Method for Analysis of TAG Formed by Reaction of Fish Oil with Hydrogenated Soybean Oil

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ABSTRACT: An HPLC method was developed for analysis of the TAG formed during interesterification of a fish oil rich in DHA residues and of a fully hydrogenated soybean oil. TAG species were separated using a three-phase (acetone/acetonitrile/chloroform) solvent system. Peak identities were assigned on the basis of a multiple linear regression analysis by using factors such as carbon number, number of double bonds, and number of PUFA in the molecule as predictors for TAG retention time. Good agreement between experimental and predicted retention times was observed when the effect of the PUFA was separated in the regression model from that of the monounsaturated FA. In addition, the new method permits one to determine tristearin at concentrations up to 3 mg/mL without encountering the problem of partial retention of this TAG in the column that was observed when chloroform is not incorporated in the mobile phase.

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Because of the important role that n-3 PUFA from marine oils play in human nutrition, the production of fats and oils enriched in n-3 PUFA for dietary purposes has significant commercial potential (1). The lipase-catalyzed production of these substances *via* several different synthetic routes has been reported, for example, *via* acidolysis (2,3), *via* glycerolysis (4,5) and *via* transesterification (6). As a consequence of these reactions, changes are observed in the overall TAG composition. Thus, it is important to monitor the evolution of the TAG composition of the interesterified products as the reaction proceeds (7).

Edible fats and oils are composed of complex mixtures of TAG, and the compositions and functional properties of these substances are determined by the profile of their FA residues. RP-HPLC has been widely used for the analysis of both natural and synthetic mixtures of TAG (8–10). For fish oils, the presence of a wide variety of FA causes major analytical difficulties in efforts to elucidate their TAG profiles. Perona and Ruiz-Gutierrez (11) have described a methodology for separation and identification of the molecular species present in the TAG of sardine oil. The order of elution of the TAG separated by RP-HPLC corresponds very closely with the chain length, the degree of unsaturation, and the presence of certain functional groups on the FA chains. To date, the methods employed to predict retention times (RT) and identities of various TAG are based on the use of empirical correlations that involve determinations of the RT of well-defined TAG. The differences between the various approaches are discussed elsewhere (12).

Gradients of acetone/acetonitrile mixtures are commonly used to separate TAG mixtures by RP-HPLC. However, our experience with this solvent system indicated that tristearin (SSS) in concentrations above 2 mg/mL leads to excessive tailing of the peaks for this species. In addition, at the indicated concentration, SSS can be partially retained by the column. The retention problem may be attributed to its poor solubility in the mobile phase and also to the long RT observed for SSS when the gradients reported in the literature were employed. The present study focused on the development of an RP-HPLC method that permits one to circumvent this problem in the analysis of the TAG mixtures present in (i) a fish oil enriched in DHA residues, (ii) a fully hydrogenated soybean oil (FHSO) rich in SSS, and (iii) the products obtained from the lipase-catalyzed interesterification reactions of these two oils. Because chloroform is a good solvent for SSS, it was introduced as a third component of the eluent to ensure complete elution of such highly apolar TAG species as SSS and tripalmitin (PPP).

For purposes of identifying the various TAG present in the samples of interest, we applied an approach similar to that used by Perona and Gutierrez (11). This procedure uses as factors for the prediction of RT the total carbon number (CN), the number of double bonds (DB), and the unsaturated FA present in the TAG molecule of interest.

