the solvent dosage on the reaction efficiency, glycerolysis was conducted in a column reactor using different solvent mixture dosages at two different reaction times. The MAG content obtained at different solvent dosages is given in **Figure 3**. In general, an increase in the MAG content was observed with increased solvent dosage and prolonged reaction time. Hardly any MAGs were formed in the solvent-free system, whereas 9–59% MAGs were formed in the solvent system with 28–61 wt % solvent. The impact of the solvent dosage on the MAG content was much more crucial

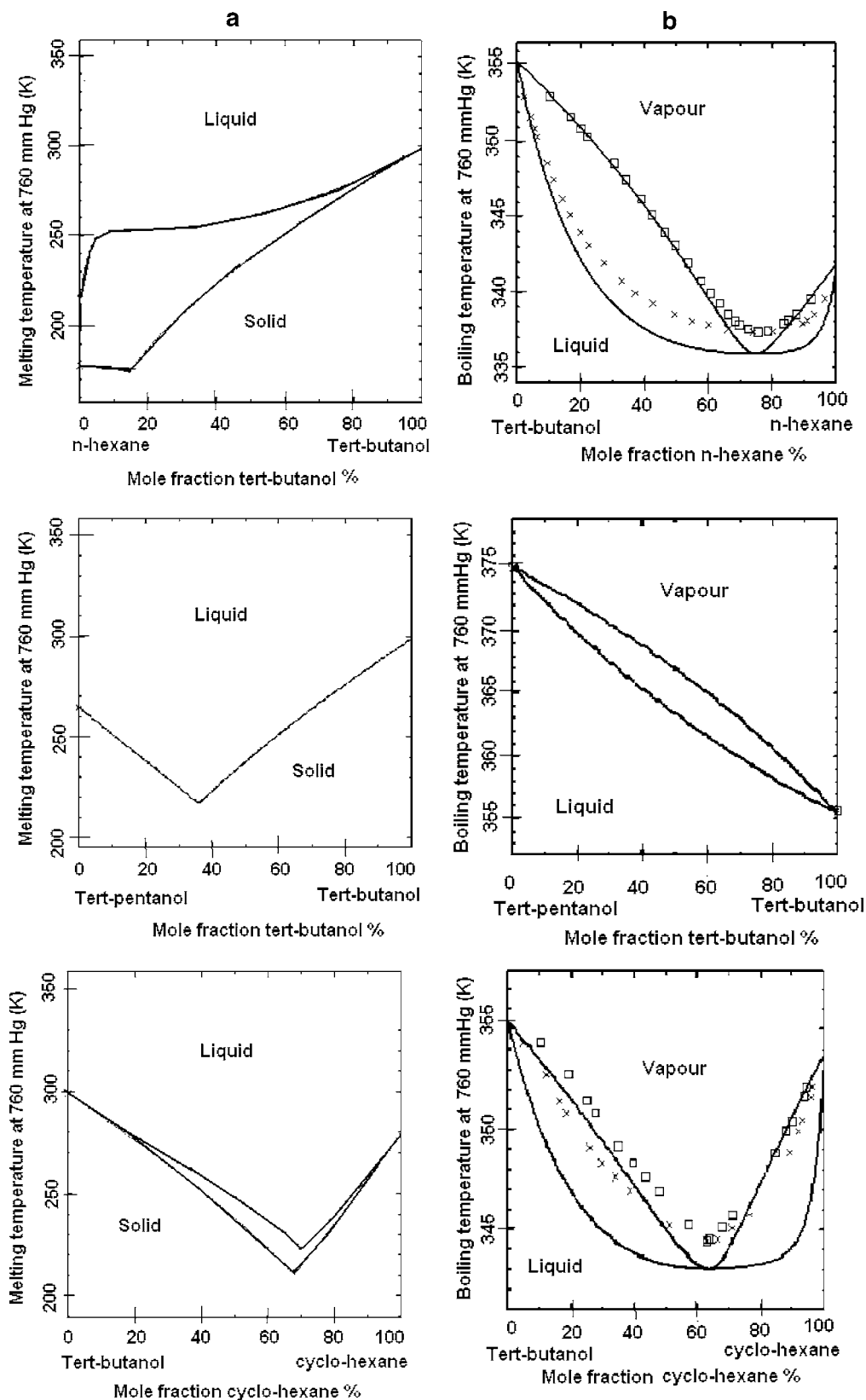


Figure 1. (a) Tx (solid/liquid) phase diagrams for binary mixtures of TB:nH, TP:TB, and TB:cH. The upper curve in the graphs represents a calculated nonideal solution behavior, and the lower curve represents a calculated ideal solution behavior. (b) Txy (vapor/liquid equilibrium) phase diagrams for binary mixtures of TB:nH, TP:TB, and TB:cH. The curves represent calculated phase equilibrium for ideal solution behavior (lower curve) and nonideal solution behavior (upper curve). Discrete points represent known literature values.

in the lower solvent range from 0 to 44% compared to the upper range from 44 to 61%. At a solvent dosage of 28%, only a relatively “low” MAG content of 9–17% was achieved, whereas desirable “high” MAG contents of 46–59% were achieved at

higher solvent dosages from 44 to 61 wt %. This indicates that a solvent dosage above a critical amount reacts as a kind of reaction activator, in this case between 28 and 44 wt % of the reaction mixture.

