Analysis of 1,2(2,3)- and 1,3-Positional Isomers of Diacylglycerols from Vegetable Oils by Reversed-Phase High-Performance Liquid Chromatography

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Abstract

Separation of 1,2(2,3)- and 1,3-positional isomers of diacylglycerols (DAG) from vegetable oils by reversed-phase high-performance liquid chromatography (RP-HPLC) is investigated. The method is based on isocratic elution using 100% acetonitrile and UV detection at 205 nm. The following elution order of DAG molecular species is identified: 1,3-dilinolein < 1,2-dilinolein < 1,3-dimyristin < 1-oleoyl-3-linoleoyl-glycerol < 1,2-dimyristoyl-*rac*-glycerol < 1(2)-oleoyl-2(3)-linoleoyl-glycerol < 1-linolenoyl-3-stearoyl-glycerol < 1(2)-linolenoyl-2(3)-stearoyl-glycerol < 1,3-diolein < 1-palmitoyl-3-oleoyl-glycerol < 1,2-dioleoyl-*sn*-glycerol < 1(2)-palmitoyl-2(3)-oleoyl-glycerol < 1-linoleoyl-3-stearoyl-glycerol < 1,3-dipalmitin < 1(2)-linoleoyl-2(3)-stearoyl-glycerol < 1-oleoyl-3-stearoyl-glycerol < 1,2-dipalmitoyl-*rac*-glycerol < 1-palmitoyl-3-stearoyl-*sn*-glycerol < 1,3-distearin < 1,2-distearoyl-*rac*-glycerol. Linearity is observed over three orders of magnitude. Limits of detection and quantitation range 0.2–0.7 μg/mL for 1,3-dilinolein to 0.6–1.9 μg/mL for 1,2-dioleoyl-*sn*-glycerol, respectively. Precision and accuracy of the method are also demonstrated. The method is developed to separate mixtures of DAG molecular species produced from edible oils.

Introduction

Diacyglycerols (DAG) or diglycerides are esters of the trihydric alcohol glycerol in which two of the hydroxyl groups are esterified with fatty acids. They can exist in three stereoisomers, namely *sn*-1,2-DAG, *sn*-2,3-DAG, and *sn*-1,3-DAG. DAG are commonly used in different degrees of purity as nonionic emulsifiers in the food, cosmetics, and pharmaceutical industries, as well as their utilization as synthetic intermediates in the chemical industry (1). DAG are also reported to have a large potential use as building blocks for organic synthesis of products such as phospholipids (2) and glycolipids (3). It can also be utilized as a starting material for synthesis of various prodrugs such as 1,3-DAG conjugated chlorambucil for treatment of lymphoma (4,5) and 1,2-DAG conjugated (*S*)-(3,4-dihydroxyphenyl)alanine (L-DOPA) for treatment of Parkinson's disease (6). In the last three years, interest in DAG has increased tremendously in the industry and academia because of the discovery of the novel application of DAG as the main ingredient in edible oils and fats capable of managing and preventing obesity. In this aspect, the applications of DAG are largely governed by its fatty acid composition, which may differ in fatty acyl chain length and degree of unsaturation.

For centuries, adulturation of oils and fats has always been a major problem for the edible oil industry (7). many organizations have published data on physical and chemical characteristics of oils and fats that can be used to assist in detection of adulteration (8). The need to characterize edible oils has caused an increase in the number of high-performance liquid chromatography (HPLC) anayltical methods for lipids. Most of the reversed-phase (RP) HPLC methods were developed for the separation of molecular species of triacylglycerols (TAG) (9–15). However, methods of analysis of DAG molecular species using RP-HPLC were not thoroughly studied. One reason is due to the limited availability of DAG reference standards. Therefore, the identification of DAG molecular species in the corresponding peaks on the chromatogram is one of the most challenging research areas in RP-HPLC of natural samples of acylglycerols.

In RP-HPLC of TAG molecular species, retention was found to be a function of both the total chain length of the fatty acyl moieties and the total number of double bonds. It has been noted that increasing the chain length of the fatty acyl moieties increases the retention time, whereas increasing the degree of unsaturation of the fatty acyl moieties reduces the retention time. This relationship had been utilized in the partition number (PN) concept (16), a general rule of thumb being that $PN = CN - 2 \times U$, where CN is the total number of carbons and U is the total number of double bonds in the fatty acyl chains. Because of its molecular similarities to TAG, the PN concept can also be applied to DAG. However, in the PN concept, elution order of the components having equal PN is not taken

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into consideration. To overcome this disadvantage, the PN concept was extended into the equivalent carbon number (ECN) concept, where differences between molecular species of equal PN were looked into. The ECN values generally define the order of elution of a homologous series of organic compounds such as TAG and DAG from an RP-HPLC column.

The application of the ECN concept to RP-HPLC was first reported (17) as ECN = CN – a' × U, where the value of the constant a' was dependent upon each chromatographic condition. Upon further calculation of the value of a' (18), it was found that the value of a' was equal to c'/b', where c' and b' are coefficients of the linear relationship log $k = q' + b'$CN + c'U, where k is the value of the capacity factor of the chromatographic peaks and q' is a constant. However, a' may take on values approaching |2|; when $a' = 2$ (which occurs in cases when saturated fatty acyl chains are present), the PN and ECN values are the same (19). This relation can be empirically derived by building a mathematical model between the retention times of a set of saturated standards and a weighted sum of the total number of carbon atoms and the number of double bonds in the fatty acyl chains present in the acylglycerol molecules (20). This had resulted in numerous works on the iden-

