Quantitative Determination of Triacylglycerol Profile of Structured Lipid by Capillary Supercritical Fluid Chromatography and High-Temperature Gas Chromatography

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ABSTRACT: Two analytical methods have been developed for the qualitative and quantitative analyses of triacylglycerol profile of structured lipid (SL)-containing medium-chain and long-chain fatty acids. Supercritical fluid chromatography (SFC) was used in the first method. The SL was dissolved in chloroform/methanol, 95:5 (vol/vol), and analyzed directly using a supercritical fluid chromatograph equipped with temperature and density programming capabilities. No derivatization was required for sample preparation. An SB-methyl-100 capillary column (10 m, 100 μ i.d., 0.25 μ film thickness) was used for the separation of the triacylglycerol species and a flame-ionization detector (FID) was used for the detection. Supercritical fluid carbon dioxide was used as the mobile phase. In the second method, the SL was hydrogenated to complete saturation prior to analysis using gas chromatography at high temperatures of up to 375°C. A DB-5HT capillary column (30 m × 0.32 mm i.d., 0.1 μ film thickness) was used for the separation. FID was used for the detection and helium gas was used as mobile phase. The triacylglycerol species were separated and identified based on their equivalent carbon number (ECN), the total carbon number of the acyl side chains. A calibration curve was constructed using a triacylglycerol mixture containing known amounts of monoacyltriacylglycerol standard materials ranging from ECN 18 (trihexanoin) to ECN 66 (tridocosanoin). The novel triacylglycerol species, ECN 32–43, created by the interesterification of medium-chain triacylglycerol (MCT) and long-chain triacylglycerol (LCT) were separated and identified based on their retention times. These triacylglycerols, ECN 32–43, were absent in the physical mixture of MCT and LCT. The unique triacylglycerol species, ECN 32–43, were therefore selected as the fingerprinting region for the qualitative identification of the SL. Quantitation of the novel triacylglycerol species in the SL was achieved by using the integrated peak area of the new species. Both methods were employed successfully to distinguish the physical mixture from the corresponding interesterified SL. Results generated by the two methods were compared and found to be in good agreement.

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KEY WORDS: Fish oil, interesterification, long-chain polyunsaturated fatty acid, long-chain triacylglycerol, medium-chain triacylglycerol, structured lipid, supercritical fluid chromatography, triacylglycerol profiles.

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Medium-chain triacylglycerol (MCT) was introduced in the 1950s for the treatment of lipid absorption disorders. MCT can be rapidly absorbed through the hepatic portal route and is used in nutritional management of infants with low birth weight or adults with compromised fat absorption conditions (1–3). However, patients fed formula and enteral nutritional products containing MCT alone may be deficient in essential fatty acids such as linoleic (18:2n-6), linolenic (18:3n-3), and other long-chain polyunsaturated fatty acids (LCPUFA), such as arachidonic (20:4n-6), eicosapentaenoic (20:5n-3), and docosahexaenoic (22:6n-3) acids. Due to the compromised metabolic condition of patients with various conditions and symptoms due to disease, long-chain triacylglycerols (LCT), such as those present in corn, soy and marine oils, are only partially hydrolyzed by pancreatic lipase, and are digested and absorbed relatively slowly (4). It has been reported that structured lipid (SL) prepared by the interesterification of MCT and LCT is better absorbed in canine and rat models than the corresponding physical mixture of these oils (5,6). The SL also was well tolerated (7) and provided beneficial effects to postsurgical cancer patient (9) animal models with thermal injury and fatty liver of sepsis (10–12). General reviews of the application of SL and MCT in medical and functional food products have been published recently (13,14).

Preparation of the novel SL containing MCT and LCT can be achieved using chemical, enzymatic, and genetic engineering processes. Some of these preparation processes have been published (15–18), and many newly developed procedures are proprietary and have been kept as trade secrets. Analysis of triacylglycerol species has been studied by many. Methods, including applications of high-performance liquid chromatography (HPLC) using refractive index and evaporative light-scattered detectors (ELSD), have been shown to be effective in the analysis of triacylglycerols (19–24). However, if ultraviolet detection is used, the response factors, peak area/unit mass of analytes, may not be easily determined for the quantitation of triacylglycerol species containing unknown degrees of unsaturation. Extensive calibration is required for the quantitative determination of the triacylglycerol profile. Other methods, such as high-temperature gas–liquid chromatography (HTGC) with flame-ionization detector (FID) and hyphenated with mass spectrometry (MS), have

