Regioselective Analysis of Triacylglycerols by Lipase Hydrolysis

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ABSTRACT: A modified procedure for the regiospecific analysis of triacylglycerols (TAG) with a 1,3-specific lipase is described. After partial lipase hydrolysis of the triacylglycerol, the released free fatty acids (FFA) and 1,2(2,3)-diacylglycerols (DAG) were isolated by thin-layer chromatography (TLC) and converted to fatty acid methyl esters (FAME). The FAME were analyzed by gas–liquid chromatography (GLC). The 1,3-specific lipases used in this study included supported preparations from strains of *Mucor miehei* and *Rhizopus oryzae*. The method also was applied to the regiospecific analyses of tung nut and Chinese melon seed oil triacyglycerols, both of which contain high proportions of α-elaeostearic acid. The TAG composition of the oils was substantiated in parallel analysis of the oils by high-performance liquid chromatography with chemical ionization mass spectrometric detection of intact TAG.
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KEY WORDS: Diacylglycerols, free fatty acids, GLC, HPLC, hydrolysis, melon seed oil, MS, 1,3-specific lipases, tung oil.

Chinese melon (*Mormordica charantia*) is a tropical crop grown throughout Asia. Its seed oil has a high percentage of its fatty acyl chains as α-elaeostearic acid (ESA), and, therefore, this oil has the potential as an alternative to tung nut oil (1). Because of this commonality of high ESA content in the oils, it was of interest to compare the regiospecific distribution of fatty acyl groups in Chinese melon and tung nut oil triglycerides. Such information would be of value when comparing the physical and chemical properties of the oils. The topic of stereospecific analyses of triacylglycerols (TAG) has received much attention in recent years. One general approach is the partial degradation of TAG with Grignard reagents to produce diacylglycerols (DG) and monoacylglycerols (MG) that are then derivatized with a chiral isocyanate to produce diastereomeric carbamates that are separable by chromatographic methods (2–4). Because acyl migration is quite facile during cleavage caused by the organometallic reagent, the conditions for this procedure are critical. Moreover, reaction readily occurs at the 2-position of the TAG, further complicating the positional analyses of the TAG in question. The Grignard method has been modified and adapted for the regiospecific analyses of specific TAG and fats and oils in general (5,6). Recently, an extremely useful procedure for determining lipase regioselectivity in reactions with homogeneous TAG was reported (7) that also has utility for determining triacyglycerol structures. A second method based on lipase selectivity requires the partial hydrolysis of TAG with highly 1,3-specific lipases, followed by analyses of the partial acylglycerols formed (8). Examples of lipases that are reported as being highly 1,3-specific include those from the genus *Rhizopus* (9,10). Other lipases also generally referred to as 1,3-specific are the lipases from *Mucor miehei* and *Pseudomonas fluorescens*, but recent evidence indicates that the latter lipases should be regarded as being regioselective and not regiospecific (10). Here we report an evaluation of the 1,3-specific lipase approach to the regiospecific analysis of specific TAG and tung nut and Chinese melon oil triacylglycerols. The procedure rests on the partial hydrolysis of TAG in organic solvent, thin-layer chromatographic (TLC) isolation of liberated fatty acids and DG and their analysis by gas–liquid chromatography (GLC). TAG structures, suggested from lipase analyses of the oils, were substantiated further by high-performance liquid chromatography–mass spectrometry (HPLC–MS). TAG molecular species of the oils was estimated by analytical HPLC.

MATERIALS AND METHODS

The tung nut and Chinese melon seed (*M. charantia* L.) oils used in this study were obtained from the Southern Regional Research Center (USDA, ARS, New Orleans, LA) and were isolated as described (1). The TAG 1,3-dipalmitoyl-3-stearoyl-*sn*-glycerol (PSP), 1,3-dioleoyl-2-lauroyl-*sn*-glycerol (OLO), and 1,3-dioleoyl-2-myristoyl-*sn*-glycerol (OMO) were prepared as described by Sonnet and Dudley (8). Fatty acid methyl esters (FAME) were prepared by reacting the oil (20 mg) with 0.5 M sodium methoxide in methanol (2 mL) at 80°C for 1 h, diluting with saturated NaCl solution (2 mL), adding hexane (1 mL), and vortexing. The upper hexane layer was separated and dried over anhydrous Na_2SO_4. FAME and butyl esters were analyzed by GLC on a Hewlett-Packard (Avondale, PA) Model 5895 chromatograph equipped with a split capillary injector, electronic pressure control (EPC), a flame-