Table I. PN, ECN, and Retention Times of Diacylglycerol Molecular Species

DAG molecular species	CN	Unsaturation	PN	ECN	Peak number	Retention time (min)
1,3-Dilinolenin	36	6	24	21.62	–	n.d.*
1,2-Dilinolenin	36	6	24	21.62	–	n.d.
1-Linoleoyl-3-linolenoyl-glycerol	36	5	26	23.54	–	n.d.
1-Linoleoyl-2-linolenoyl-glycerol	36	5	26	23.54	–	n.d.
1-Myristoyl-3-linolenoyl-glycerol	32	3	26	24.81	–	n.d.
1-Myristoyl-2-linolenoyl-glycerol	32	3	26	24.81	–	n.d.
1,3-Dilinolein†	36	4	28	25.46	1	13.01
1-Oleoyl-3-linolenoyl-glycerol	36	4	28	25.86	–	n.d.
1-Oleoyl-2-linolenoyl-glycerol	36	4	28	25.86	–	n.d.
1,2-Dilinolein†	36	4	28	25.46	2	13.75
1-Myristoyl-3-linoleoyl-glycerol	32	2	28	26.73	–	n.d.
1-Myristoyl-2-linoleoyl-glycerol	32	2	28	26.73	–	n.d.
1-Palmitoyl-3-linolenoyl-glycerol	34	3	28	26.81	–	n.d.
1-Palmitoyl-2-linolenoyl-glycerol	34	3	28	26.81	–	n.d.
1,3-Dimyristin†	28	0	28	28.00	–	16.92
1-Oleoyl-3-linoleoyl-glycerol	36	3	30	27.78	3	17.28
1,2-Dimyristoyl-rac-glycerol†	28	0	28	28.00	–	18.22
1-Oleoyl-2-linoleoyl-glycerol	36	3	30	27.78	4	18.35
1-Palmitoyl-3-linoleoyl-glycerol	34	2	30	28.73	–	n.d.
1-Palmitoyl-2-linoleoyl-glycerol	34	2	30	28.73	–	n.d.
1-Linolenoyl-3-stearoyl-glycerol	36	3	30	28.81	5	19.38
1-Linolenoyl-2-stearoyl-glycerol	36	3	30	28.81	6	20.03
1-Myristoyl-3-oleoyl-glycerol	32	1	30	29.05	–	n.d.
1-Myristoyl-2-oleoyl-glycerol	32	1	30	29.05	–	n.d.
1-Myristoyl-3-palmitoyl-glycerol	30	0	30	30.00	–	n.d.
1-Myristoyl-2-palmitoyl-glycerol	30	0	30	30.00	–	n.d.
1,3-Diolein†	36	2	32	30.10	7	24.33
1-Palmitoyl-3-oleoyl-glycerol	34	1	32	31.05	8	25.29
1,2-Dioleoyl-*sn*-glycerol†	36	2	32	30.10	9	26.17
1-Palmitoyl-2-oleoyl-glycerol	34	1	32	31.05	10	27.29
1-Linoleoyl-3-stearoyl-glycerol	36	2	32	30.73	11	29.46
1-Myristoyl-3-stearoyl-glycerol	32	0	32	32.00	–	n.d.
1-Myristoyl-2-stearoyl-glycerol	32	0	32	32.00	–	n.d.
1,3-Dipalmitin†	32	0	32	32.00	12	35.18
1-Linoleoyl-2-stearoyl-glycerol	36	2	32	30.73	13	36.28
1-Oleoyl-3-stearoyl-glycerol	36	1	34	33.05	14	37.52
1,2-Dipalmitoyl-*rac*-glycerol†	32	0	32	32.00	15	39.92
1-Oleoyl-2-stearoyl-glycerol	36	1	34	33.05	–	n.d.
1-Palmitoyl-3-stearoyl-*sn*-glycerol	34	0	34	34.00	–	53.12
1-Palmitoyl-2-stearoyl-glycerol	34	0	34	34.00	–	n.d.
1,3-Distearin†	36	0	36	36.00	–	80.92
1,2-Distearoyl-*rac*-glycerol†	36	0	36	36.00	–	86.85

* Not detected.
† Identified by using reference standards.

tification of TAG using the ECN concept (20–24), unfortunately work on ECN values of DAG molecular species was not found. Nevertheless, findings by Podlaha and Töregård (23) on the relationship of TAG and their ECN values were used in the prediction of the elution order of 1,2- and 1,3-positional isomers of DAG discussed in this paper. Podlaha and Töregård (25) had shown that the ECN values of TAG are the sum of their fatty acid ECN values. Their finding was used to calculate the ECN values of DAG molecular species presented here. In addition, enzymatically synthesized DAG from palm, soybean, canola, and corn oils, as well as their binary blends, were also used to predict the peaks of the chromatogram that were not identifiable using available reference standards. These synthesized DAG samples composed of fatty acids are commonly found in edible oils. In this paper, an analytical method for the separation of 1,2- or 2,3- and 1,3-isoforms of DAG using RP-HPLC is described.

Experimental

Materials

Acetonitrile for HPLC, sodium methoxide, and petroleum ether for GLC were purchased from Merck KgaA (Darmstadt, Germany). The following reference standards were obtained from Sigma (St. Louis, MO): 1,3-dimyristin; 1,2-dimyristoyl-*rac*-glycerol; 1,3-dipalmitin; 1,2-dipalmitoyl-*rac*-glycerol; 1-palmitoyl-3-stearoyl-*sn*-glycerol; 1,3-distearin; 1,2-distearoyl-*rac*-glycerol; 1,3-diolein; 1,2-dioleoyl-*sn*-glycerol; 1,3-dilinolein; and 1,2-dilinolein.

RP-HPLC was performed on a Shimadzu LC-10AD liquid chromatograph (Kyoto, Japan) equipped with a Shimadzu SPD-10AV UV detector set at 205 nm, a pump (Shimadzu LC-10AT), an auto-injector (Shimadzu SIL-10ADVP), and a column oven (Shimadzu CTO-10AVP) set at 40ºC. A Merck KgaA (Darmstadt, Germany) LiChrospher 100 RP-18e 5 µm (250 × 4 mm)

Table II. Comparison of the Predicted Elution Order of 1,3-Diacylgycerol Molecular Species with that of Triacylglycerols Based on the Polar Differences of 1-and 3-Position Fatty Acids

Source	Elution order
1,3-DAG molecular species of present work	1,3LnLn < 1,3LLn < 1,3MLn < 1,3LL < 1,3OLn < 1,3ML < 1,3PLn < 1,3MM < 1,3OL < 1,3PL < 1,3SLn < 1,3MO < 1,3MP < 1,3OO < 1,3PO < 1,3SL < 1,3PP ≤ 1,3MS < 1,3SO < 1,3PS < 1,3SS
Bergqvist and Kaufmann (17)	LnLnLn < LLnLn < OLnLn < PLnLn < SLnLn < SLnL LLLn < LLL < OLL < PLL < SLL OOLn < POLn < OOL < POL < SOLn < OOO < SOL < POO < SOO PPLn < PPL < SPLn < SPL < PPO < PPP < SPO < PPS SSLn < SSL < SSO < PSS < SSS
Hierro et al. (23)	OLL < PLL POLn < OOL < POL < OOO < SOL < POO < SOO PPLn < PPL < PPO < PPP < SPO < PPS
Řezanka and Marě (20)	LnLnLn < LLnLn < OLnLn < PLnLn < SLnLn LLLn < LLL < OLLn < MLL < PLLn < OLL < PLL < SLLn < SLL OOLn < MOL < POLn < OOL < POL < MOO < OOO < SOL < POO < SOO MPL < PPL < MPO < MPP < PPO < PPP < PPS PSL < SSLn < SSL < PSO < SSO < PSS < SSS
Podlaha and Töregård (22)	LLL < PLL MMLn < MMP MPL < PPL < MPO < MPP < PPO < PPP < PPS OOL < POL < MOO < OOO < SOL < POO < SOO PSO < SSO < PSS < SSS