also been reported to be effective in the separation, identification and quantitation of triacylglycerols (25). However, the HTGC method thermally degrades triacylglycerol species that contain polyunsaturated fatty acids. Recovery of the polyunsaturated triacylglycerols is poor, sometimes as low as <50%. The recovery of the polyunsaturated triacylglycerols could be improved if the material is first hydrogenated, and the triacylglycerol species are then analyzed as their corresponding saturated species. Supercritical fluid chromatography (SFC) has been used in our laboratory for the determination of mono- and diacylglycerols with and without derivatization (26). The use of supercritical carbon dioxide as mobile phase allows the use of a relatively low temperature, ≤150°C, to achieve the elution of triacylglycerols of high molecular weight, 300–900 Da, and relatively low volatility. Eliminating the use of a high temperature also prevents the thermal degradation of the polyunsaturated triacylglycerol species during chromatography analysis. This is particularly important in the analysis of fish and fungal oils which contain high levels of LCPUFA. Using supercritical carbon dioxide as a mobile phase in SFC, a temperature of ≤150°C was sufficient to facilitate the elution of triacylglycerols in most of the common edible fats and oils. The detector of the SFC was a FID, similar to that used in HTGC. The FID offers the advantage of quantitation without extensive calibration, especially for triacylglycerol species containing a mixture of saturated and unsaturated fatty acids (27). Separation of triacylglycerols using a micropacked argentation column using supercritical media as mobile phases has also been reported (28). The commercial availability of polar and nonpolar capillary SFC columns greatly facilitates the separation of the triacylglycerols by carbon number as well as by degree of unsaturation. In this study, it has been our goal to establish a routine method for the qualitative and quantitative determination of novel SL derived from edible oils containing medium- and long-chain polyunsaturated fatty acids. Since the fatty acid composition of the SL is identical to that of the corresponding physical mixture, the analysis of triacylglycerol species is a more specific assay to differentiate the SL from the physical mixture of the same oil blend.

EXPERIMENTAL PROCEDURES

Materials and methods. The supercritical fluid chromatograph (SFC Dionex 602 Series; Dionex Inc. Westmont, IL) used in this study was equipped with the capability of density and temperature programming, an autosampler, time-split injector, and a FID. Peak integration, calibration, and data handling were achieved using a Hewlett-Packard ChemStation and G2070AA software (Hewlett-Packard, Wilmington, DE). A SFC column, SB-methyl-100 (Dionex Inc.), 10 m, 100 μ i.d., and 25 μ film thickness, was used for the separation. A frit restrictor, 100 μ (Dionex Inc.), was used to connect the analytical column to the FID. Both temperature and density programs were used to optimize the separation of the triacylglycerols. Initial density was 0.3 g/mL; density ramp, 0.0050 g/mL/min; final density, 0.6000 g/mL; and final hold time was 5 min. Initial oven temperature was 125°C; temperature ramp, 5°C/min; final temperature, 150°C; final hold time, 62 min; and detector temperature, 375°C. The sample preparation was dissolved in chloroform/methanol (95:5, vol/vol). A supercritical carbon dioxide (99.99% purity) cylinder with dip tube and no helium head space was purchased from AGA, Scott Specialty Gas (Wood Dale, IL) or equivalent. Hydrogen and air were generated using hydrogen and air generators (Summit Industries Inc., Dayton, OH, or equivalent). Nitrogen was prepurified grade and free from organic impurities. A gas chromatograph, HP6890 equipped with a FID, gas flow/pressure control sensors, and a split injection port (split ratio, 30:1), was used in the study. A capillary column, DB-5HT (J&W Scientific, 30 m, 0.32 mm i.d., and 0.1 μ film thickness) was used for the separation. The following are operating parameters for the HTGC: initial oven temperature, 100°C; initial time, 0 min; ramp rate, 8°C/min; final temperature, 375°C; final time, 21 min; equilibrium time, 1 min; maximal temperature, 400°C. Total run time was 55.38 min. Split injector temperature was 300°C, with helium as the carrier gas. Detector temperature was set at 390°C. Sample was hydrogenated using PtO_2 as catalyst. Monoacyltriacylglycerol standard materials that contain fatty acids with carbon numbers ranging from 6 to 22 were purchased from Sigma (St. Louis, MO) and Nu-Chek-Prep Inc. (Elysian, MN). All organic solvents were distilled in glass (Burdick and Jackson, or equivalent). All other chemicals used were reagent grade or better.

