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Acyl Migration Kinetics of Vegetable Oil 1,2-Diacylglycerols

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Abstract The acyl migration kinetics of long-chain 1,2-diacylglycerol (1,2-DAG) to form 1,3-diacylglycerol (1,3-DAG) over the temperature range of 25–80 °C were examined using ^{1}H-NMR spectroscopy. Lipase-catalyzed ethanolysis of high-oleic sunflower oil, followed by a series of solvent extraction steps, generated high purity 1,2-DAG (0.93 mol fraction of the DAG content). The 1,2-DAG mole fraction of 0.32 at equilibrium was found to be insensitive to temperature, indicating that long-chain acyl group migration is neither endothermic nor exothermic. Determination of the temperature-dependent, first-order reaction kinetic parameters revealed a 1,2-DAG half life ($t_{1/2}$) of 3,425 h and 15.8 h at 25 and 80 °C, respectively. A comparison of 1,2-DAG with 2-monoacylglycerol indicated that there is no difference between the two in the potential energy state ($\Delta G^{\ddagger}$) of their respective transitions states or cyclic intermediates.

Keywords Acyl migration · Structured lipids · 1,2-Diacylglycerol · Alcoholysis · NMR · Lipase

Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

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Introduction

Structured acylglycerols are useful in a broad range of food, cosmeceutical and pharmaceutical applications [1]. Conventional chemical means of synthesizing triacylglycerols with regiospecific placement of acyl groups are well known but can be prohibitively expensive for non-pharmaceutical applications given the need to proceed through a series of protection and deprotection steps [2–5]. Enzymatic production of structured acylglycerols can avoid the protection/deprotection steps, but is still quite challenging because of the need to control so many variables associated with biocatalytic processing on a large scale [6,7]. If the desired product is either a monoacylglycerol (MAG) or diacylglycerol (DAG) of a particular isomeric form, then further complications arise as spontaneous acyl migration can occur.

The phenomenon of acyl migration in glycerols and sugars has been studied since the dawn of stereochemical research [8]. MAG reaches an equilibrium resulting in a 2-MAG:1-MAG ratio of ~1:9, while long-chain DAGs typically display a ~1:2 ratio of 1(3),2-DAG to 1,3-DAG at equilibrium [2,9,10]. MAG and DAG acyl migration rates are affected by temperature and solvent, as well as acid and base, which catalyze the migration [11]. In an effort to draw new water from an old well, we have reexamined aspects of acyl migration in MAGs and DAGs derived from lipase-catalyzed alcoholysis of vegetable oils.

We recently described a ^{1}H-NMR spectroscopy technique to rapidly and accurately determine 2-MAG:1-MAG ratios [10]. Herein, we report the acyl migration kinetics of DAG using this same method. Because of glycerol's prochiral central carbon, DAG has three isomeric forms, *sn*-1,2-DAG, *sn*-1,3-DAG and *sn*-2,3-DAG. These isomers are distinguishable enzymatically and metabolically, and are

separable chromatographically [12]. However, the physicochemical properties of enantiomers *sn*-1,2-DAG and *sn*-2,3-DAG are not expected to be different. Rather than the usual collective for these two isomers (i.e., 1(3),2-DAG), in the present work they are referred to simply as 1,2-DAG.

Materials and Methods

Materials

High oleic sunflower oil (HOSO; 87% oleic fatty acid composition) was obtained from Archer Daniels Midland Company (Decatur, IL). Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on porous silica) was a gift from Novozymes North America (Franklinton, NC). Novozym 435 (immobilized *Candida antarctica* lipase B) was purchased from Novozymes through Brenntag Great Lakes (Chicago, IL). 2-MAG was prepared as described previously [10]. Solvents were reagent grade and purchased from Sigma–Aldrich (St. Louis, MO).

Methods

1,2-DAG Synthesis

HOSO was the starting oil for producing 1,2-DAG. Partial deacylation of HOSO was conducted in a jacketed chromatography column (2.5 × 30 cm) containing 40 g of Lipozyme TL IM. The enzyme bed (20-cm bed height) was maintained at 40 °C. HOSO and 1-propanol were mixed in a 1:1 mol ratio and passed through the enzyme bed at a flow rate of 1.0 ml min^{-1}, achieving a substrate enzyme bed residence time of ~60 min. The column eluate was collected and stored at 4 °C. Liquid carbon dioxide extraction (25 °C, 11.0 MPa) was used to remove fatty acid propyl esters quantitatively from the reaction product [13]. From the raffinate mixture (50 g), the 1,2-DAG fraction was isolated by extraction with three 200-ml portions of methanol. Methanol from the extracts was removed under vacuum at 25 °C. The residue was combined with 200 mL of 95:5 acetonitrile/water (v:v). This solution was extracted with an equal volume of hexane. The hexane phase was reduced in volume under vacuum (house vacuum initially and then 10 Pa overnight) at 25 °C to yield 15 g of a light-yellow oil.