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ionization detector, and a Hewlett-Packard Model 3396 integrator. FAME and butyl ester separations were made on an SP-2340 column (0.25 mm i.d. × 60 m × 0.25 μm) from Supelco Inc. (Bellefonte, PA). The carrier gas was He with linear velocity of 22.9 cm sec^{-1} at a 60:1 split ratio. Elution was carried out with temperature programming from 140 to 155°C at 0.5°C min^{-1}; then from 155 to 200°C at 2°C min^{-1}; and hold 20 min. FAME were identified by comparison with standards, obtained from Nu-Chek-Prep (Elysian, MN). Butyl esters required for identification by GLC were prepared by reaction of TAG with sodium butoxide in butanol as described above for FAME. Analytical TLC was conducted on 0.25-mm silica gel 60 plates (5 × 20 cm); preparative TLC was done on 1-mm silica gel 60 plates (20 × 20 cm), both obtained from Analtech (Newark, DE). Plates were developed with hexane/ether/formic acid (80:20:2), air-dried, and developed by either charring with 50% H_2SO_4 or exposure to I_2 vapor.

HPLC was carried out with a Hewlett-Packard Model 1050 solvent delivery module equipped with an autosampler with variable volume injector and a Varex (Burtonville, MD) ELSD II mass detector. The detector output was routed to a Hewlett-Packard Vectra XM2 4/100i data module. Tung and melon seed oil TAG were analyzed by nonaqueous reverse-phase HPLC on a Beckman/Altex (Rainin, Woburn, MA) Ultrasphere ODS 5 μm (4.6 × 25 cm) column. Separations were obtained with acetone (solvent A)/acetonitrile (solvent B) as eluant at a flow rate of 0.8 mL min^{-1}, and the following gradient profile: initial condition 70:30, A/B, hold 5 min; to 100% A over 18 min; hold for 5 min; return to original conditions (11). Total run time was 33 min. Estimation of total TAG carbon number for individual peaks was made by comparison with standard triacylglycerol mix G-1 obtained from Nu-Chek-Prep. HPLC–MS was carried out on a Hewlett-Packard Model 5989 LC-MS with HP 59987A electrospray LC-MS interface and HP 59980B particle beam interface under the HPLC conditions described earlier. Ammonia gas was used for all chemical ionization spectra recorded.

The enzymes used in this study included a commercial immobilized preparation of the 1,3-specific lipase from *M. miehei* (Lipozyme IM) obtained from Novo Nordisk (Franklinton, NC) with an activity of 2613 μmol free fatty acid (FFA) min^{-1} g^{-1}. A second 1,3-specific lipase was from *Rhizopus oryzae* (Lipase FAP) obtained from Amano Enzyme Co. (Troy, VA) and was supported on silica gel as described previously (12), with an activity of 1572 μmol FFA min^{-1} g^{-1}. Activity of the enzyme preparations are expressed as meq of oleic acid released from olive oil per gram of supported lipase. Titrations were conducted with a Radiometer AGU Autoburette (Copenhagen, Denmark), and reaction mixtures were agitated with a Lab-Line shaker bath (Wheeling, IL). Moisture content of the supported enzymes was adjusted by drying the enzyme preparations at 60°C for 4 h in a Perkin-Elmer (Norwalk, CT) infrared drying balance and adding water to a level of 10% by weight of supported enzyme.

Characterization of TAG by enzyme-catalyzed hydrolysis. The equivalent weight of the tung nut and melon seed oils was determined by titrating the FFA, obtained by saponification with alcoholic KOH, with .1 N NaOH to pH 12. The average value (n = 3) was 280 for both oils, and this formed the basis for determination of percent hydrolysis in the enzyme-catalyzed reactions. The following procedure is typical: the specific TAG OMO (about .5 mmol) was carefully weighed into a 5-mL volumetric flask and diluted to the mark with isooctane. Aliquots (2 mL) were placed into two 5-mL tubes, the supported lipase (25 mg) was added, and the mixtures were shaken (170 rpm) at 30°C for 2 h. The samples were filtered to remove catalyst, and the solvent was removed under nitrogen. One sample was taken up in 20 mL of ether/ethanol/water (3:3:2) and titrated to pH 11 with .1 N NaOH to obtain percent conversion (11). The second sample was taken up in hexane (5 mL) and washed with water (2 × 2 mL), dried ($MgSO_4$), and pumped free of solvent to constant weight. The residue was dissolved in hexane, transferred to a 1-mL volumetric flask, and analyzed by TLC: TAG (R_f .73); FFA (R_f .60); 1,3-DG (R_f .30); 1,2(2,3)-DG (R_f .25); 2-MG (R_f .12). The FFA and 1,2(2,3)-diacylglycerols (DAG) were isolated by preparative TLC. FFA and DG were converted to FAME with diazomethane and NaOMe/methanol respectively, and analyzed by GLC.