MATERIALS AND METHODS

Samples and standards. A commercial preparation of fish oil (03/55 TG) was obtained from Ocean Nutrition Canada Ltd. (Halifax, Nova Scotia) and FHSO was a gift from AC Humko Oil Products (Cordova, TN). The following pure TAG were used as standards: SSS, 99% (ICN Biomedicals, Irvine, CA); PPP, *ca.* 99% (Sigma, St. Louis, MO); TAG mixture (Supelco, Bellefonte, PA) containing tricaprin (CCC), tricaprylin (CaCaCa), trilaurin (LLL), trimyristin (MMM), and tripalmitin

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TABLE 1
Solvent Gradient Used to Separate TAG (mobile phase content expressed as vol%)

Time (min)	Chloroform (%)	Acetone (%)	Acetonitrile (%)
0	2	0	98
10	2	0	98
50	2	40	58
65	2	55	43
90	20	80	0
95	20	80	0
97	2	0	98
110	2	0	98

(PPP); olive oil standard mixture (Supelco) containing 1,2-dilinoleoyl-3-oleoyl-*rac*-glycerol (LLO), 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (OOP), 1,2-dioleoyl-3-stearoyl-*rac*-glycerol (OOS), trilinolein (LLL), and triolein (OOO); tridocosahexaenoin (DhDhDh) >99% purity from Nu-Chek-Prep (Elysian, MN); and triarachidonin (AAA) >99% purity from Nu-Chek-Prep.

RP-HPLC analyses. The chromatographic system consisted of Waters 600 quaternary pump, an Econosil C18 column (250 × 3.2 mm; Alltech, Deerfield, IL), and an Alltech 500 Evaporative Light Scattering Detector (ELSD). The system was controlled by a computer through Millenium System software (Waters). All solvents were HPLC grade and were used without further purification. The mobile phase gradient employed to separate the TAG species is shown in Table 1. A constant flow rate of 1 mL/min was used.

Calibration curves for each of the standards mentioned were generated by injecting solutions of these TAG in chloroform at concentrations ranging from 0.1 to 3 mg/mL. The same standard solutions were also used to determine the RT of the pure TAG and in conducting multiple linear regression analyses to provide a basis for prediction of the RT of those TAG newly formed by interesterification reactions.

GC analyses. Profiles of the FA residues of fish oil and FHSO were determined using an HP5890 (Hewlett-Packard, Palo Alto, CA) gas chromatograph fitted with a 60 m × 0.32 mm × 0.25 μm Supelcowax-10 capillary column (Supelco) and FID. Samples had previously been derivatized to form the methyl esters by treatment with 0.1 N methanolic NaOH for 30 min at 60°C. The oven temperature was programmed as follows: starting at 150°C, heated to 225°C at 20°C/min, and then held at 225°C for 46.25 min. The injector temperature was 220°C, and the detector temperature was 250°C. Sample (1 μL) was injected, and a split ratio of 20:1 was employed. FAME were identified by comparing RT with those obtained for a PUFA standard mixture (Supelco).

Multiple linear regression analyses. To establish a predictive correlation for the RT of the TAG species present in fish oil and FHSO, as well as those formed as a consequence of the transesterification reaction, we used the RT observed for the pure TAG standards. Multiple linear regression analysis of the data leads to the coefficients of the following expression for the RT:

$$RT = b_0 + b_1[CN] + b_2[DB] + b_3[UFA] \quad [1]$$

where [CN] is the sum of the carbon numbers of the FA residues attached to the glycerol backbone, [DB] is the number of double bonds contained in these residues, and [UFA] is the number of monounsaturated FA and PUFA present in the TAG molecule of interest. All regression analyses were carried out using the commercial software Minitab Release 12 for Windows® (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

RP-HPLC analysis of TAG standards. For all of the pure TAG investigated, characteristic sigmoidal response curves were observed from the ELSD. All standards showed correlation factors (r^2) equal to or near 1 when a cubic or fourth-order regression was employed. A second-order polynomial expression was obtained by taking logarithms of both the injected concentration and the response of the detector. In all cases, the correlation coefficient (r^2) was greater than 0.99. This approach was used for quantitative purposes. As examples, calibration curves for SSS are shown in Figure 1.