The sample for the SFC analysis was prepared as follows: Approximately 150 mg of the oil samples was weighed and dissolved in 10 mL of chloroform/methanol, 95:5 (vol/vol). The dissolved sample was injected directly into the SFC, either manually or with an autosampler. No derivatization of the sample was required. Sample preparation for the HTGC analysis was similar to a method previously reported (29,30). Approximately 150 mg of the oil sample was dissolved in reagent (alcohol/hexane, 1:1, vol/vol). Hydrogenation was accomplished by bubbling hydrogen through the solution in the presence of platinum oxide as catalyst. The reaction was completed after 15 min at room temperature. The sample was filtered prior to the HTGC analysis.

Fatty acid compositions were analyzed using the gas chromatography method similar to the procedure described previously (31,32). A HP5890 gas chromatograph was used for the analysis. Fatty acid methyl esters (FAME) were prepared by boron trifluoride (BF_3) in methanol and analyzed by gas chromatography. An Omegawax 320 column (Supelco Inc., Bellefonte, PA) was used for the analysis of FAME.

RESULTS AND DISCUSSION

Separation of triacylglycerol standard mixture by SFC and HTGC. Typical SFC and HTGC chromatograms of a calibration standard mixture are shown in Figure 1A and B, respectively. The nomenclature for the equivalent carbon number (ECN) is defined as the sum of carbon number in the acyl side