Characterization of TAG by Grignard hydrolysis. The partial hydrolysis of TAG was done by following the procedure of Becker *et al.* (5) with ethyl magnesium bromide as the Grignard reagent. The resulting mixture of partial acylglycerols were separated by preparative TLC, and the 1(3)-MG were isolated. The MG fraction was converted to FAME with NaOMe/methanol and analyzed by GLC.

Competitive enzymatic esterification of tung nut FFA. Esterification reactions were carried out at 25°C in 10-mL Erlenmeyer flasks by mixing 1 mL of 250 mM tung nut oil FFA (82% α-elaeostearic acid) in hexane, 1 mL of 200 mM oleic acid in hexane with 4 mL of 500 mM 1-butanol in hexane. The supported enzyme (20–60 mg) was added, and the mixture was shaken (200 rpm) for 3 h at 25°C . Reactions were quenched by filtering through Celite to remove the catalyst, followed by the addition of 2 mL of 2 N HCl. The hexane layer was separated, and the aqueous phase was extracted with 2 mL hexane. The extracts were combined, and the solvent was removed under nitrogen. The unreacted FFA were converted to FAME, and the butyl and methyl ester mixture was analyzed by GLC.

RESULTS AND DISCUSSION

The results of the lipase-catalyzed hydrolysis of the symmetrical triacylglycerols PSP, OLO, and OMO with the supported 1,3-selective lipases used in this study are given in Table 1. One lipase, Lipozyme™ IM, was a commercially available immobilized preparation of *M. miehei*, whereas the silica-supported *R. oryzae* lipase was a laboratory preparation (12). Our previous work on supported lipases has shown that the essential character of a given enzyme, as judged by fatty acid selectivity or stereoselectivity, is not impaired by immobiliza-

TABLE 1
Fatty Acid Composition of Free Fatty Acid (FFA) and 1,2,(2,3)-Diacylglycerol (DAG) Fractions from Partial Hydrolysis of Triacylglycerols (TAG) with 1,3-Selective Lipases[a]

TAG[b]	Fatty acid[c]	*M. miehei*[d] FFA	*M. miehei*[d] DG	*R. oryzae*[e] TAG	*R. oryzae*[e] FFA	*R. oryzae*[e] DG	EMB[f] 1,3-DG[g]
PSP	$C_{16:0}$	90	55	66	93	53	—
	$C_{18:0}$	10	45	34	7	47	—
OLO	$C_{18:1}$	95	52	67	96	51	—
	$C_{12:0}$	5	48	33	4	49	—
OMO	$C_{18:1}$	92	52	66	96	51	93
	$C_{14:0}$	8	48	34	4	49	7

[a]Lipase reactions were carried out to 12–15% hydrolysis with supported lipases in isooctane for 2 h; reaction temperature for PSP was 45°C, for OLO and OMO, 30°C. See Materials and Methods section for details.
[b]TAG designation: PSP = 1,3-dipalmitoyl-2-stearoyl-*sn*-glycerol; OLO = 1,3-dioleoyl-2-lauroyl-*sn*-glycerol; OMO = 1,3-dioleoyl-2-myristoyl-*sn*-glycerol.
[c]Fatty acid designations: $C_{16:0}$ = palmitic acid; $C_{18:0}$ = stearic acid; $C_{18:1}$ = oleic acid; $C_{12:0}$ = lauric acid; and $C_{14:0}$ = myristic acid.
[d]Lipozyme IM.
[e]*Rhizopus oryzae* supported on silica gel.
[f]EMB = ethylmagnesium bromide partial deacylation of OMO.
[g]1,3-Diacylglycerol determined as described by Becker *et al.* (Ref. 5).