FA profile (GC analysis). The results of the FA analyses of the original feedstocks are presented in Table 2. The most abundant FA residue in the fish was DHA (66.21% w/w), followed

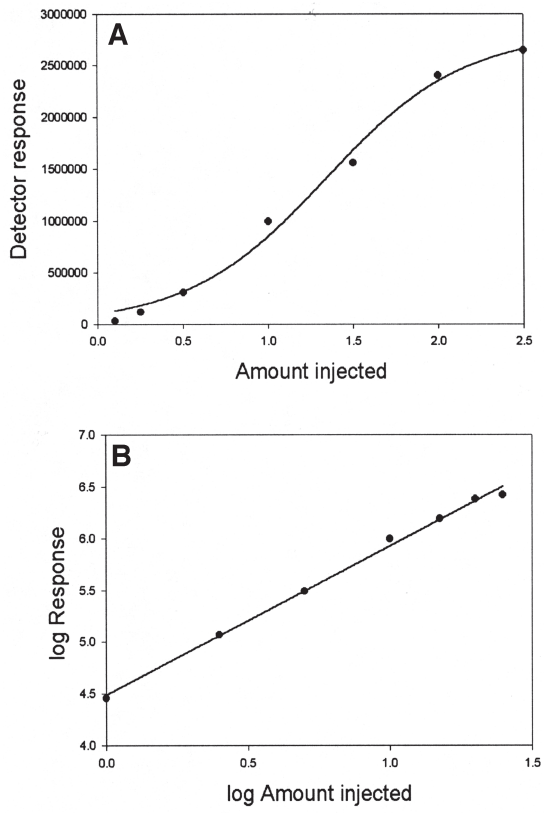

FIG. 1. Calibration curves for the tristearin (SSS) standard. The response of the detector (mV) is plotted against the amount injected (micrograms), either directly (A) or in logarithmic form (B).

TABLE 2
Composition of FA residues[a] (in wt%) in Fish Oil and Fully Hydrogenated Soybean Oil (FHSO)

FA	Fish oil	FHSO
14:0	0.42	
16:0	1.20	12.88
16:1	1.82	
18:0	0.44	87.12
18:1	2.62	
18:2n-6	1.65	
20:1n-9	0.40	
20:4	0.56	
20:5n-3	10.15	
22:1	1.88	
22:5n-3	12.63	
22:6n-3	66.21	
Total SFA	2.06	100
MUFA	6.72	
PUFA	91.20	

[a]SFA, saturated FA; MUFA, monounsaturated FA.

by docosapentaenoic acid (DPA) (12.5%) and EPA (10.15%), while low concentrations of myristic (14:0), stearic (18:0), 20:1, and 20:4 acids were also observed. The FHSO contained residues of stearic (87.12%) and palmitic (12.88%) acids only.

Multiple linear regression analyses. Several multiple linear regression analyses were conducted. The accuracy of each resulting expression was tested for its ability to predict the RT of the various TAG species. When all of the standards employed for the regression analyses were taken into account, significant differences were observed between predicted and experimental RT. Consequently, new values for the constants were calculated by conducting additional regression analyses of data for samples in which those TAG species that are of little relevance in the mixtures of interest here were omitted, e.g., CCC, CaCaCa, and LLL. When this new data set was used, the fit of the predicted values to the experimental RT improved significantly. When only those standards containing FA with carbon numbers ranging from 14 (myristic) to 22 (DHA) were used in the regression, very acceptable agreement of predicted and experimental values was obtained. The corresponding values of the constants in Equation 1 for RT in minutes are $b_0 = -14.4$, $b_1 = 1.77$, $b_2 = -4.10$, and $b_3 = -0.364$.

TABLE 3
Experimental and Predicted Retention Times (in min) for TAG Standards[a]

TAG	Equation 1	Equation 2	Experimental
DhDhDh	27.53	28.11	28.17
AAA	41.51	40.11	40.37
LLL	55.49	56.46	56.92
OOO	67.79	67.77	67.32
OOP	68.71	68.70	68.10
PPP	70.56	70.56	70.65
OOS	72.25	72.24	73.33
SSS	81.18	81.18	81.19

[a]A, arachidonic; Dh, docosahexaenoic; L, linoleic; O, oleic; P, palmitic; S, stearic.