NMR

Proton spectra were obtained on a Bruker Avance 500 spectrometer (500 MHz ^{1}H) using a 5-mm BBI probe. All samples were dissolved in $CDCl_3$, and all spectra were acquired at 27 °C. Chemical shifts are reported as ppm from tetramethylsilane calculated from the lock signal.

1,2-DAG (1.0 ml) samples were sealed in HPLC vials and held at 25, 40, 60, and 80 ± 0.1 °C in a constant temperature silicone oil bath. Aliquots (~20 µl) were taken periodically and dispensed into NMR tubes. The tubes were sealed and stored at −25 °C. The aliquots were dissolved in ~0.75 ml of $CDCl_3$ at room temperature, and the ^{1}H-NMR spectra were obtained within 2 min of dilution. Experiments were performed in triplicate at each temperature.

FFA Analysis

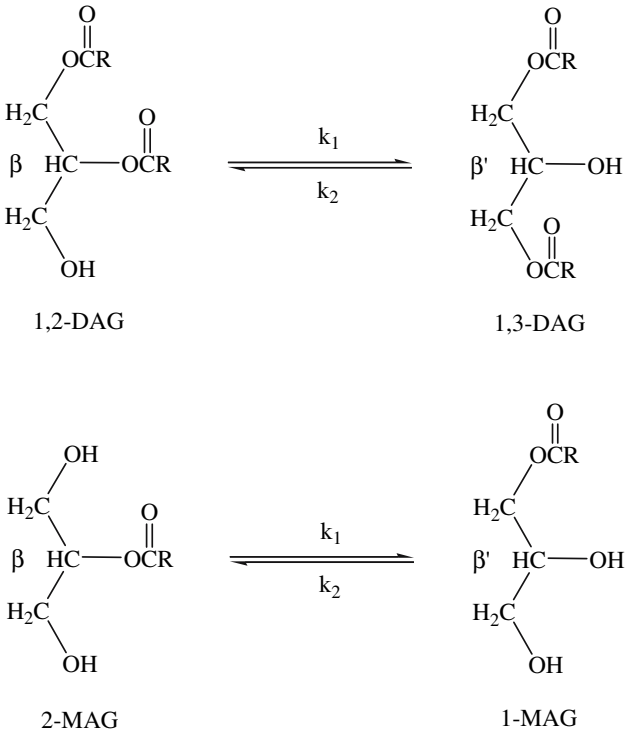
Percent FFA in the 1,2-DAG was determined using a Metrohm Ltd. (Herisau, Switzerland) 751 GPD Titrino, following the AOCS Method Te 2a-64 [14] with ethanol substituted for methanol. The average of duplicate measurements is reported.

Results

1,2-DAG Characterization

The ^{1}H-NMR of the crude propanolysis reaction product consisted of spectra containing multiplets downfield of 5.3 ppm and upfield of 3.8 ppm attributable to the protons of the fatty acid acyl groups. The region of interest, between 3.8 and 5.3 ppm, consisted of the signals attributed to the α- and β-protons of the acylglycerol backbone (see Scheme 1 for structures). The signals attributed to the α- and β-protons of the acyl glycerol species have been previously assigned [10,15–17]. The convoluted α-proton signals of the various acylglycerol species were found between 4.13 and 4.40 and at 3.75 ppm. Included among the α and β glycerol backbone proton signals was the triplet assigned to the α-methylene protons of the propyl group of the fatty acid propyl ester at 4.03 ppm.

Partial deacylation of HOSO using a 1,2-specific lipase followed by solvent fractionation produced high purity 1,2-DAG (Table 1) suitable for acyl migration analysis. The ^{1}H-NMR spectrum (Fig. 1) shows the acylglycerol species present, as well as a small amount of residual fatty acid propyl ester content (5 mol% of glycerides). DAG represented 80.6 mol% of the acylglycerols, 93.0 mol% of which was the 1,2-DAG regioisomer. The FFA content of the isolated material was determined to be 1.46 ± 0.04 w/w%. Low FFA content was desired since acids can catalyze the acyl migration.