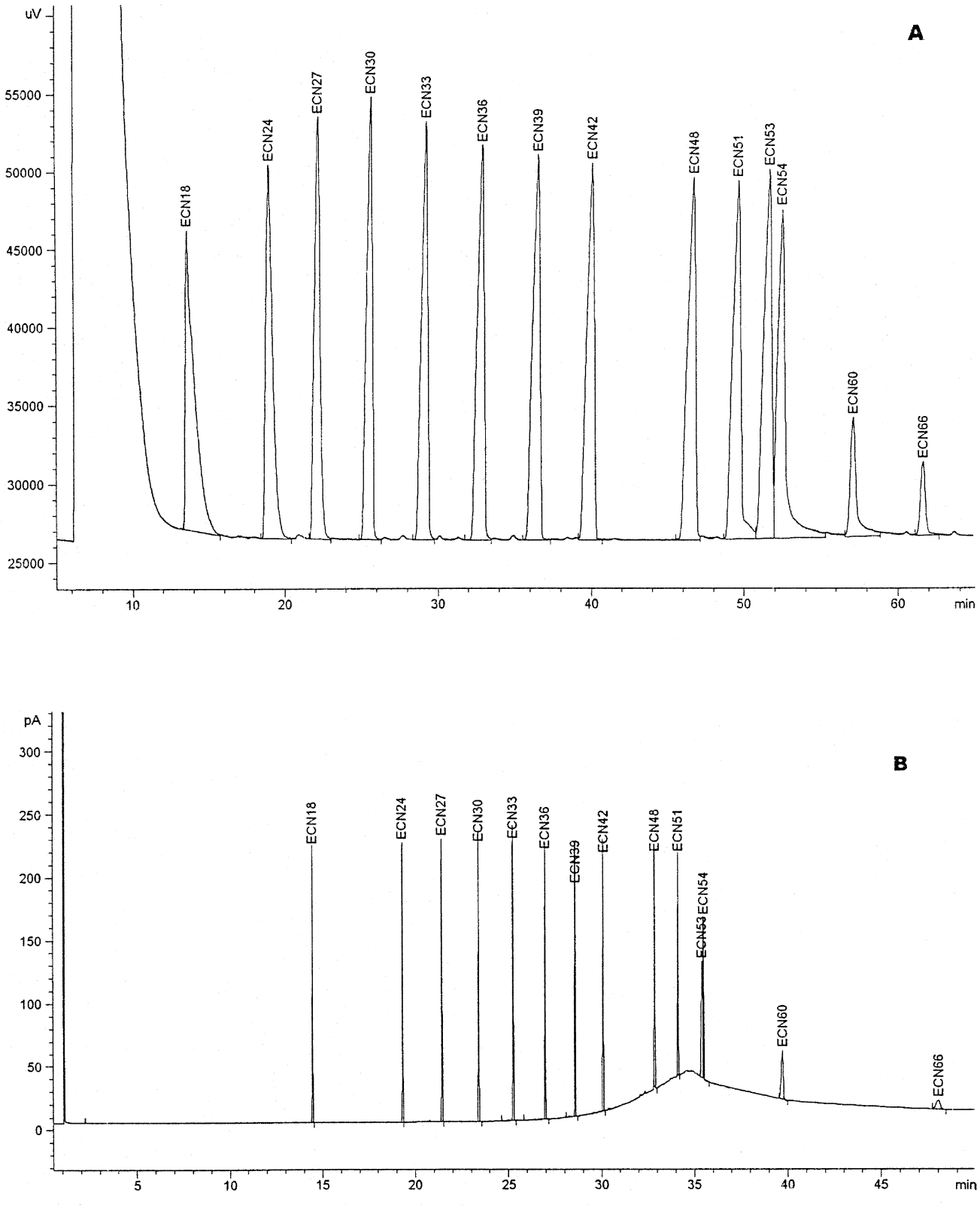

FIG. 1. Typical chromatogram of triacylglycerol standard mixture. (A) Supercritical fluid chromatography (SFC);(B) high-temperature gas chromatography (HTGC). ECN, equivalent carbon number.

chains of the triacylglycerol molecule. For example, trihexanoin is ECN 18, and trioctadecanoin (tristearin) is ECN 54. The triacylglycerol species with three carbon number differences were well resolved by both methods as shown in Figure 1A and 1B. In the SFC analysis, critical pairs (triacylglycerol species with same carbon numbers but different degrees of unsaturation) were partially resolved. Thus, trioctadecadienoin (trilinolein) is eluted immediately before trioctadecanoin and they are identified as ECN 53 and ECN 54, respectively. Nevertheless, for the HTGC method, trioctadecadienoin and trioctadecanoin were poorly resolved. Since all the samples will be hydrogenated prior to the HTGC analysis, the separation of critical pairs was not important in the analysis. The application of different column stationary phases also was investigated. Not suprisingly, the use of more polar columns resulted in better resolution of the unsaturated species (data not shown). However, the overlapping of unsaturated species of lower molecular weight components to the saturated species of higher molecular weight components, such as ECN 54:6, ECN 54:3, and ECN 60:0, makes it more difficult to identify the carbon numbers of the triacylglycerol species of the test samples. Since our goal was to develop a routine analytical method and to establish a characteristic profile for the interesterified products, a nonpolar column, better suited for our application, was selected.

Separation and qualitative identification of novel triacylglycerol species in SL preparations. Two samples, a physical mixture (PM) and a SL derived from a mixture of MCT and fish oil containing LCPUFA, were analyzed using SFC and HTGC procedures. The fatty acid composition of these two samples are practically identical as shown in Table 1. Typical SFC chromatograms of the PM and the SL are displayed in Figure 2A and B. Likewise, typical HTGC chromatograms of the two samples are displayed in Figure 3A and B. The chromatograms of the triacylglycerol profiles unequivocally identified the physical mixture from its SL preparation based on the appearance of the novel triacylglycerol species (ECN 32–43). By using both methods, the triacylglycerol species in the physical mixture and SL were separated and identified according to their retention times. For the triacylglycerol species of unknown fatty acid composition, the ECN assignment is based on the expected retention time calculated from the retention time of two known standards. For example, the retention times for ECN 27 (trinonanoin) and ECN 30 (tridecanoin) were 22.23 and 25.71 min (Fig. 1A), respectively. A linear relationship for the retention time and carbon number is assumed between two known triacylglycerol standards. Thus, the expected retention times for ECN 28 and ECN 29 were calculated to be 23.39 and 24.55 min, respectively. Peaks in the unknown sample eluted at 23.39 and 24.55 min and were identified as ECN 28 and ECN 29, respectively. A 3% window was used for the retention time allowance. MCT contains more than 95% of C8 and C10 fatty acids, thus, ECN 24, ECN 26, ECN 28, and ECN 30 made up almost the entirety of the triacylglycerol profile. ECN 18 and ECN 32 were detected only in trace levels in the physical mixture. The fatty acids in the fish oil are ≥14 carbons, thus all of the triacylglycerols in the fish oil are >ECN 42. The results, shown in Figures 2A and 3A, clearly demonstrate that the triacylglycerol species in the physical mixture are either ECN < 32 or ECN > 42. In the SL preparation, new triacylglycerol species eluted at the retention time of ECN 32–43 were detected as shown in Figures 2B and 3B. The ECN 32–43 represent the novel triacylglycerol species made up of two molecules of medium-chain fatty acids (C6–C10) and one molecule of LCPUFA (C20:4, C20:5, and C22:6) in the SL. The appearance of these new triacylglycerol species, ECN 32–43, provides a unique fingerprinting region for the qualitative identification of SL.