Subsequently, we tried to improve the fit by introducing a new variable in the regression model, namely, the number of PUFA residues in the TAG. This approach was intended to separate the effects of the long-chain PUFA (arachidonic acid, DHA, and EPA) from those of monounsaturated FA (MUFA) on the elution times of the various TAG species. This modified regression model has the following form:

$$RT = b_0 + b_1[CN] + b_2[DB] + b_3[MUFA] + b_4[PUFA] \quad [2]$$

The regression analysis leads to the following values of the parameters in Equation 2: $b_0 = -14.4$, $b_1 = 1.77$, $b_2 = -3.77$, $b_3 = -0.699$, and $b_4 = -2.15$. Statistical significance at the 95% confidence level was obtained for all parameters in each of the two regression models. Both expressions were used to estimate RT for the standards, and these estimates were then compared with the corresponding experimental values. Inspection of the entries in Table 3 reveals that introduction of that additional factor for the long-chain PUFA residues in the regression analysis

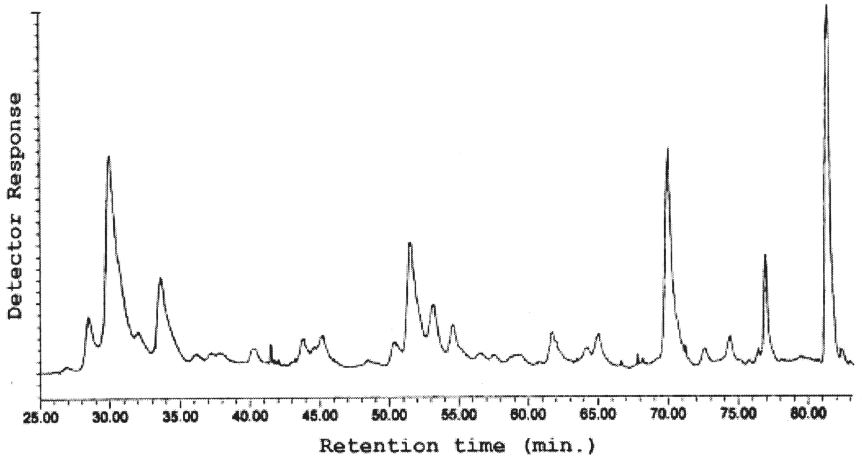

FIG. 2. Separation of the TAG species present in a transesterified mixture of fish oil and fully hydrogenated soybean oil (FHSO). Transesterification was carried out at 70°C for 12 h using lipase PS (Amano Enzymes, Inc., Nagoya, Japan) and a fish oil/FHSO weight ratio of 80:20.

TABLE 4
TAG Species[a] Found in Fish Oil and a Transesterified Mixture of This Oil with FHSO

Fish oil TAG	% Area	Experimental RT (min)	Predicted RT (min)
DhDhDh	6.11	28.24	28.11
DpDhDh	43.37	31.81	31.88
EEDp	16.49	32.43	32.34
EDpDp	2.68	36.00	35.88
PoDhDh	4.68	37.67	37.79
DpDpDp	3.86	39.42	39.42
PEE	1.32	42.23	42.72
ODpDh	2.53	44.64	45.10
ODpDp	0.87	48.02	48.87
PoODh	0.77	50.66	51.01
SDpDp	5.32	53.24	53.34
PPDh	3.33	56.47	56.41
PODp/PoSDp/SOE	0.82	59.23	59.25
SODp	5.69	62.75	62.79
PoOO	1.24	64.25	64.23
PPPo	0.91	66.83	66.09