* Abbreviations: *1,3-Diacylglycerols*: 1,3LL, 1,3-dilinolein; 1,3LLn, 1-linoleoyl-3-linolenoyl-glycerol; 1,3LnLn, 1,3-dilinolenin; 1,3ML, 1-myristoyl-3-linoleoyl-glycerol; 1,3MLn, 1-myristoyl-3-linolenoyl-glycerol; 1,3MM, 1,3-dimyristin; 1,3MO, 1-myristoyl-3-oleoyl-glycerol; 1,3MP, 1-myristoyl-3-palmitoyl-glycerol; 1,3MS, 1-myristoyl-3-stearoyl-glycerol; 1,3OL, 1-oleoyl-3-linoleoyl-glycerol; 1,3OLn, 1-oleoyl-3-linolenoyl-glycerol; 1,3OO, 1,3-diolein; 1,3PL, 1-palmitoyl-3-linoleoyl-glycerol; 1,3PLn, 1-palmitoyl-3-linolenoyl-glycerol; 1,3PO, 1-palmitoyl-3-oleoyl-glycerol; 1,3PP, 1,3-dipalmitin; 1,3PS, 1-palmitoyl-3-stearoyl-glycerol; 1,3SL, 1-stearoyl-3-linoleoyl-glycerol; 1,3SLn, 1-stearoyl-3-linolenoyl-glycerol; 1,3SO, 1-stearoyl-3-oleoyl-glycerol; 1,3SS, 1,3-distearin.
Triacylglycerols: LLL, trilinolein; LLLn, 1,2-dilinoleoyl-3-linolenoyl-*sn*-glycerol; LLnLn, 1,2-dilinolenoyl-3-linoleoyl-*sn*-glycerol; LnLnLn, trilinolenin; MLL, 1-myristoyl-2,3-dilinoleoyl-*sn*-glycerol; MMLn, 1,2-dimyristoyl-3-linolenoyl-*sn*-glycerol; MMP, 1,2-dimyristoyl-3-palmitoyl-*sn*-glycerol; MOL, 1-myristoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol; MOO, 1-myristoyl-2,3-dioleoyl-*sn*-glycerol; MPL, 1-myristoyl-2-palmitoyl-3-linoleoyl-*rac*-glycerol; MPO, 1-myristoyl-2-palmitoyl-3-oleoyl-*rac*-glycerol; MPP, 1-myristoyl-2,3-dipalmitoyl-*sn*-glycerol; OLL, 1-oleoyl-2,3-dilinoleoyl-*sn*-glycerol; OLLn, 1-oleoyl-2-linoleoyl-3-linolenoyl-*rac*-glycerol; OLnLn, 1-oleoyl-2,3-dilinolenoyl-*sn*-glycerol; OOL, 1,2-dioleoyl-3-linoleoyl-*sn*-glycerol; OOLn, 1,2-dioleoyl-3-*sn*-glycerol; OOO, triolein; PLL, 1-palmitoyl-2,3-dilinoleoyl-*sn*-glycerol; PLLn, 1-palmitoyl-2-linoleoyl-3-linolenoyl-*rac*-glycerol; PLnLn, 1-palmitoyl-2,3-dilinolenoyl-*sn*-glycerol; POL, 1-palmitoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol; POLn, 1-palmitoyl-2-oleoyl-3-linolenoyl-*rac*-glycerol; POO, 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol; PPL, 1,2-dipalmitoyl-3-linoleoyl-*sn*-glycerol; PPLn, 1,2-dipalmitoyl-3-linolenoyl-*sn*-glycerol; PPO, 1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol; PPP, tripalmitin; PPS, 1,2-dipalmitoyl-3-stearoyl-*sn*-glycerol; PSL, 1-palmitoyl-2-stearoyl-3-linoleoyl-*rac*-glycerol; PSO, 1-palmitoyl-2-stearoyl-3-oleoyl-*rac*-glycerol; PSS, 1-palmitoyl-2,3-distearoyl-*sn*-glycerol; SLL, 1-stearoyl-2,3-dilinoleoyl-*sn*-glycerol; SLLn, 1-stearoyl-2-linoleoyl-3-linolenoyl-*rac*-glycerol; SLnL, 1-stearoyl-2-linolenoyl-3-linoleoyl-*rac*-glycerol; SLnLn, 1-stearoyl-2,3-dilinolenoyl-*sn*-glycerol; SOL, 1-stearoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol; SOLn, 1-stearoyl-2-oleoyl-3-linolenoyl-*rac*-glycerol; SOO, 1-stearoyl-2,3-dioleoyl-*sn*-glycerol; SPL, 1-stearoyl-2-palmitoyl-3-linoleoyl-*rac*-glycerol; SPLn, 1-stearoyl-2-palmitoyl-3-linolenoyl-*rac*-glycerol; SSL, 1,2-distearoyl-3-linoleoyl-*sn*-glycerol; SSLn, 1,2-distearoyl-3-linolenoyl-*sn*-glycerol; SSO, 1,2-distearoyl-3-oleoyl-*sn*-glycerol; SSS, tristearin.

analytical C_{18} column was used. A Shimadzu GC-17A gas-liquid equipped with a flame ionization detector and a 70% cyanopropyl polysilphenylene–siloxane-coated capillary column, BPX70 (30-m × 0.32-mm i.d., 0.25-µm film thickness), from SGE International Pty. Ltd. (Victoria, Australia) was used to determine the fatty acid composition of the DAG samples by means of analyzing the methylated fatty acids. The temperature at the injector and detector ports was set at 240ºC, and the carrier gas flow rate was at 50 mL/min. The oven temperature was set to increase at 10°C/min from 120ºC to 160ºC and, thereafter, at 3ºC/min to 240ºC.