tion onto this support (13). Both supported enzymes contained sufficient bound water (10%) to reach the degree of hydrolysis (>15%) needed to assess the regioselectivity of the lipases in organic solvent (isooctane was used in this study). For the lipase reactions, the FFA and DAG fractions, after isolation by preparative TLC and conversion to FAME, were analyzed for their distribution of fatty acids. As shown in Table 1, both the FFA and DG distribution of fatty acids were predictive of the position of acyl groups on the glycerol backbone for each TAG studied. In general, the data obtained with the supported *R. oryzae* lipase suggested that it was more 1,3-selective than the *M. miehei* lipase, but these differences were within experimental errors. The apparent lower 1,3-selectivity of both lipases toward PSP, however, was consistently different from that observed for the other TAG, but this may be a temperature effect. Because of the general feature of lower solubility of saturated TAG in isooctane, the PSP reactions were conducted at 45°C, whereas the reactions with the unsaturated TAG were conducted at 30°C. In short, a simple but direct regiospecific analyses of the TAG structures could be made by analysis of the FFA fraction alone. However, if a more rigorous proof of structure is needed, analysis of both the FFA and DG fractions should be done. Also given in Table 1 are data obtained for the regioselective analysis of OMO by the organometallic procedure (5). A comparison of the chemical deacylation and enzymatic hydrolysis data shows that both methods are predictive for TAG structure. In general, we prefer the lipase method because in our hands the reactions are easier to carry out and results are more reproducible.

We then turned our attention to using the above methods for the regiospecific analysis of the TAG of tung and melon seed oils. Briefly, attempts at partial hydrolysis of these oils by the Grignard method proved unsuccessful because we could not detect nor isolate distinct monoglyceride fractions in TLC of the reaction products. Presumably, the Grignard reagent reacted not only with the acyl functionality of the TAG but also with the highly reactive 1,3-conjugated diene function of ESA. On the other hand, partial hydrolysis of Chinese melon seed oil with the *R. oryzae* lipase gave a reaction product from which the FFA fraction was readily isolated by TLC. The fatty acid compositions of Chinese melon and tung nut oils and the FFA fraction isolated from the *R. oryzae* hydrolysis product are given in Table 2. The predominant fatty acid in both oils is ESA. The major difference between the oils is the larger stearic acid (SA) content of Chinese melon seed oil, 24 vs. 3% in tung oil, with the rest of the fatty acids listed being present in similar amounts in both oils. For the FFA fraction, the fatty acids were almost exclusively ESA and SA (>80%) which strongly suggested that, in Chinese melon seed oil, the major TAG structures have either an ESA or SA residue at the 1 and 3 positions. The major fatty acid in the DG fraction was ESA (<85%, data not shown). These data, in conjunction with the total fatty acid profile of the oil, indicated that the predominant TAG in this oil is 1,2-dielaeostearoyl-3-stearoylglycerol (EES), with minor amounts of other EEX TAG, where X is one of the other fatty acids listed in Table 2. The lipase-catalyzed hydrolysis reaction of Chinese melon seed oil proceeded more slowly, as judged by percent conversion (10 vs. 15%), than did the TAG studied in Table 1. Also, the FFA data in Table 2 appeared biased toward ESA and skewed in favor of the other fatty acids listed because of the higher than expected ratio of total other acids to ESA in the FFA fraction. It has been pointed out that the use of lipases in fatty acid positional analyses of TAG needs to be examined carefully because a given lipase may express a selectivity for or against a given fatty acid structure (14). For example, pancreatic lipase is known to hydrolyze short- and medium-chain fatty acids in preference to long-chain fatty acids (15). Other research on the topic of fatty acid substrate selectivities of lipases in regard to chainlength or unsatura-

TABLE 2
Fatty Acid Composition of Tung Nut (TN) and Chinese Melon Seed (CMS) Oils[a] and Free Fatty Acid (FFA) Fraction from *Rhizopus oryzae* Lipase Partial Hydrolysis of Oils[b]

Fatty acid	TN	FFA[c] from TN	CMS	FFA[c] from CMS
Palmitic acid	2.5	8.0	1.6	4.0
Stearic acid	3.0	8.0	24.3	45.6
Oleic acid	6.5	20.5	4.1	7.1
Linoleic acid	5.5	17.3	3.6	7.8
α-Elaeostearic acid	82.5	46.2	65.2	36.2

[a]Weight percent fatty acid composition of tung nut and Chinese melon seed oils determined by gas–liquid chromatography of FAME. See Materials and Methods section for details.
[b]Lipase-catalyzed reaction conducted as described in Materials and Methods section. Percent hydrolysis 8–10%.
[c]Weight percent fatty acid composition of free fatty acid fraction from *R. oryzae* hydrolysis of oils of tung and Chinese melon seed oils. See the Materials and Methods section for details.