Quantitative determination of novel triacylglycerol species in SL linearity. The new triacylglycerol species were detected by the FID of the SFC and HTGC. The amount of these triacylglycerols was quantitated using the integrated peak area of the peaks eluted at ECN 32–43. The linearity of the quantitation was investigated using a standard mixture containing known amounts of triacylglycerol standard materials, i.e., tridodecanoin (ECN 36) and tritridecanoin (ECN 39). The linearity of the area response for the concentration ranging from

TABLE 1
Fatty Acid Composition of a Physical Mixture and a SL Prepared from a Mixture of MCT and Fish Oil[a]

Fatty acid	Physical mixture (%)	SL (%)
8:0	45.2	43.9
10:0	18.2	18.3
12:0	0.1	0.1
14:0	2.5	2.6
15:0	0.1	0.1
16:0	3.6	3.7
16:1	3.7	3.9
16:2	0.1	0.7
17:0	0.1	0.1
16:3	1	1
16:4	1.8	1.8
18:0	0.4	0.4
18:1	3.8	4
18:2n-6	0.4	0.4
18:3n-6	0.1	0.1
18:3n-3	0.3	0.3
18:4n-3	1.4	1.4
20:0	0	0
20:1	0.2	0.2
20:3	0.1	0.1
20:4n-6	0.4	0.4
20:4n-3	0.3	0.3
20:5n-3	10	10.2
22:0	0	0.1
22:1	0.1	0
21:5	0.4	0.4
22:5	1	1.1
22:6n-3	3.9	4
Total MCT	63.5	62.3
Total fish oil	36.4	37.3

[a]SL, structured lipid; MCT, medium-chain triacylglycerol.