Preparation of the standard and sample solutions

Reference standard solutions of DAG were prepared by dissolving each compound in chloroform, resulting in a final concentration of 5% (w/w).

DAG synthesized from palm, soybean, canola, and corn oils and their binary blends were prepared by enzymatic esterification from hydrolyzed fatty acids of the oils. These oils were enzymatically hydrolyzed by reacting a mixture of oil and deionized water 1:1 (w/w) in the presence of 10% (w/v) *Candida rugosa* lipase at 40ºC for 24 h. After hydrolysis, the oily fraction containing free fatty acids (FFA) was separated by centrifugation at 5000 rpm for 5 min at 40ºC. Enzymatic esterification was carried out by reacting the FFA with glycerol (at a 2:1 molar ratio) catalyzed by 10% (w/w) *Rhizomucor miehei* lipase (Lipozyme RM IM) at 40ºC for 8 h. DAG synthesis from binary blends of these oils was carried out at ratios of 1:9, 3:7, 5:5, 7:3, and 9:1. After esterification, the DAG products were centrifuged at 40ºC for 5 min to remove the lipase preparation. RP-HPLC samples of these DAG samples were then prepared in chloroform at a final concentration of 5% (w/w).

RP-HPLC analysis

Ten microlitres of the prepared standard and sample solutions were injected into the chromatograph. An isocratic flow of 100% acetonitrile at 1.1 mL/min was used as the eluent for the separation of 1,2(2,3)- and 1,3-DAG. The retention times of the reference standards and predicted molecular species of DAG are shown in Table I. DAG synthesized from palm, soy-

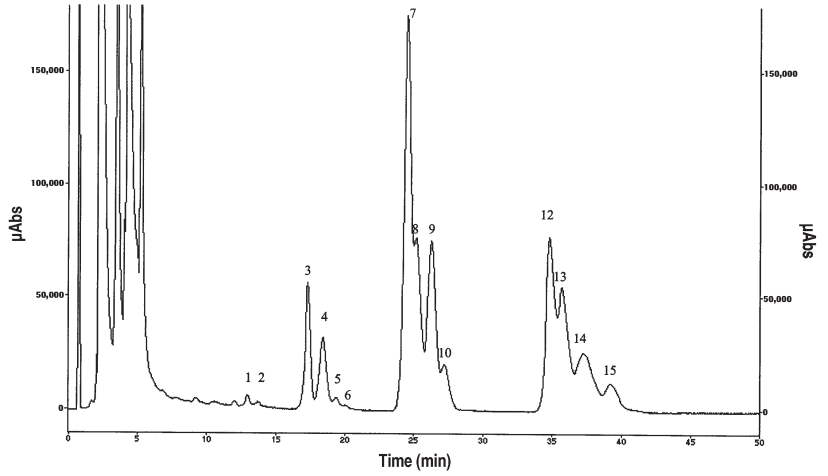

Figure 1. RP-HPLC separation of diacylglycerols produced from palm oil. A C_{18} column and UV detector set at 205 nm were used with an isocratic flow of 100% acetonitrile at a flow rate of 1.1 mL/min. See Table I for peak identification.

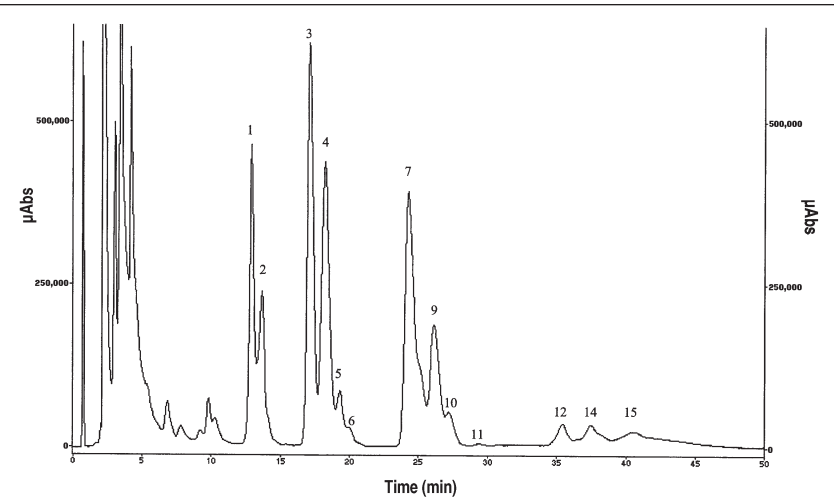

Figure 2. RP-HPLC separation of diacylglycerols produced from soybean oil. See Figure 1 for RP-HPLC conditions and Table I for peak identification.

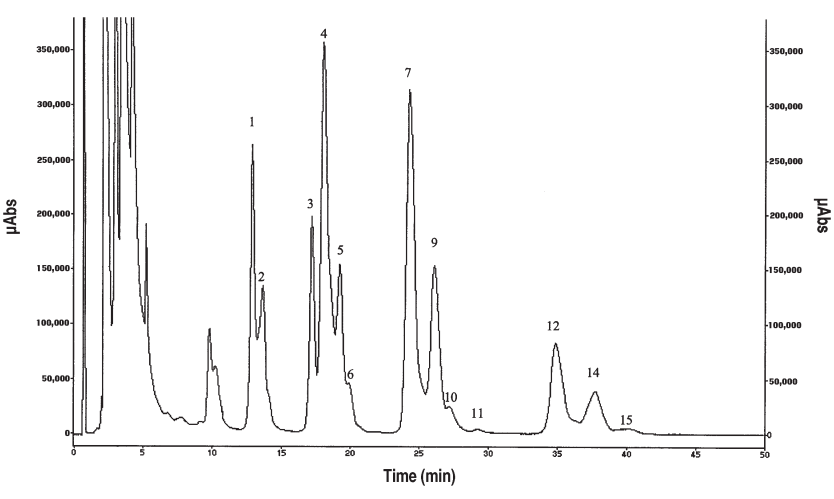

Figure 3. RP-HPLC separation of diacylglycerols produced from canola oil. See Figure 1 for RP-HPLC conditions and Table I for peak identification.