Scheme 1 DAG and MAG isomerization (acyl migration)

Table 1 Acylglycerol composition of product isolated from partially deacylated HOSO determined by ^{1}H-NMR

Acylglycerol	Composition (mol%)
TAG	5.7
1,2-DAG	74.9
1,3-DAG	5.7
2-MAG	9.2
1-MAG	4.5

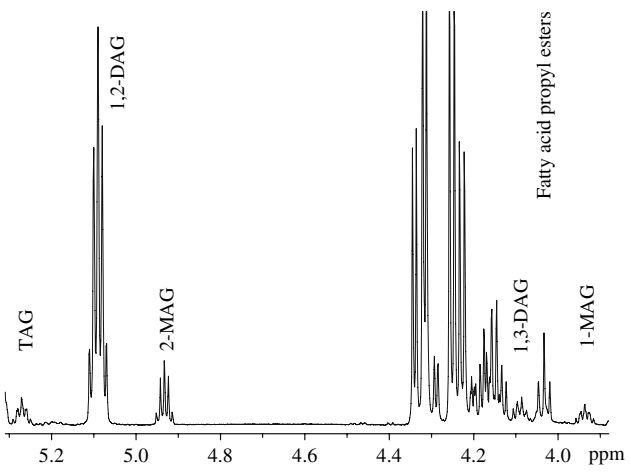


Fig. 1 ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of DAG sample after solvent purification. Labeled acylglycerol peaks are the β-protons of those species

Solventless (NEAT) Acyl Migration Kinetics

^{1}H-NMR was employed to monitor changes over time in acylglycerol distribution (Fig. 2). The β- and β'-protons (Scheme 1) are unique in the fact that each are equimolar to the moles of each acylglycerol species. Therefore, the relative ratio of the integration values of the β- and β'-protons can be used to determine the relative molar ratio of 1,2-DAG to 1,3-DAG. The mole fraction of 1,2-DAG ($X_{1,2\text{-DAG}}$) was calculated using Eq. 1 where H_β is the integration value for the β-proton of 1,2-DAG and $H_{\beta'}$ is the integration value for the β'-proton of 1,3-DAG. The mole percent of 1,2-DAG was obtained by multiplying $X_{1,2\text{-DAG}}$ by 100.

$$X_{1,2\text{-DAG}} = H_\beta/(H_\beta + H_{\beta'}). \quad (1)$$

This protocol has been validated previously with MAG acyl migration [10]. Conversion of 1,2-DAG to 1,3-DAG, depicted in Scheme 1, follows the same acyl migration pathway as 2-MAG conversion to 1-MAG (Scheme 2). Obviously, TAG is not subject to acyl migration and its amount was constant throughout the study.

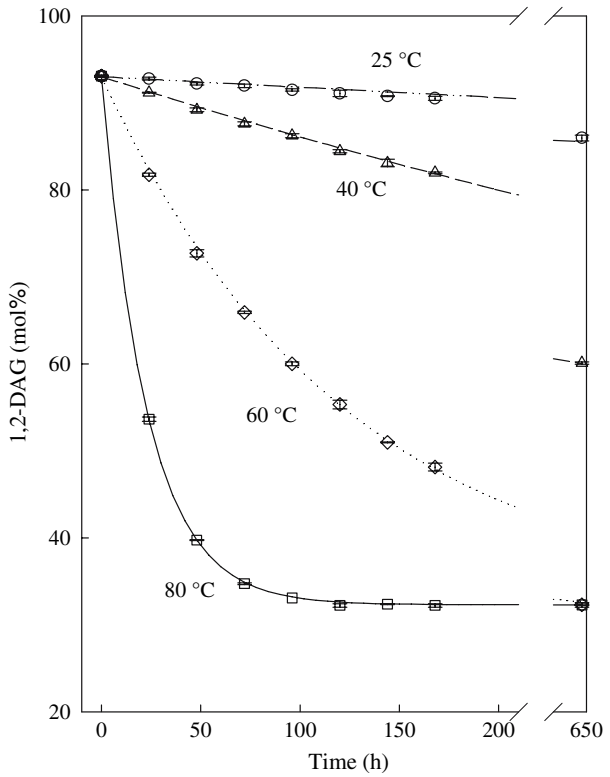


Fig. 2 The influence of temperature on the acyl migration in 1,2-DAG to form 1,3-DAG: 25 °C (*open circles*), 40 °C (*open triangles*), 60 °C (*open diamonds*), 80 °C (*open squares*). Data are the mean of three measurements, *error bars* denote one standard deviation from the mean and lines represent values derived from the kinetic model (Eq. 4) and fitted parameters (Table 2)