Table 1. Measured Melting Point for Different Pure and Binary Solvent Mixtures Compared to Predicted and Accessible Literature Values and Price Index for the Different Solvent Combinations Obtained from an Industrial Supplier

solvent 1:solvent 2	solvent ratio v/v	measured by DSC ^a	predicted by UNIFAC method (8)		literature values		price index ^b
		MP (°C)	BP (°C)	MP (°C)	BP (°C)	MP (°C)	
TB	100	21.9 ± 2.2	82	24.9	82.2 (9)	25.5 (9)	107
TP	100	-12.0 ± 0.6	101	-9.1	103 (9)	-9 (9)	235
(1) TB:TP	90-10	15.1 ± 1.4	83	17			120
(2) TB:TP	80-20	8.8 ± 0.9	85	7			132
(3) TB:nH	80-20	10.9 ± 0.6	72	10	73 (10)		144
(4) TB:cH	80-20	7.9 ± 0.5	77	7	75 (11)		100

^a Average value of triple determinations. "Endset" values are used as expression for complete melted sample. ^b Calculated as relative price index between solvent amounts of 5 tons with purity of 99% for TB, *n*-hexane, and cyclohexane and purity of 98% for TP. Prices were obtained from DANISCO A/S, Brabrand, Denmark (2005). Price index = 100 is set for cheapest combination.

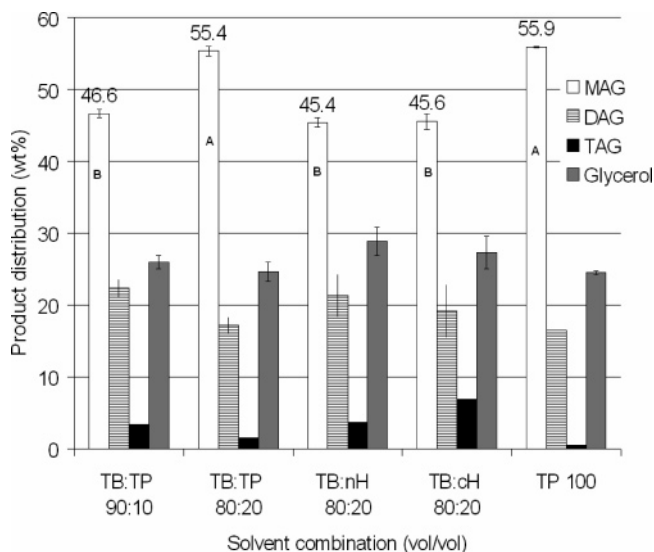


Figure 2. Measured product distribution (weight percent) after continuous glycerolysis in different binary solvent systems. Reaction conditions: glycerol to oil ratio, 5:1 mol/mol; temperature, 40 °C; solvent dosage, 20 mL/10 g of oil; reaction weight time, 31 min; and flow, 0.6 mL/min. Statistically significant different samples are represented by A and B. Error bars represent the standard deviation based on triple determinations.