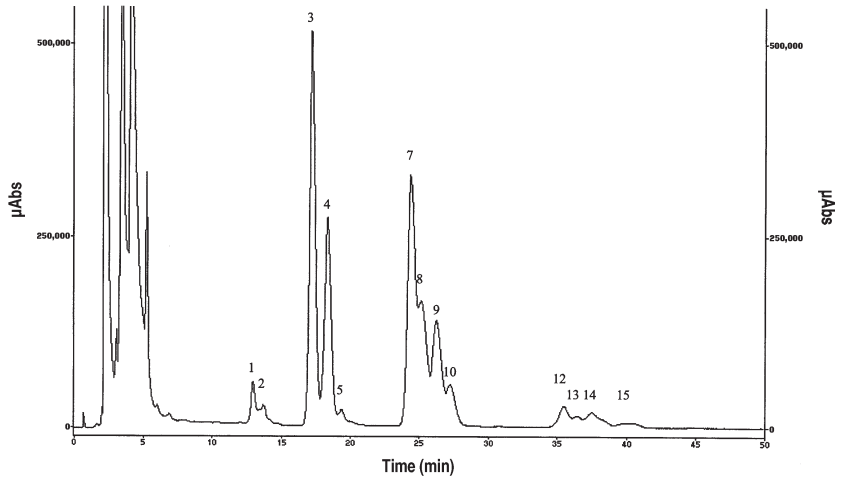

Figure 4. RP-HPLC separation of diacylglycerols produced from corn oil. See Figure 1 for RP-HPLC conditions and Table I for peak identification.

bean, canola, and corn oils were used as representative samples for DAG that may consist, in any combination, of palmitic, stearic, oleic, linoleic, and linolenic acids commonly found in natural edible oils.

These distinct fatty acid profiles of DAG obtained from these oils, together with the observed changes in fatty acid composition and percentage peak areas of the unknown RP-HPLC peaks of the binary blends of these oils as the blend ratio shifts from 1:9 to 9:1, the elution order of DAG molecular species based on the calculated ECN values, and the relative retention time of the unknown peak to a known reference standard peak, the unknown RP-HPLC peaks of DAG molecular species can be identified.

The predicted elution order of DAG

Table III. RP-HPLC Peak Areas of Diacylglycerol Samples

		%Peak area at retention time								
Blend type	Blend ratio %	17.28 ± 0.03 min*	18.35 ± 0.03 min*	19.38 ± 0.04 min*	20.03 ± 0.05 min*	25.29 ± 0.04 min*	27.29 ± 0.05 min*	29.46 ± 0.04 min*	36.28 ± 0.05 min*	37.52 ± 0.05 min*
Palm:Soybean	1:9	22.33	17.84	2.29	1.06	9.45	3.84	0.54	0.06	1.41
	3:7	19.56	15.95	2.14	0.78	14.41	3.19	n.d.†	0.83	1.79
	5:5	15.57	13.45	1.93	0.78	21.88	3.87	n.d.	1.49	1.78
	7:3	11.17	10.39	1.67	0.59	28.63	3.23	n.d.	2.12	2.15
	9:1	5.10	6.03	0.84	0.62	43.15	5.26	n.d.	6.74	3.20
Palm:Canola	1:9	7.38	21.67	6.75	2.17	4.10	1.29	n.d.	n.d.	2.97
	3:7	5.87	19.28	5.48	2.52	14.09	2.86	0.26	1.63	3.91
	5:5	5.13	16.85	4.40	2.14	22.52	3.03	0.32	2.71	4.14
	7:3	4.36	13.86	3.18	1.82	34.11	3.63	n.d.	4.56	0.16
	9:1	3.38	7.14	1.41	0.71	48.34	3.46	n.d.	6.19	0.34
Palm:Corn	1:9	25.86	15.77	0.73	n.d.	10.68	3.78	n.d.	0.87	2.05
	3:7	22.44	13.00	0.65	n.d.	18.73	3.54	n.d.	1.44	1.99
	5:5	17.85	10.42	0.76	0.14	24.08	3.61	n.d.	1.66	1.90
	7:3	13.40	7.81	0.47	n.d.	31.79	3.36	n.d.	1.56	0.07
	9:1	6.37	4.22	0.34	n.d.	41.10	2.61	n.d.	2.01	5.18
Soybean:Canola	1:9	9.25	20.25	6.06	1.57	n.d.	1.44	0.49	n.d.	2.21
	3:7	12.25	18.26	4.65	1.44	n.d.	1.65	0.54	n.d.	3.77
	5:5	15.40	17.35	3.77	1.30	n.d.	2.15	0.83	n.d.	1.61
	7:3	18.49	17.18	3.09	1.18	n.d.	2.43	0.76	n.d.	1.72
	9:1	20.75	16.74	2.34	1.04	n.d.	2.49	0.70	n.d.	1.65
Soybean:Corn	1:9	27.33	17.04	1.19	0.18	7.57	3.33	n.d.	0.16	1.24
	3:7	23.97	16.14	1.38	0.31	7.47	5.47	n.d.	0.51	2.02
	5:5	24.66	17.00	1.58	0.39	7.73	4.48	n.d.	0.06	1.46
	7:3	24.35	17.24	1.87	0.69	7.57	4.49	n.d.	n.d.	1.50
	9:1	23.21	17.05	2.00	0.77	7.13	4.21	n.d.	n.d.	1.62
Canola:Corn	1:9	24.26	15.67	1.34	0.23	n.d.	3.92	n.d.	n.d.	1.12
	3:7	22.27	16.59	2.30	0.76	n.d.	1.93	n.d.	n.d.	1.35
	5:5	17.51	16.74	3.18	0.95	n.d.	1.62	n.d.	n.d.	1.55
	7:3	12.53	16.70	4.06	1.21	n.d.	1.21	n.d.	0.30	2.22
	9:1	9.50	19.83	5.62	1.53	n.d.	1.25	0.81	n.d.	2.17

* Retention time of the unknown peak is calculated based on the average retention times determined from all binary oil blend types.
† Not detected.