tion has been reported recently (16). To our knowledge, the reactivity of ESA with lipases has not been examined; therefore, the relative reactivity of ESA compared to oleic acid was determined. Because a lipase's selectivity toward a given fatty acid is more strongly expressed in the esterification mode than in hydrolysis (13,16), we measured the relative reactivity of ESA and oleic acid in lipase-catalyzed esterification reactions with 1-butanol. Both supported *M. miehei* and *R. oryzae* lipases esterified oleic acid faster than ESA (relative reactivity of oleic acid/ESA, 5:1). For the other fatty acids listed in Table 2, their relative reactivities in lipase-catalyzed esterification reactions are comparable (17). Because of this observed lipase discrimination toward ESA, we opted to confirm the TAG structures suggested from the lipase regiospecific analyses of Chinese melon seed oil by HPLC with MS detection.

Figure 1A shows the molecular species separation of a standard TAG mixture that consisted of a series of saturated TAG of trioctanoin ($C_{24:0}$) through tristearin ($C_{54:0}$) and the unsaturated TAG tripalmitolein ($C_{48:3}$); triolein ($C_{54:3}$), trilinolein ($C_{54:6}$), and trilinolenin ($C_{54:9}$). The nonaqueous reverse-phase HPLC separation of this homologous series of TAG is based on the concept of equivalent carbon number (ECN), where ECN is defined as the sum of the carbon atoms in the fatty acyl chains of a TAG minus two carbon atoms for each carbon double bond in the acyl chains (18). A more detailed discussion on the HPLC separation of complex mixtures of natural fats and oils by ECN, including critical pair separations, can be found elsewhere (19). Suffice it to say that the molecular species separations shown in Figure 1A allowed for detailed analyses of the TAG present in tung and Chinese melon seed oils. The HPLC separations of Chinese melon seed and tung nut oils are shown in Figures 1B and 1C, respectively. A comparison of Figures 1B and 1C shows that both oils contain TAG with similar ECN, albeit in different relative proportions. For example, in tung nut oil, the major TAG peak (Fig. 1C, peak A) elutes at a retention time similar to that of a homologous TAG with an ECN of 36 carbon atoms. This peak would therefore correspond to the TAG trieleostearin, EEE, which is known to be the major TAG in tung oil (20). Similarly, in Chinese melon seed oil, the major TAG peak (Fig. 1B, peak E) eluted at a retention time between trilinolein ($C_{54:6}$) and trimyristin ($C_{42:0}$). The lipase analysis indicated that the major TAG in this oil was EES ($C_{54:6}$). The TAG trilinolein and EES are a critical pair because they have the same calculated ECN; however, there is a difference in ECN because of the *trans* double bonds in ESA. The TAG peaks labeled A to F in Figures 1B and 1C were characterized by HPLC–MS (Table 3). The assignment of TAG structure for the individual peaks was made from an analysis of fragmentation ions in comparison with literature data on the chemical ionization mass spectrometry of TAG (21). The composition (area %) of the separated TAG was deduced from analytical HPLC with mass detection by evaporative light-scattering detector. The chemical ionization (ammonia gas) molecular ions listed in Table 3 correspond to either a tristearin or tripalmitin molecule deficient in the number of hydrogen atoms indicative for the double bonds present in the acylglycerol structure. These HPLC–MS data, when combined with the lipase hydrolysis data of Table 2, en-