The solventless DAG acyl migration rate increased with temperature (Fig. 2), but the equilibrium ratio between 1,2-DAG and 1,3-DAG was negligibly so influenced. After 648 h of treatment at 60 or 80 °C, the equilibrium amount of 1,2-DAG was 32.0 mol% in either sample. As samples at 25 and 40 °C had not reached equilibrium after 648 h, samples equilibrated at 60 and 80 °C were treated for an additional 168 h at 25 and 40 °C. No change in 1,2-DAG mol% was observed. Because reversible acyl migration should lead to equilibrium from either direction, this finding indicated the 1,2-DAG:1,3-DAG equilibrium ratio was constant over the 25–80 °C range.

As previously detailed for MAG acyl migration kinetics [10], DAG acyl migration kinetics were modeled employing a reversible first-order reaction scheme in which [1,2-DAG] and [1,3-DAG] are the concentration of 1,2-DAG and 1,3-DAG, respectively, and k_1 and k_2 are the respective forward and reverse rate constants:

$$[1,2\text{-DAG}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [1,3\text{-DAG}]. \quad (2)$$

The rate law is

$$d[1,2\text{-DAG}]/dt = -k_1[1,2\text{-DAG}] + k_2[1,3\text{-DAG}]. \quad (3)$$

Direct substitution of diacylglycerols species for monoacylglycerols in the prior derivation [10] gave an explicit expression of [1,2-DAG] as a function of time

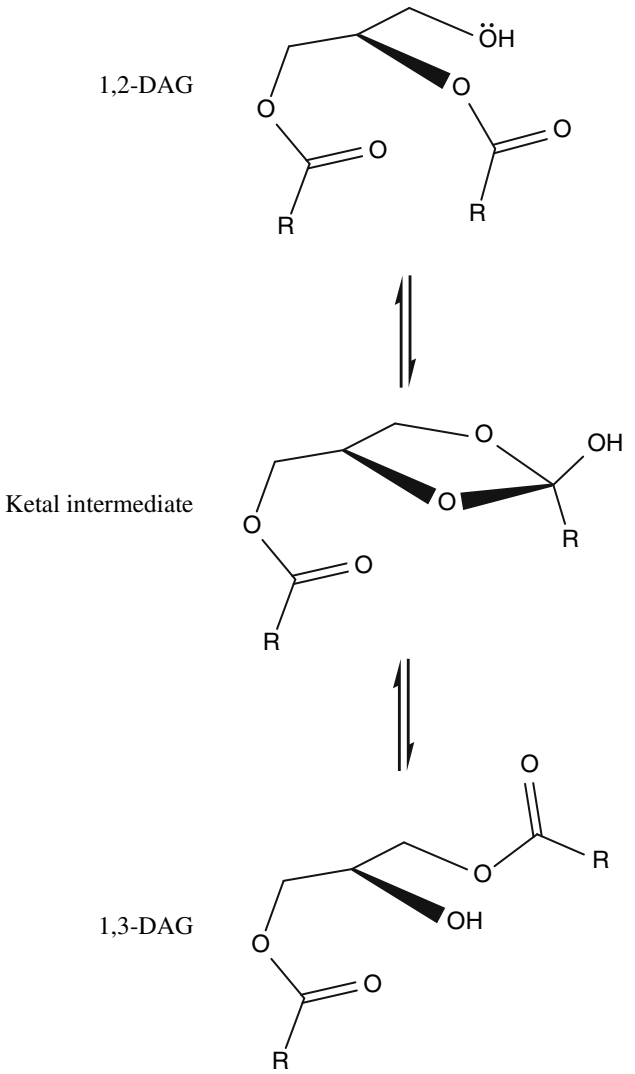
$$[1,2\text{-DAG}]_t = [([1,2\text{-DAG}]_0 - [1,2\text{-DAG}]_e)/\exp(Ck_1t)] + [1,2\text{-DAG}]_e \quad (4)$$