molecular species based on their calculated ECN values was then checked for its accuracy by comparing the predicted elution order of the 1,3-DAG molecular species with the reported ECN-based elution orders of TAG (20,23,25,26) having the same type of 1- and 3-position fatty acids as the 1,3-DAG molecular species and the same type of 2-position fatty acid for all the TAG in the series to be compared. In this manner, the effect of the equipolar functional groups at the 2-position in 1,3-DAG (i.e., the hydroxy group) and TAG (i.e., the fatty acyl group) on their respective elution orders can be eliminated, thereby enabling the comparison of elution orders of the 1,3-DAG and TAG to be based only on their polar differences of the 1- and 3-position fatty acyl groups. For example, the elution order of a series of 1,3-DAG molecular species [1-oleoyl-3-linoleoyl glycerol (1,3OL), 1-palmitoyl-3-linoleoyl glycerol (1,3PL), 1-myristoyl-3-oleoyl glycerol (1,3MO), 1,3-diolein (1,3OO), 1-stearoyl-3-linoleoyl glycerol (1,3SL), 1-palmitoyl-3-oleoyl glycerol (1,3PO), and 1-stearoyl-3-oleoyl glycerol (1,3SO)] was compared with that of TAG molecular species [1,2-dioleoyl-3-linoleoyl-*sn*-glycerol (OOL), 1-palmitoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol (POL), 1-myristoyl-2,3-dioleoyl-*sn*-glycerol (MOO), triolein (OOO), 1-stearoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol (SOL), 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol (POO), and 1-stearoyl-2,3-dioleoyl-*sn*-glycerol (SOO)].

GLC analysis

Samples for GLC analysis were melted thoroughly and weighed for the preparation of a 5% (w/v) sample in petroleum ether. Five percent (v/v) of sodium methoxide was added into the solution, vortexed, and left to stand for 5 min. Two-tenths

Table IV. Fatty Acid Composition of Diacylglycerol Samples

Oil source	Blend ratio	Palmitic acid (%)	Stearic acid (%)	Oleic acid (%)	Linoleic acid (%)	Linolenic acid (%)
Palm	–	45.35	3.86	41.31	8.17	n.d.*
Soybean	–	8.20	3.34	31.99	50.60	5.49
Canola	–	4.65	2.46	61.87	23.77	6.55
Corn	–	13.62	2.47	32.35	50.10	0.52
Palm:Soybean	1:9	17.95	4.77	27.92	44.97	3.66
	3:7	23.88	4.78	29.26	37.95	3.04
	5:5	29.70	4.33	33.24	29.55	1.92
	7:3	33.78	3.91	37.83	21.52	1.58
	9:1	42.46	4.26	41.15	9.55	0.84
Palm:Canola	1:9	9.82	2.61	59.16	20.51	6.30
	3:7	18.78	2.75	55.85	17.72	3.74
	5:5	25.42	3.22	51.25	16.25	2.61
	7:3	32.95	4.10	46.85	12.55	1.89
	9:1	40.41	4.93	42.84	9.54	0.60
Palm:Corn	1:9	15.88	2.66	34.30	45.60	0.62
	3:7	25.36	3.26	34.53	35.15	0.46
	5:5	29.09	3.47	36.88	28.78	0.47
	7:3	35.43	3.73	38.00	20.86	0.55
	9:1	41.06	3.86	40.50	12.66	0.33
Soybean:Canola	1:9	4.95	2.54	57.09	26.73	7.29
	3:7	5.68	3.07	53.02	31.87	5.68
	5:5	6.55	3.41	46.06	38.24	5.12
	7:3	7.08	4.00	40.23	43.37	4.64
	9:1	7.54	4.35	33.53	49.71	4.26
Soybean:Corn	1:9	13.15	2.75	32.19	49.98	1.09
	3:7	12.05	3.13	32.20	50.21	1.68
	5:5	10.85	4.02	32.33	50.30	1.83
	7:3	9.75	4.91	32.02	50.25	2.37
	9:1	8.59	4.76	30.85	50.36	4.93
Canola:Corn	1:9	12.55	2.32	36.00	47.25	1.45
	3:7	11.04	2.30	39.61	43.34	3.29
	5:5	9.09	2.23	47.72	37.70	3.07
	7:3	7.25	2.08	54.05	32.35	4.11
	9:1	5.55	2.15	59.81	26.43	5.92

* Not detected.

microliter of the upper organic layer was injected into the GLC system.

Linearity

Linearity was investigated over four orders of magnitude of concentrations in the 0.06–100 μg/mL range. Fifteen concentration levels of standard solutions were prepared from the standard compounds. Three replicated injections were performed at each point. A linear regression was performed on the data obtained.

Limits of detection and quantitation

Limits of detection (LOD) and quantitation (LOQ) for 1,3-dilinolein, 1,2-dilinolein, 1,3-diolein, 1,2-dioleoyl-*sn*-glycerol, 1,3-dipalmitin, and 1,2-dipalmitoyl-*rac*-glycerol were determined. Standard solutions containing 20 μg/mL of standard compound were prepared. Ten injections were performed for each compound. The LOD was calculated as three times the standard deviation of the mean, and the LOQ was calculated as ten times the standard deviation of the mean of the 10 measurements.

Precision

In order to investigate the precision of teh method, samples of DAG were prepared at 100 μg/mL concentration and analyzed for intraday repeatability and interday reproducibility. Results from six consecutive measurements were used to determine intraday repeatability, whereas results from six measurements performed on three different days were used to calculate interday reproducibility. Intraday repeatability and interday reproducibility were calculated in terms of relative standard deviation as percentage of the mean.

Recovery

Evaluation of recovery was performed by analyzing spiked samples of DAG containing known amounts of standard compounds. Standard compounds were added into the DAG sample at a concentration that was approximately equal to that determined from analysis of the DAG sample. Recovery was calculated as follows:

$$\%R = (M_{sc} - M_c \times 100)/M_s \qquad \text{Eq. 1}$$

where $\%R$ is percent recovery, M_{sc} is the raw amount of component analyzed in the spiked sample, M_c is the raw amount of component in the unspiked sample, and M_s is the amount of spiked sample.

Statistical analysis

The Statistical Analysis System (SAS, Cary, NC) was used to perform statistical analyses (27). Data were analyzed by one-way analysis of variance (ANOVA). Significance was determined at $P < 0.05$.

Results and Discussion

Because most natural edible oils contain palmitic, stearic, oleic, linoleic, and linolenic acids at different proportions, DAG oil produced from natural edible oils will be comprised of some or all of these fatty acids. A separation method required for the analysis of 1,2- or 2,3- and 1,3-DAG molecular species synthesized from natural edible oil sources is reported here. The RP-HPLC separation reported here used DAG samples comprising of fatty acid constituents that are commonly found in natural edible oils, unlike previous studies that only reported on the separation of DAG based on limited available reference standards (28,29).