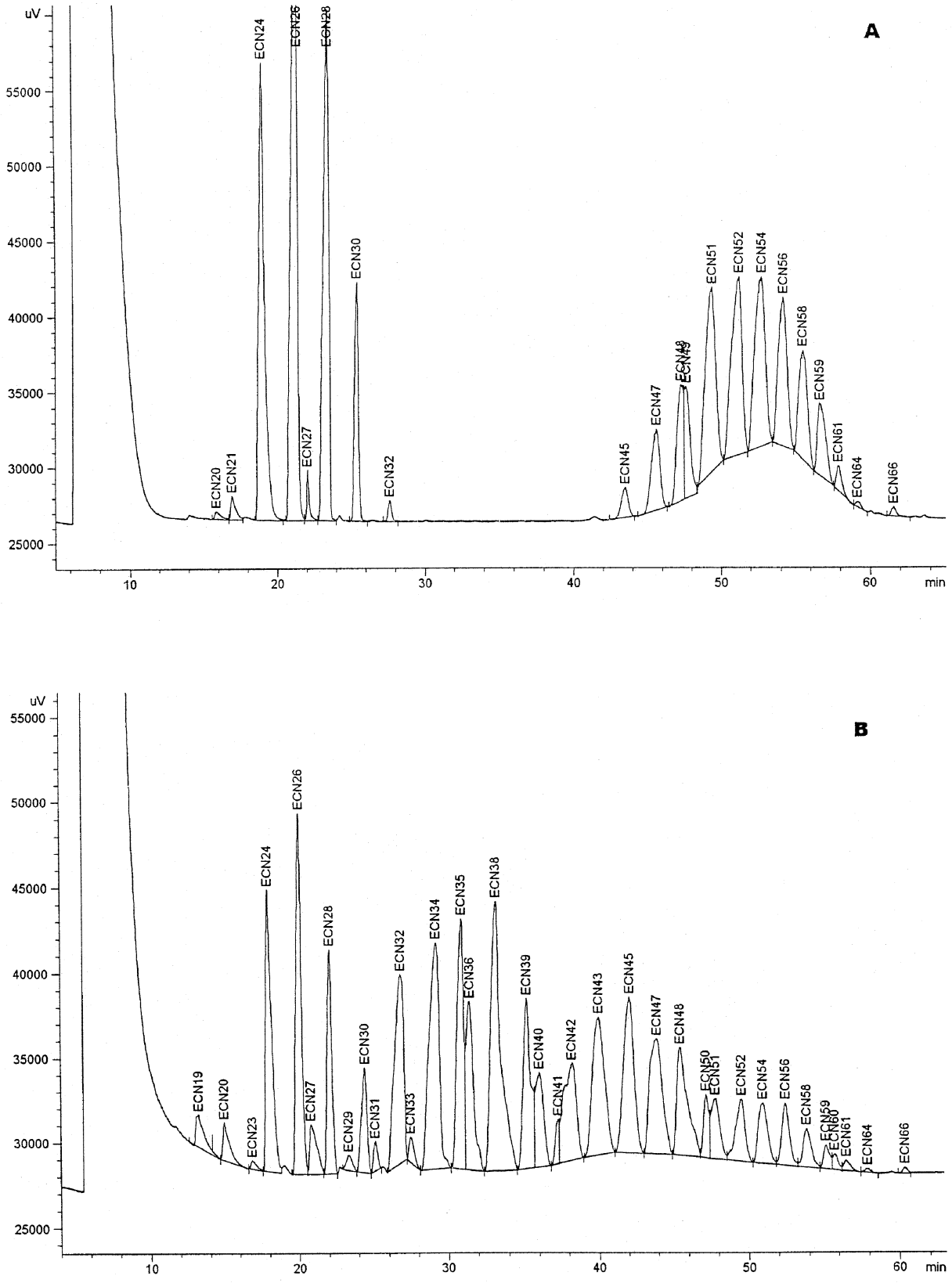

FIG. 2. Typical SFC chromatogram of medium triglyceride (MCT) and fish oil. (A) Physical mixture and (B) structured lipid (SL). See Figure 1 for abbreviations.

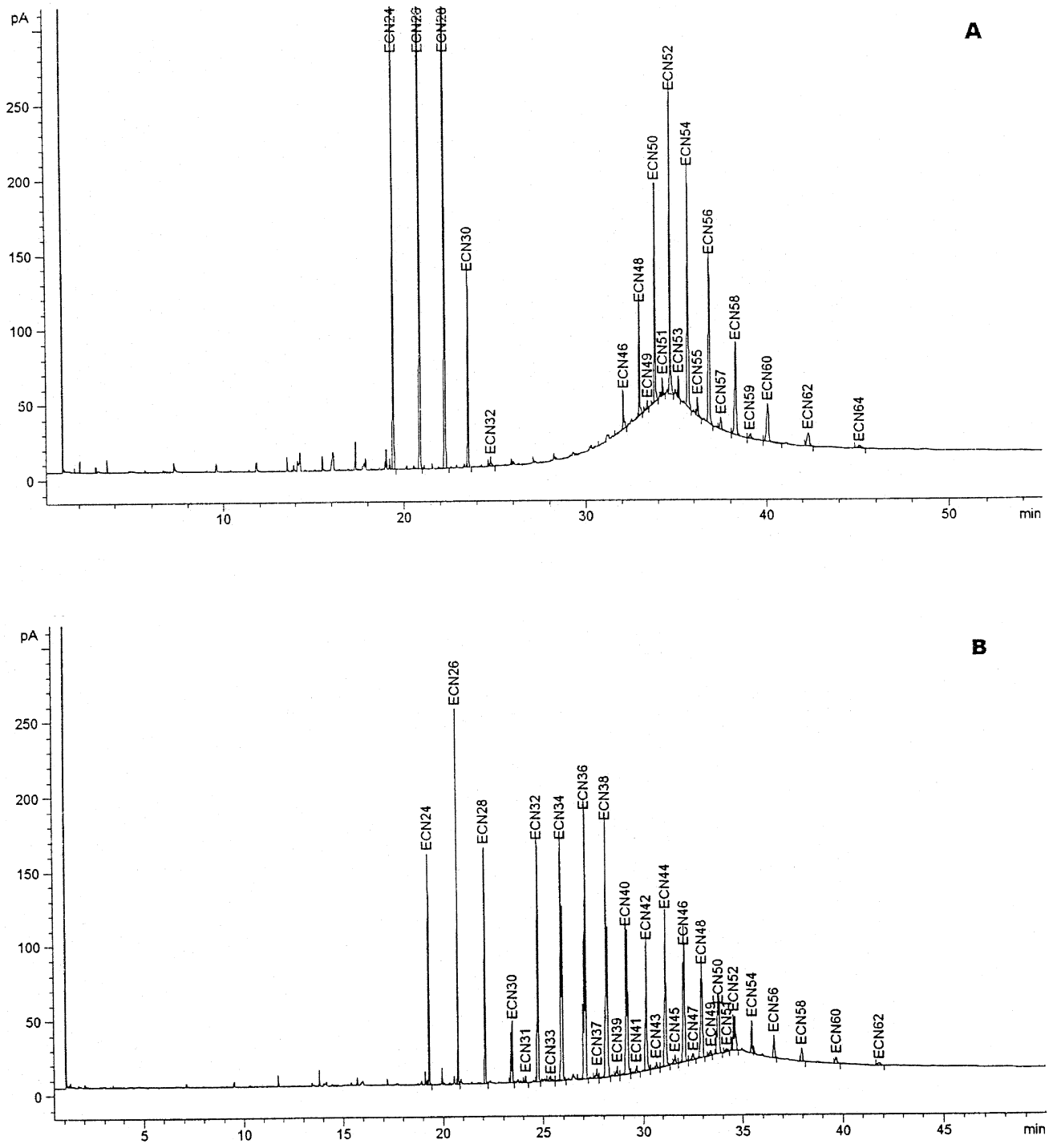

FIG. 3. Typical HTGC chromatogram of MCT and fish oil. (A) Physical mixture and (B) SL. See Figures 1 and 2 for abbreviations.