An increased solvent dosage from 44 to 54% significantly enhanced the MAG content by 9 and 5% for reaction times of 20 and 40 min, respectively. In contrast, no significant effect on the MAG content was achieved with increased solvent dosage from 54 to 61%. A significant effect of the reaction time was obtained independent of the solvent dosage. A prolonged reaction time from 20 to 40 min enhanced the MAG content by 2–8% depending on the solvent dosage from 28 to 61%.

To identify the most optimal reaction time and solvent dosage, the MAG content per space–time was calculated, shown in **Table 2**. From **Table 2** it can be seen that a reaction time of 20 min and a solvent dosage of 44 or 54% yielded similar and highest possible space–time MAG contents. Prolonged reaction time and increased solvent dosage just lowers the MAG content per space–time, making these conditions less attractive. A solvent dosage of 54 wt % is believed to be most attractive to obtain the highest possible MAG content in the product mixture, whereas 44% solvent has the benefit of lower solvent consumption. Subsequently, purification is required for both reaction systems to obtain high-purity MAG products. Hence, whether varied MAG content and solvent dosages influence the purification process differently should be considered to identify the most attractive setup. Anyhow, a required solvent amount of 44–54 wt % is regarded as a relatively high dosage, which emphasizes

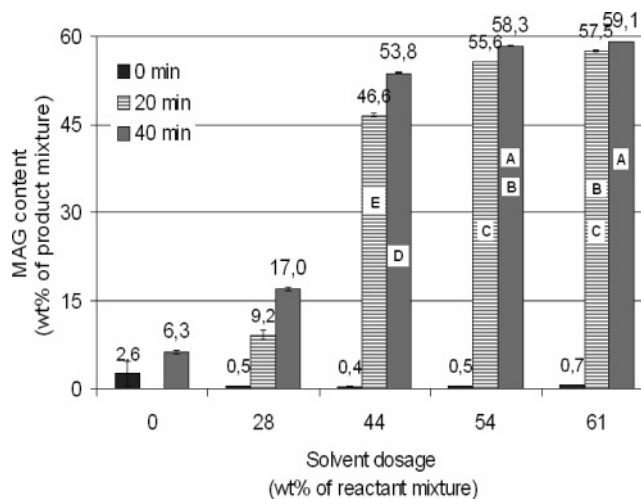


Figure 3. MAG content after continuous glycerolysis in varied dosages of a binary mixture of TB:TP 80:20 (volume percent) at reaction weight times of 20 and 40 min. The solvent-free system was added 5 wt % DimodanHR (high-purity MAG). Reaction conditions: glycerol to oil ratio, 3:1 mol/mol (apart from solvent-free system with molar ratio of 5); temperature, 40 °C; and flow, 0.47 or 0.93 mL/min. A–E indicate statistically significant different samples. Error bars represent the standard deviation based on double determinations.

Table 2. MAG Content per Space Time (Weight Percent/Minute) after Glycerolysis in Varied Dosages of the Binary TB:TP 80:20% Mixture and Different Reaction Times Calculated as Weight Percent MAG Content Withdrawn Solvent Amount and Divided by the Reaction Time (Results Are Based on Double Determinations)

solvent dosage (wt %)	reaction time	
	20 min	40 min
28	0.33	0.31
44	1.30	0.75
54	1.28	0.67
61	1.12	0.58

the importance of recycle considerations to make the system industrially feasible/sustainable.

Phase Split Behavior. Glycerol and oil are immiscible. Polarity differences can help explain this finding: Glycerol has a very polar structure with three hydroxyl groups, indicated by a log *P* value of -1.76. Oil has a very nonpolar structure indicated by high log *P* values of 7.64 and 7.05 for oleic (C18:1) and linoleic acids (C18:2), respectively (17). Pure triolein and pure glycerol have been predicted to be completely soluble in TB at temperatures above 78 °C and in TP above 28 °C (6).