The predicted elution order of 1,3-DAG was found to follow that of the corresponding TAG, which was used in the comparison (Table II). Comparison of the predicted elution order of 1,2-DAG was not done because stereospecific isomers of 1,2-DAG (i.e., *sn*-1,2-DAG and *sn*-2,3-DAG) may coexist in the DAG samples. Although the effect of 1,2-and 2,3-stereospecific isomers of DAG on the retention time has not been previously reported, the relative positions of the functional groups on the glycerol backbone may have minor effects on their respective retention times. It was also shown previously that certain unsaturated fatty acids at the *sn*-2 position of TAG had higher polarity than at the *sn*-1(3) positions (28). Based on these findings, 1,2- and 2,3-stereospecific isomers of DAG may appear to possess different degrees of polarity; for example, 1-palmitoyl-2-oleoyl-*sn*-glycerol may be slightly more polar and, hence, elutes slightly earlier than 2-palmitoyl-3-oleoyl-*sn*-glycerol. This apparent difference in polarity may cause the 1,2- and 2,3-stereospecific isomers of DAG to have different retention times. Unfortunately, such minute differences in polarity of 1,2- and 2,3-DAG were too close to be resolved with current RP-HPLC columns. For this reason, the comparison of predicted elution order of 1,2(2,3)-DAG was not made.

The calculated ECN values and retention times of the DAG molecular species are tabulated in Table I. Eleven molecular species of DAG were identified by matching the corresponding retention times with those of the reference standards. The identities of nine molecular species of DAG (namely, 1-oleoyl-3-linoleoyl-glycerol, 1(2)-oleoyl-2(3)-linoleoyl-glycerol, 1-linolenoyl-3-stearoyl-glycerol, 1(2)-linolenoyl-2(3)-stearoyl-glycerol, 1-palmitoyl-3-oleoyl-glycerol, 1(2)-palmitoyl-2(3)-oleoyl-glycerol, 1-linoleoyl-3-stearoyl-glycerol, 1(2)-linoleoyl-2(3)-stearoyl-glycerol, and 1-oleoyl-3-stearoyl- glycerol) were predicted, and their separation and retention times

Table V. Linear Regression Equations of Calibration Graphs for 1,2- and 1,3-Positional Isomers of DAG (n = 3)

DAG molecular species	Linearity range (μg/mL)	Slope, (b)	y-Intercept (a)	r^2
1,3-Dilinolein	0.1–100	222588	13897	0.9975
1,2-Dilinolein	0.1–100	170477	7965	0.9977
1,3-Diolein	1.0–100	218203	39740	0.9962
1,2-Dioleoyl-*sn*-glycerol	1.3–100	169956	6505	0.9973
1,3-Dipalmitin	0.4–100	137826	1043	0.9985
1,2-Dipalmitoyl-*rac*-glycerol	0.3–100	139242	4122	0.9989

have not been previously reported. Their retention times show good reproducibility. Figures 1–4 show the RP-HPLC chromatograms of DAG molecular species synthesized from palm, soybean, canola, and corn oils. Changes in peak area of the predicted DAG molecular species as the binary oil blend reatio shifts from 1:9 to 9:1 are shown in Table III.

DAG obtained from palm oil consisted predominantly of palmitic acid (Table IV), whereas that from soybean oil contained the highest amount of linoleic acid among the four DAG oils. Oleic acid was the major fatty acid in DAG synthesized from canola oil. Both DAG obtained from soybean and canola oils contained approximately 5–6% of linolenic acid. The fatty acid profile of DAG synthesized from corn oil was similar to that from soybean oil, except that its linolenic acid content was approximately ten times lower.

Changes in composition of palmitic, stearic, oleic, linoleic, and linolenic acids, as the binary blend ratio shifted from 1:9 to 9:1, are shown in Table IV. In order to predict the identity of the unknown peaks of the DAG samples, the relative retention time of the unknown peak to a known reference standard peak (Table I), predicted elution order of DAG based on calculated ECN values (Table II), and changes in RP-HPLC percentage peak area of unknown peaks (Table III) in relation to changes in fatty acid composition in the DAG samples from the binary blends as the blend ratio shifted from 1:9 to 9:1 (Table IV) are taken into consideration. For example, to predict the identity of an unknown peak having a retention time of 17.28 min in the DAG sample synthesized from soybean oil, we know that the unknown peak lies between the reference standards 1,3-dimyristin and 1,2-dimyristoyl-*rac*-glycerol. However, because myristic acid is not detectable in soybean oil, it is very unlikely that the unidentified DAG molecular species, having a relatively large percentage RP-HPLC peak area, will be comprised of any myristoyl group. The next nearest reference standard peaks flanking the unknown peak are 1,2-dilinolein and 1,3-diolein. Based on the predicted elution order of DAG molecular species from calculated ECN values, DAG molecular species that are possible to elute between the 1,2-dilinolein and 1,3-diolein reference standards are 1-palmitoyl-3-linolenoyl-glycerol,1(2)-palmitoyl-2(3)-linolenoyl-glycerol, 1,3-dimyristin, 1,2-dimyristoyl-*rac*-glycerol, 1-oleoyl-3-linoleoyl-glycerol, 1(2)-oleoyl-2(3)-linoleoyl-glycerol, 1-palmitoyl-3-linoleoyl-glycerol, 1(2)-palmitoyl-2(3)-linoleoyl-glycerol, 1-linolenoyl-3-stearoyl-glycerol, 1(2)-linolenoyl-2(3)-stearoyl-glycerol, 1-myristoyl-3-oleoyl-glycerol, 1(2)-myristoyl-2(3)-oleoyl-glycerol, 1-myristoyl-3-palmitoyl-glycerol, and 1(2)-myristoyl-2(3)-palmitoyl-glycerol. Because oleic (31.99%) and linoleic (50.60%) acids are present in relatively large amounts, whereas palmitic

Table VI. LODs and LOQs of 1,2- and 1,3-Positional Isomers of DAG (*n* = 10)

DAG molecular Species	LOD (mg/mL)	LOQ (mg/mL)
1,3-Dilinolein	0.22	0.72
1,2-Dilinolein	0.33	1.10
1,3-Diolein	0.44	1.46
1,2-Dioleoyl-*sn*-glycerol	0.56	1.87
1,3-Dipalmitin	0.35	1.15
1,2-Dipalmitoyl-*rac*-glycerol	0.37	1.22