in which $[1,2\text{-DAG}]_0$ and $[1,2\text{-DAG}]_e$ are the initial and equilibrium 1,2-DAG concentrations, respectively. The term Ck_1 is related to the equilibrium constant by the expression

$$k_1 + k_2 = (K+1)k_1/K = Ck_1. \quad (5)$$

Table 2 provides the first-order rate constants and half-life values determined for 1,2-DAG acyl migration based on fitting the data shown in Fig. 2 to Eq. 4. A comparison with 2-MAG acyl migration performed under identical conditions, including a similar amount of free fatty acids (1.23 w/w% in the 2-MAG sample compared to 1.46 w/w% in the DAG sample), indicated that the rates of migration of the 2-position fatty acid moiety are effectively the same as those of 1,2-DAG at a given temperature (Table 2).

The acyl migration data presented were based on material prepared by enzymatic treatment of vegetable oil followed by solvent fractionation. There was the expectation that 1,2-DAG derived by conventional organic synthesis would display similar acyl migration rates. This was not the case however with a commercial (Sigma–Aldrich) sample of 1,2-DAG (98 mol% initially), which showed a threefold higher rate of acyl migration (at 60 °C) than the enzymatically derived one described herein (data not shown). The reason for the disparity is unknown.



Scheme 2 Ketal intermediate formation during DAG isomerization. R is an alkyl group

Table 2 Comparison of first-order reaction constants and half-life values for solventless 1,2-DAG and 2-MAG acyl migration

Temperature (°C)	1,2-DAG		2-MAG[a]	
	k_1 (h^{-1})	$t_{1/2}$[b] (h)	k_1 (h^{-1})	$t_{1/2}$[b] (h)
25	0.000184	3,425	0.000180	3,500
40	0.00110	573	0.00141	447
60	0.00737	85.5	0.00752	83.8
80	0.0398	15.8	0.0276	22.8

[a] Values from [10]

[b] $t_{1/2} = (k_1 + k_2)^{-1}\ln(2)$

Influence of Soybean Oil as a Solvent on Acyl Migration

Although the influence of various solvents on acyl migration has been reported, the effect of vegetable oil has not. The rate of 2-MAG acyl migration was found to increase by 27% in the presence of 50% v/v of soybean oil at 60 °C (Fig. 3). The soybean oil had a very low free fatty acid content (0.11 w/w%), so the acceleration of acyl migration was not due to an increased acid catalyst concentration.

Discussion

The proposed mechanism for either 2-MAG or 1,2-DAG acyl migration in the liquid state, as set forth by Fischer [8] and others [5,11,18], involves the formation of a five-member intramolecular ring intermediate initiated by a nucleophilic "attack" of a primary hydroxyl oxygen on the secondary acyl carbonyl group (Scheme 2). Subsequent ring-opening leads to formation of the more thermodynamically stable, unbranched, isomers of MAG and DAG (1-MAG and 1,3-DAG).