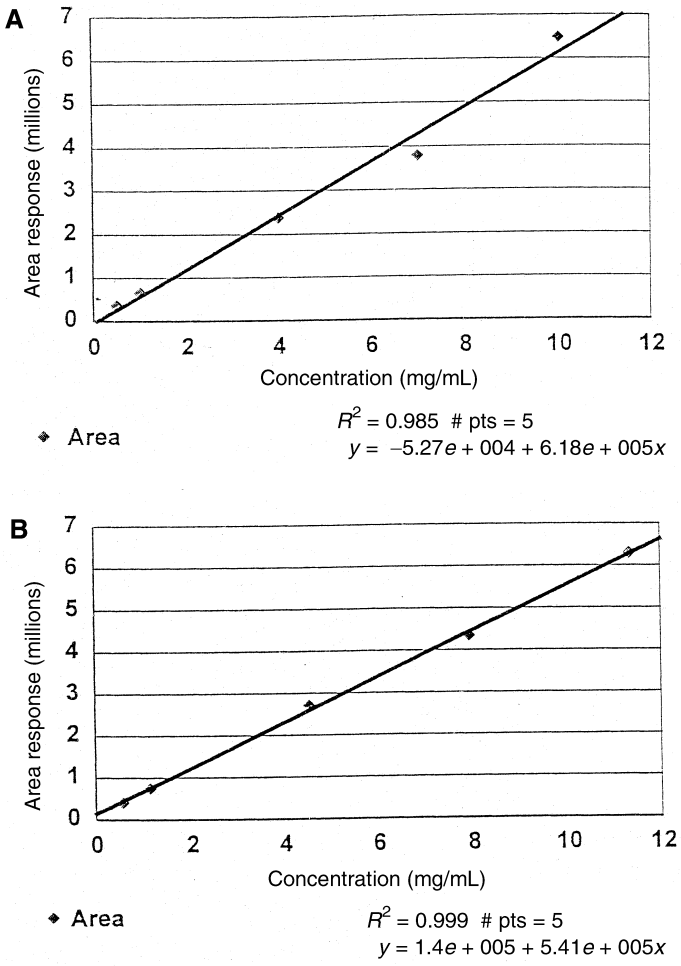

FIG. 4. Linear plot of detector response for SFC; (A) ECN 36 and (B) ECN 39. See Figures 1 and 2 for other abbreviation.

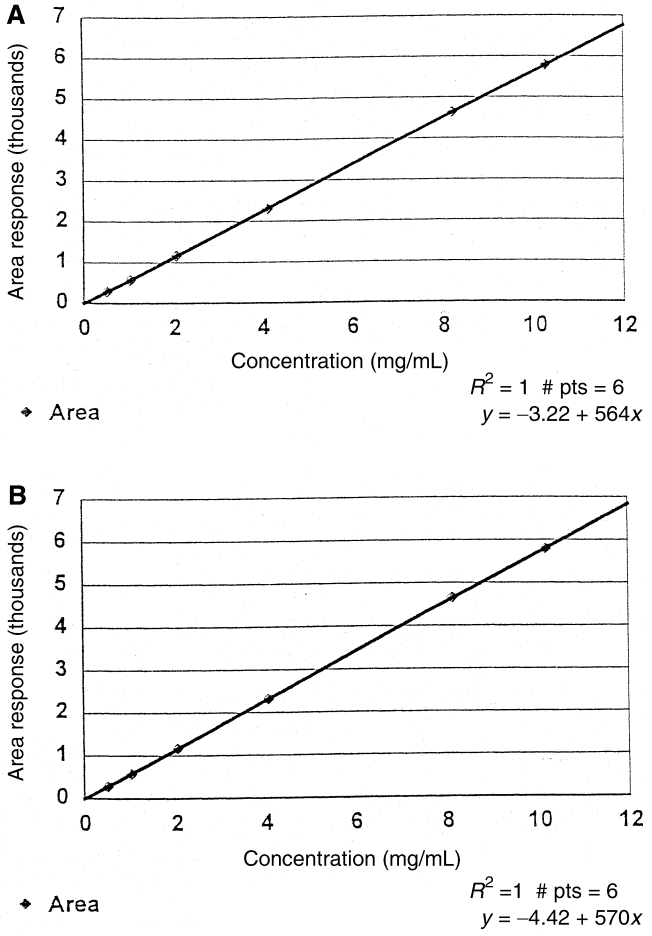

FIG. 5. Linear plot of detector response for HTGC; (A) ECN 36 and (B) ECN 39. See Figure 1 for abbreviations.