Table 3. Compound Distribution in Upper and Lower Phase of a Reaction Mixture of Glycerol, Oil, and Binary TB:TP 80:20% Mixture at Different Temperatures (Based on Double Determinations)

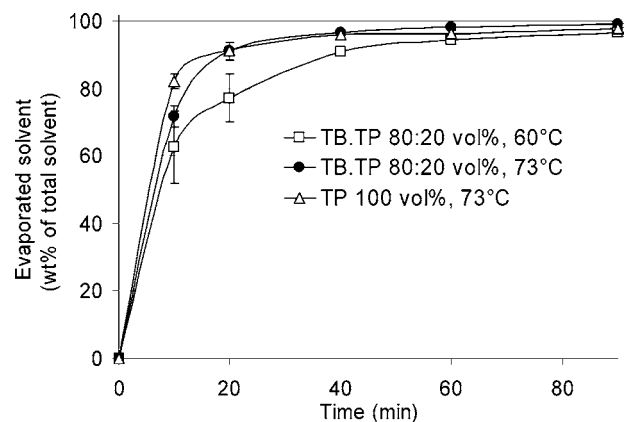
temperature (°C)	compound	weighted out (wt %)	distribution measured		
			upper phase (wt %)	lower phase (wt %)	total (wt %)
40	oil	36.0	34.6	1.7	36.3
	glycerol	13.8	1.4	15.4	16.8
	solvent	50.2	43.4	3.5	46.9
	total	100	79.4	20.6	100
75	oil	36.0	40.5		40.5
	glycerol	13.9	1.2	12.3	13.5
	solvent	50.1	44.6	1.4	46.0
	total	100	86.3	13.7	100

From this prediction, it was assumed that a binary mixture of TB:TP would result in a homogeneous reaction mixture somewhere in the temperature ranges from 40 to 100 °C (6). To verify this assumption, the miscibility behavior at temperatures from 40 to 100 °C was experimentally observed. In the solvent system, a two-phase system, with glycerol- and oil-rich phases, was surprisingly observed regardless of the temperature tested (40, 60, 75, 80, or 100 °C). A phase split occurred immediately after the stirrer was stopped, even though all of the mixtures seemed to be nicely mixed during stirring. Thus, contrary to our expectations, it was not possible to experimentally validate an upper critical solution temperature.

Glycerol and sunflower oil were mixed separately with TB:TP 80:20 vol % to test whether the phase split was caused by immiscibility of either glycerol or oil in the binary solvent mixture. Results showed that glycerol as well as sunflower oil, in pure form, was completely miscible in the binary solvent mixture even at relatively low temperatures of 20 °C. This confirms the predicted ability of the binary solvent mixture to dissolve polar as well as nonpolar compounds.

However, the observed phase split in the reaction mixture suggests a cosolute effect or indicates that the solvent mixture prefers one compound over the other when brought together. Thus, a closer investigation of the solvent and compound distribution in each phase was performed. Results are shown in **Table 3**. Measured values deviated by only <5% from amounts weighted out, which is ascribed to inaccuracies of the method. A clear separation of oil and glycerol in an upper and a lower phase was achieved. In the upper phase, the less dense oil accounted for 96–97 wt %, whereas the denser glycerol accounted for only <4 wt %. In the lower phase, the opposite tendency was obtained with 90–100 wt % of the dense glycerol and <10 wt % of the oil situated. This corresponds to oil:glycerol mole fractions of 72–78:22–28% in the upper phase and oil:glycerol mole fractions in the lower phase of 0–1:99–100%. The solvent distribution with >92% of the solvent situated in the upper oily phase and <3.5% in the lower glycerol phase clearly indicates that the solvent mixture has greater preference for oil compared to glycerol.

This obvious phase split behavior does not suggest particularly improved reactant miscibility in the solvent system compared to the solvent-free system. This suggests that the enhanced reaction efficiency in the solvent system, shown in **Figure 3**, also relates to aspects other than the improved system homogeneity. The activating effect of the solvent is believed to be due to sufficiently changed polarity of the system and reduced viscosity of the reactant mixture. In the presence of an organic

**Figure 4.** Time course for solvent removal during rotation evaporation under vacuum at 60 and 73 °C. Reaction mixture consisted of 10 g of oil mixed with 8.7 g of glycerol (glycerol to oil molar ratio of 8) and 20 mL of solvent of either 100% TP or a TB:TP 80:20% mixture. Error bars represent the standard deviation based on double determinations.