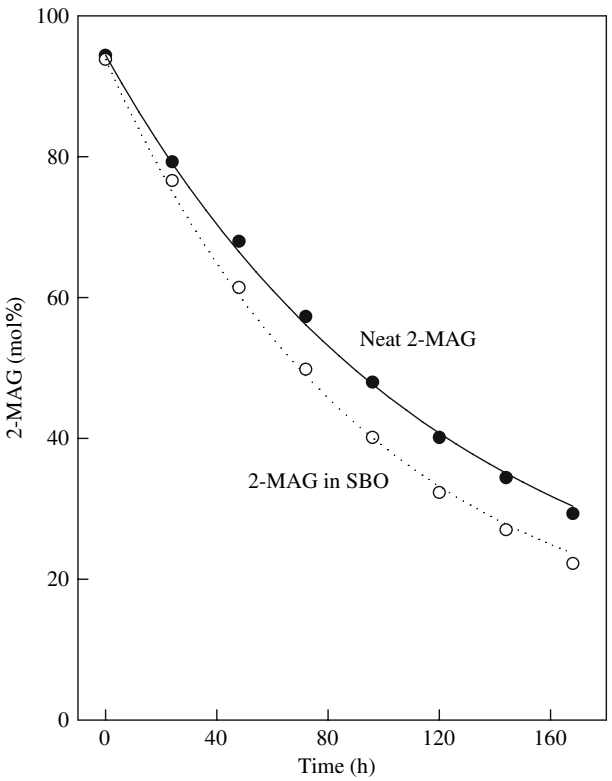


Fig. 3 The influence of SBO on 2-MAG acyl migration at 60 °C: neat (no SBO) (*open rectangles*), or in SBO (*open circles*). Data are the mean of three measurements. Lines represent values derived from the kinetic model (Eq. 4) and fitted parameters (neat $k_1 = 0.00752\ h^{-1}$; in SBO: $k_1 = 0.00954\ h^{-1}$; $K = 9.1$ for both fitted lines)

The length, degree of unsaturation and branching of the acyl (R) group(s) influence the equilibrium distribution of MAGs and DAGs [9,19]. For long-chain DAGs the typical distribution is 30–40% 1,2-DAG isomer [18,20], while the 2-MAG comprises just 9% of an equilibrated sample (i.e., 91% 1-MAG) [10]. Our finding that 1,2-DAG prepared from high-oleic sunflower oil comprised 32% of DAG species in fully thermally equilibrated state is thus consistent with literature reports. Freeman and Morton [20] observed that the equilibrium distribution of dipalmitin changed only minimally with temperature, 31% 1,2-dipalmitin at 80 °C to 38% 1,2-dipalmitin at 300 °C, so the apparently invariant DAG distribution observed herein over the much narrower temperature range (25–85 °C) was unexceptional.

The observed 1,2-DAG to 1,3-DAG ratio at equilibrium was not equal to that expected for a simple statistical distribution of acyl groups (i.e., 2:1 1,2-DAG:1,3-DAG). This clearly indicates that there must be a difference in free energy for the two isomers. The Van't Hoff relationship asserts that there must be a response in the equilibrium constant with a change in temperature (Eq. 6).

$$\mathrm{d}(\ln\ K)/\mathrm{d}T = \Delta H^{\circ}/RT^2. \tag{6}$$

If the enthalpy change (ΔH°) is zero, because K is constant with temperature, then the relationship between the Gibbs free energy (ΔG°) and the equilibrium constant ($\Delta G^{\circ} = \Delta H^{\circ} - T\,\Delta S^{\circ}$ and $\Delta G^{\circ} = -RT\ln(K)$) simplifies to $\Delta S^{\circ} = R\ln(K)$. Acyl migration is neither endothermic nor exothermic. Therefore, acyl migration is entropically driven, indicating that 1,3-DAG and 1-MAG have greater rotational, vibrational and torsional freedom than their isomeric counterparts (1,2-DAG and 2-MAG).

The rate of acyl migration of MAG and DAG is known to be influenced by acids, base, solvent, and chemical nature of the migrating group. Acids and bases facilitate the initial nucleophilic attack of the primary hydroxyl group (Scheme 2) [18]. The solvent influence is well documented, if not completely understood [5,11]. Short-chain fatty acids migrate faster than long-chain fatty acids [9,19]; branched-chain groups move faster than straight-chains [18]. The 1,2-DAG examined herein contained a small amount of free fatty acids (1.46 w/w%) that may act as a catalyst for acyl migration. The small residual amount of triacylglycerol (Table 1) in the material is unlikely to have accelerated DAG acyl migration as the "solvent" influence of triacylglycerol on 2-MAG isomerization was minimal even at a 50% w/w composition.

In the present work it was demonstrated that the rate of acyl migration is approximately the same with 1,2-DAG and 2-MAG in which the acylglycerols are similarly derived from unsaturated, unbranched vegetable oils. The activation energy for 2-MAG isomerization was previously

estimated to be 79 kJ mol^{-1}, calculated from the Arrhenius relationship (ln k_1 vs. $1/RT$) [10]; 1,2-DAG isomerization would therefore have the same value. Serdarevich [18] alludes to the assumption that acyl migration with 1,2-DAG should be slower than with 2-MAG because the presence of two fatty acid groups in DAG results in a larger deformation of the cyclic intermediate, thus raising the transition state energy barrier ($\Delta G^{\ddagger}$). Furthermore, based on simple statistical reasoning, 2-MAG with its two free primary hydroxyl groups should be more reactive than 1,2-DAG with just one free primary hydroxyl group. However, we have not found literature reports that verify this hypothesis and the present findings do not support it.

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