Transesterified mixture TAG	% Area	Experimental RT (min)	Predicted RT (min)
DhDhDh	2.58	28.48	28.11
DpDhDh	17.63	31.03	31.88
EEDp	6.12	32.65	32.34
EDpDp	0.98	36.00	35.88
PoDhDh	1.09	37.67	37.79
PoEDh	0.99	37.85	38.02
DpDpDp	2.31	39.47	39.42
OEDh	0.59	41.57	41.56
PEE	1.78	42.86	42.72
PoDpDp/OEDp	3.09	45.24	45.33
PDpDp/SEDp	1.45	50.12	49.80
PoODh	1.08	50.44	51.01
PPoDh	7.61	51.53	51.94
SDpDp	3.80	53.23	53.34
PPoDp/POE/PoSE	2.90	54.63	55.71
PPE	1.20	56.65	56.64
PoPoPo	1.02	57.50	57.15
PODp/PoSDp/SOE	1.86	59.23	59.25
SODp	2.92	62.75	62.79
SSE	1.30	63.70	64.23
PoPoS	2.20	65.06	65.16
SSDp	1.35	67.30	67.77
PPO/PPoS/PPP	9.94	70.03	69.63
PSO	2.45	72.63	73.17
PPS	3.32	74.85	74.10
PSS	4.23	77.38	77.64
SSS	14.21	81.46	81.18

[a]Dp, docosapentaenoic; E, eicosapentaenoic; Po, palmitoleic; RT, retention time. For other abbreviations see Table 3.

permits a slight improvement of the correlation. Because only two of the standards included in the regression analysis contained PUFA residues, the improvement in the predicted values could not be quantified in a definitive manner. Analyses of additional standard samples of TAG mixtures containing other PUFA would be necessary to assess the improvements resulting from incorporation of this factor definitively. Nonetheless, inspection of the entries in Table 3 indicates that the most significant improvements in the predicted RT were observed for those TAG containing long-chain PUFA. This result indicates that this factor should be included in analyses of TAG mixtures containing PUFA. When the predicted values are plotted vs. the experimental RT, there is a small improvement in the r^2 value from 0.998 to 0.999 when Equation 2 is used rather than Equation 1.

RP-HPLC of mixtures formed during transesterification. To predict the RT of those TAG species expected to be formed during transesterification of fish oil and FHSO, we combined, three by three, the FA present in these oils with concentration levels greater than 2% (w/w). Positional isomers were considered as the same species since the technique described here

does not discriminate between such isomers (11). Only three peaks were observed in the chromatogram of the FHSO. SSS is the major component (72.5%, w/w), and the other two peaks correspond to SSP (25.5%) and PPS (2%), as predicted by the model. When solutions containing 20 mg/mL fish oil were injected for TAG analysis, 16 peaks were observed. The associated RT of these peaks were compared with those predicted by the regression analysis for the combination of the FA present in each sample. Figure 2, a typical chromatogram, corresponds to a sample transesterified at 70°C for 12 h using lipase PS (Amano Enzymes Inc., Nagoya, Japan) as a biocatalyst and using a fish oil/FHSO weight ratio of 80:20. The TAG compositions of the original fish oil and a sample of this oil transesterified with FHSO are presented in Table 4.

Usually, only a single TAG was assigned to an individual chromatographic peak, although sometimes the RT predicted by the model is the same for two or three different combinations of FA residues that have similar total CN, similar numbers of DB, and/or similar MUFA and PUFA values. For example, in a fish oil sample, we observed one peak with an RT very close to that predicted for following species: PODp, PoSDp, and SOE (where Dp is docosapentaenoic acid, E is eicosapentaenoic acid, and Po is palmitoleic acid). As expected from the FA profile, the major TAG species present in the particular fish oil analyzed were rich in DHA, whereas other significant species contained EPA and DPA residues. The major effect of the transesterification reaction was observed in the formation of new palmitic and stearic acid-enriched species, although the presence of other newly formed TAG species may be attributed to rearrangements of the FA residues present in the original fish oil TAG.

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