medium a better emulsion of glycerol and oil with greater surface area accessible for the enzyme is most likely formed. It is also plausible that changes in the polarity prevent the adherence of glycerol to the enzyme, thereby avoiding a restricted contact between oil and enzyme. The solvent's capability to access the active site of the enzyme most likely favors the enzyme activity, too (18). Reduced viscosity of the reaction mixture makes the flow and thereby the access/contact to the enzyme easier with reduced mass transfer limitations. However, it should be noted that an inhomogeneous system still has the risk of mass transfer limitations due to inconsistent flow of the reactants. Finally, it should be noted that the phase split behavior was observed in the reaction mixture only. The conversion of TAG and glycerol to more amphiphilic MAG and DAG molecules, which have emulsifying properties, is believed to be among the reasons for a homogeneous product mixture.

Solvent Removal by Rotation Evaporation. The ability to remove a TB:TP mixture and pure TP from a product mixture after glycerolysis was tested by rotation evaporation under vacuum at 60 and 73 °C. The results are given in **Figure 4**. Of the TB:TP 80:20 vol % mixture, 97 wt % was evaporated at 60 °C, whereas 99 wt % was evaporated at 73 °C. In comparison, 98 wt % of the pure TP was removed at 73 °C (**Figure 4**). The condensation processing was successfully conducted with no indication of crystallization in the equipment. Thus, the solvent removal was found to be fairly easy, and implementation of a more careful temperature and pressure control is expected to overcome the problems with the minor leftover in the product mixture.

The possibility of complete solvent removal at more controlled conditions was tested by conducting purification processing at industrial pilot plant facilities at DANISCO A/S. A product mixture obtained from continuous enzymatic glycerolysis reaction in a pure TP system (MAG, DAG, excess glycerol, and TAG and 50 wt % TP) was used for stripping and distillation processing. After this purification process, no solvent was left in the remaining product mixture. This verifies that solvent can be relatively easily removed from the product mixture after glycerolysis by currently used industrial purification techniques.

In conclusion, the TB:TP 80:20 vol % was shown to be a competitive alternative organic medium to pure TB or TP for efficient continuous enzymatic glycerolysis. The binary mixture

ensures high MAG formation in a short time, reasonable solvent price, and easy handling during distillation/condensation processing. A relatively high solvent dosage of minimum 44% was found to be necessary to achieve MAG yields attractive to industrial applications. However, reusability of the solvent and fast continuous operation is believed to overcome some of the problems with lowered space–time product yield. Even though solvents represent extra challenges from a safety point of view, the benefits of the solvents should by far exceed the drawbacks. Enhanced enzyme activity with only a very short required reaction time of 20 min makes the solvent system very attractive to industrial facilities compared to the much slower and more inefficient solvent-free system. Thus, implementation of the enzymatic glycerolysis in a binary mixture of TB:TP 80:20 vol % into industrial plants is believed to be the future production method for selected high-value MAG products for which a gentle technology at ambient temperatures is required.

ABBREVIATIONS USED

BP, boiling point; cH, cyclohexane; DAG, diacylglycerol; MAG, monoacylglycerol; MP, melting point; nH, *n*-hexane; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; TB, *tert*-butanol; TP, *tert*-pentanol.

SAFETY

As described under Results and Discussion highly flammable and harmful risks are related to hexane and TB as well as TP (12–15). According to European standards the maximum allowable exposure limits for the four solvents are *tert*-butanol, 150 mg/m³, 50 ppm; *tert*-pentanol, 360 mg/m³, 100 ppm; cyclohexane, 172 mg/m³, 50 ppm; and *n*-hexane, 90 mg/m³, 25 ppm (12–15). The inhaled vapor solvents can be accumulated in tissues with high lipid content (e.g., nerves, brain, bone marrow, adipose tissue, liver, and kidneys) and cause damage to the cells. Direct solvent–skin contact can lead to dissolving of the natural fatty layer, causing cracking of the skin with enhanced risk of infections or direct inflammations and blistering (13). Thus, special precautionary handling procedures should be taken when working with the solvents or solvent-containing preparations. Good ventilation is required, and gloves should be worn (12, 13).

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