0.5 to 10 mg/mL were excellent for both methods as shown by the regression coefficient of ≥0.99. See Figures 4 and 5.

Accuracy. The accuracy of the method was determined using a standard mixture containing known amount of triacylglycerol, trioctanoin (ECN 24), tridecanoin (ECN 30), tridodecanoin (ECN 36), tritridecanoin (ECN 39), trihexadecanoin (ECN 48), and triheptadecanoin (ECN 51). The tritridecanoin (ECN 39) was used as an internal standard, and the area under the peak of each triacylglycerol species was used for the quantitation. The results of the chromatography were compared with the true weights of the standard materials. The agreements were 88–103% and 85–98% for SFC and HTGC, respectively. The results demonstrated that the integrated peak area can be used to adequately quantitate the profile of the triacylglycerol species of SL. The summary is shown in Table 2.

Precision. The relative standard deviations for within-day, between-day, and between-operators in our laboratory were ≤5% throughout the method development and routine applications.

Comparison of the analytical results of SL using SFC and HTGC methods. The triacylglycerol profiles of a SL preparation were analyzed using SFC and HTGC, and the results are compared and summarized in Table 3. It should be mentioned that the triacylglycerol profile as shown in the SFC and HTGC were not identical. The SFC samples contained the natural triacylglycerol species with unsaturated fatty acids, whereas the HTGC sample had been hydrogenated and the triacylglycerol contained only saturated fatty acids. However, by using the summation of the fingerprinting region, ECN 32–43, the quantitation of the novel triacylglycerol species was measured. The agreement between the two methods was excellent.

Both SFC and HTGC are satisfactory for the qualitative and quantitative analyses of interesterified triacylglycerol containing MCT and LCT. The advantage of SFC resides in the simplicity of the sample preparation. However, SFC is not widely available in most analytical laboratories. Alternately, the HTGC method proved to be capable of providing a rugged, accurate, and precise method for the analysis of triacylglycerol profiles. The availability of new heat-stable stationery phases for the gas chromatography (GC) column is a much welcomed improvement to overcome the thermal degradation of GC columns in routine analysis. The quantitation of triacylglycerol profiles for ECN 24–51 is excellent as

TABLE 2
Determination of Accuracy Based on Triacylglycerol Standards by SFC and HTGC Using ECN 39 as IS[a]

Triacylglycerol (ECN)	Actual standard weight (g)	Calculated weight by SFC (g)	Calculated weight by HTGC (g)	Agreement with actual weight by SFC (%)	Agreement with actual weight by HTGC (%)
24	0.2063	0.1974	0.1816	88	88
30	0.2042	0.1831	0.1967	90	96
36	0.2033	0.2033	0.1999	100	98
39 (IS)	0.2033	0.2033	0.2033	100 by definition	100 by definition
48	0.2050	0.2138	0.1851	104	90
51	0.2091	0.2161	0.1781	103	85

[a]SFC, supercritical fluid chromatography; HTGC, high-temperature gas–liquid chromatography; ECN, equivalent carbon number; IS, internal standard

TABLE 3
Triacylglycerol Profile of a Physical Mixture and a SL Prepared from a Mixture of MCT and Fish Oil by HTGC and SFC[a]

ECN	Physical mixture (% peak area)	SL by SFC (% peak area)	SL by HTGC (% peak area)
32	0	7.1	7.2
33	0	0.4	0.3
34	0	8.5	9.7
35	0	5.9	0.3
36	0	3.9	10.9
37	0	8.7	0.4
38	0	0	10.7
39	0	3.7	0.4
40	0	2.5	7.2
41	0	0.6	0.3
42	0	4.1	4.5
43	0.2	5.7	0.3
Total % peak area	0.2	51.5	51.8

[a]See Figures 1 and 2 for abbreviations.

shown in our results. However, higher molecular weight compounds (ECN > 60) may require calibration to compensate for the less efficient elution and lower detector response. The methods presented herein provide useful and practical means for manufacturing optimization, quality assurance, and nutritional investigation of lipids containing natural and synthetic triacylglycerol species.

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