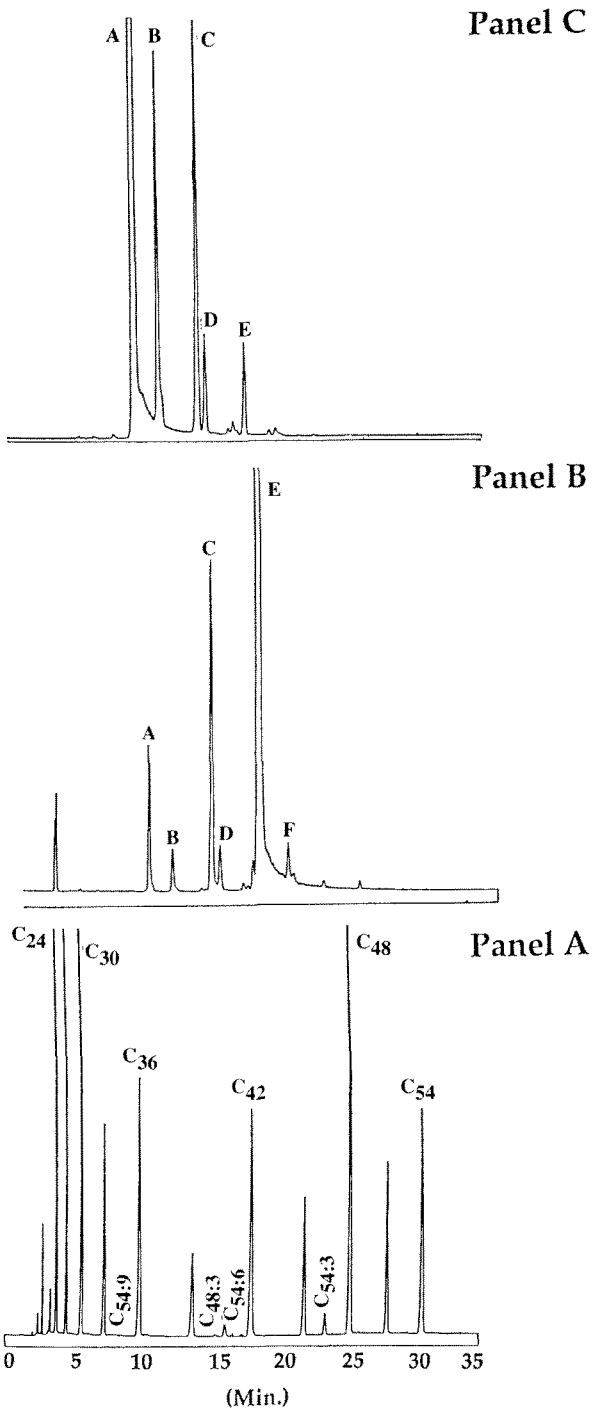

FIG. 1. Nonaqueous reversed-phase high-performance liquid chromatographic separation of triacylglycerols. For conditions see Materials and Methods section. Panel A, triacylglycerol standard containing saturated triacylglycerols from trioctanoin (C_{24}) through tristearin (C_{54}) and the unsaturated triacylglycerols tripalmitolin ($C_{48:3}$); triolein ($C_{54:3}$), trilinolein ($C_{54:6}$), and trilinolenin ($C_{54:9}$). Panel B, Chinese melon seed oil triacylglycerols; and panel C, tung nut oil triacylglycerols. See Table 3 for designation of triacylglycerols marked A through F in Panels B and C.

TABLE 3
High-Performance Liquid Chromatographic–Mass Spectrometric (HPLC–MS) Identification of Tung Nut and Chinese Melon Seed Oils Triacylglycerols (TAG)

HPLC peak[a]	Molecular ion[b]	No. double bonds[c]	TAG[d]	% TN[e]	% CM[e]
A	873	9	EEE	62	5
B	875	8	ELE	10	3
C	877	7	EEO	14	13
D	851[f]	6	EEP	6	4
E	879	6	EES	8	66
F	881	5	ELS	—	5

[a]Peak identification for Chinese melon seed and tung nut oils shown in Figures 1B and 1C, respectively.
[b]Molecular ion [M + 18] obtained from chemical ionization (ammonia gas) mass spectrometry of HPLC separated oils.
[c]Number of double bonds calculated from tristearin or tripalmitin minus the experimental molecular weight of triacylglycerol peak.
[d]Triacylglycerol structure for peak component: E = elaeostearoyl; L = linoleoyl; O = oleoyl; P = palmitoyl; S = stearoyl fatty acyl residue on triacylglycerol backbone.
[e]TN = tung nut oil; CM = Chinese melon seed oil.
[f]Molecular weight based on SSP triacylglycerol.

abled us to assign the TAG structures shown in Table 3 for the TAG peaks separated by HPLC. From this analysis, it was confirmed that the major TAG in tung nut oil was EEE, whereas for Chinese melon seed oil the major TAG were EES and EEO.

From the foregoing, it can be concluded that partial hydrolysis of TAG with a highly 1,3-specific lipase, such as from *R. oryzae*, can be used for the regiospecific analysis of TAG, provided that the fatty acyl residues within the TAG are not highly discriminated against by the lipase used. If the latter is the situation, as found in this study with oils that contained high amounts of elaeostearic acid, a complimentary procedure is needed to aid in the determination of the TAG structure in question. In this study, we were able to delineate the TAG composition of the tung nut and Chinese melon seed oils by the combined use of partial lipase hydrolysis of the oils with high-performance liquid chromatographic separation and mass spectrometric detection of the TAG.

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