Table VII. Intraday Repeatability of Quantitative Data for 1,2- and 1,3-Positional Isomers of DAG (*n* = 6)

Diacylglycerol molecular species (%, w/w) in sample	Analysis						Mean	SD	RSD (%)
	1	2	3	4	5	6			
1,3-Dilinolein	6.121	6.213	6.286	6.195	6.251	6.283	6.225	0.063	1.01
1,2-Dilinolein	3.984	4.123	4.053	4.195	4.168	3.897	4.070	0.115	2.82
1,3-Diolein	17.922	17.568	17.925	17.832	17.568	17.699	17.752	0.165	0.93
1,2-Dioleoyl-*sn*-glycerol	12.536	12.411	12.845	12.778	12.356	12.654	12.597	0.197	1.56
1,3-Dipalmitin	2.285	2.258	2.545	2.485	2.388	2.459	2.403	0.114	4.75
1,2-Dipalmitoyl-*rac*-glycerol	3.878	3.916	3.748	3.874	4.125	3.864	3.901	0.124	3.17

* Abbreviations: SD = standard deviation and RSD = relative standard deiviation.

Table VIII. Interday reproducibility of Quantitative Data for 1,2- and 1,3-Positional Isomers of DAG (*n* = 6)

Diacylglycerol molecular species (%, w/w) in sample	Analysis						Mean	SD	RSD (%)
	1	2	3	4	5	6			
1,3-Dilinolein	6.087	6.228	6.164	6.115	6.302	6.231	6.188	0.081	1.30
1,2-Dilinolein	3.912	3.854	3.959	4.068	4.113	4.091	4.000	0.106	2.66
1,3-Diolein	17.912	18.021	17.869	17.648	17.884	17.955	17.882	0.127	0.71
1,2-Dioleoyl-*sn*-glycerol	12.657	12.726	12.345	12.764	12.388	12.648	12.588	0.177	1.41
1,3-Dipalmitin	2.442	2.235	2.344	2.477	2.487	2.235	2.370	0.116	4.90
1,2-Dipalmitoyl-*rac*-glycerol	3.884	3.768	3.899	3.857	3.922	4.132	3.910	0.121	3.09

(8.20%), stearic (3.34%), and linolenic (5.49%) acids are found in relatively lower amounts in soybean oil, it is possible that the unknown DAG molecular species contains both oleoyl and linoleoyl moieties (i.e., 1-oleoyl-3-linoleoyl-glycerol or 1(2)-oleoyl-2(3)-linoleoyl-glycerol). A previous report (28) had shown that DAG with a hydroxy group at the *sn*-2 position elute slightly earlier than their respective *sn*-1(3) isomers. This was also observed in the DAG reference standards, as shown in Table I. With this observation, we know that 1-oleoyl-3-linoleoyl-glycerol will elute earlier than 1(2)-oleoyl-2(3)-linoleoyl-glycerol. Therefore, it is probable that the identity of the unknown peak is 1-oleoyl-3-linoleoyl-glycerol. The same reasoning was used in the prediction of the other unidentified RP-HPLC peaks of DAG samples.

The prediction of elution order of positional isomers of DAG based on the ECN concept cannot be performed because the ECN concept only takes into consideration the number of carbon atoms and the degree of unsaturation in the acylglycerol molecule but not the location and unsaturation of the fatty acyl groups on the acylglycerol molecule. Podlaha and Töregård (25) reported that the position of the unsaturated fatty acid in four isomeric pairs of TAG has no measurable influence on the ECN value.

Linear regression equations of the calibration graphs are tabulated in Table V. Linear response was confirmed in the range from 0.7–100 μg/mL (1,3-dilinolein) to 1.9–100 μg/mL (1,2-dioleoyl-*sn*-glycerol). LOD and LOQ of DAG reference standards were calculated on teh basis of a signal-to-noise ration of 3. Table VI shows the LOD and LOQ for 1,3-dilinolein, 1,2-dilinolein, 1,3-diolein, 1,2-dioleoyl-*sn*-glycerol, 1,3-dipalmitin, and 1,2-dipalmitoyl-*rac*-glycerol. The standard components were able to be detected at concentrations of 0.6 μg/mL and below. The method was most sensitive for 1,3-dilinolein (0.22 μg/mL) and least sensitive for 1,2-dioleoyl-*sn*-glycerol (0.56 μg/mL). The LOQ ranged from 0.72 μg/mL for 1,3-dilinolein to 1.87 μg/mL for 1,2-dioleoyl-*sn*-glycerol.

Precision of the method was checked by intraday repeatability and interday reproducibility studies of a prepared sample of DAG oil. the quantitative data for intraday repeatability are shown in Table VII. Interday reproducibility of the detector response was evaluated by six independent samples of the same DAG oil. Te results are summarizedin Table VIII. The data in Tables VII and VIII show good intrday repeatability and interday reproducibility of the results by this method.

Recovery of spiked standard compounds is shown in Table IX. 1,3-Dilinolein, 1,2-dilinolein, 1,3-diolein, 1,2-dioleoyl-*sn*-glycerol, 1,3-dipalmitin, and 1,2-dipalmitoyl-*rac*-glycerol were observed to be quantitative.

In conclusion, we have developed a RP-HPLC method for the separation of critical 1,2- and 1,3-isomers of DAG present in DAG oils that are produced from natural edible oils. Prediction of identity of unknown DAG molecular species was done based on predicted elution order of DAG based on calculated ECN values, relative retention time of the unknown peak to a known reference standard peak, and changes in RP-HPLC percentage peak area of unknown peaks in relation to changes in fatty acid composition in the DAG samples from the binary blends as the blend ratio shifted. The elution characteristics shown in this paper can be helpful in identifying DAG molecular species, for which DAG reference standards are unavailable.

Table IX. Recovery of 1,2- and 1,3-Positional Isomers of DAG (*n* = 3)

DAG molecular species	Amount spiked (mg)	Average recovery (%)
1,3-Dilinolein	6.1	104
1,2-Dilinolein	4.2	101
1,3-Diolein	17.5	105
1,2-Dioleoyl-*sn*-glycerol	12.8	103
1,3-Dipalmitin	2.5	101
1,2-Dipalmitoyl-*rac*-glycerol	3.8	106

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