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Application of Partition Chromatographic Theory for the Analysis of Marine Triglyceride Molecular Species*[1]

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The relative retention value of the individual triglyceride molecular species on reverse phase high performance liquid chromatography was considered to be dependent principally on the addition theorem of chemical potentials of the three fatty acid residues.

It was demonstrated that the chemical potential of each fatty acid residue is proportional to the relative retention potential index i.e. the logarithm of the relative retention value of each fatty acid residue; and that the addition of the relative retention potential indexes of the three fatty acid residues denoted the logarithm of the relative retention value, the relative retention time of each individual molecular species of triglyceride.

Triglyceride molecular species from sand flounder was analyzed and compared with the theoretically predicted values.

There is no doubt that the physical properties of triglyceride (TG), the susceptibility of TG to hydrolytic enzymes or oxygen attack and preventive effect of fish oil TG against thrombosis, are considered to be dependent not only on the fatty acid composition, but also on the molecular species of TG. But the thorough analysis of TG molecular species especially from marine sources has not been fully exploited.

The first attempt to analyze the TG molecular species from marine sources was done by Dolev and Olcott,[1] and by Bottino[2] using silver nitrate impregnated thin layer chromatography. They have succeeded in separating the TG homologues into 7–25 fractions according to their total double bonds (DB).

Matsui *et al.*[3] used preparative gas liquid chromatography (GLC) for the analysis of total acyl carbon number (CN) of TGs' from four kinds of fresh water fish, and predicted their TG molecular species.

In the latter 1970s', reverse phase high performance liquid chromatography (HPLC) was widely used for the analysis of lipid molecular species.

Wada *et al.* discovered the partition number theory[4–7] on HPLC and applied it for TG molecular species analysis. He used five kinds of fish namely: black cod,[8,9] jack mackerel,[10] sablefish,[11] hagfish[12] and red seabream.[13] They had succeeded in analyzing the TG molecular species composition by excluding the eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) rich TG fraction. When the TG molecules contain highly unsaturated fatty acids such as EPA or DHA, they exceed the quantitative reproducible limit of the CN analysis that can be done by GLC. This restricts the determination of fatty acid residue combination i.e. acyl group combination of the TG molecule, and this has been an inherent problem in the analysis of TG molecular species from marine sources that are rich in EPA and DHA.

The authors tackled the aforementioned problem from an entirely new viewpoint. The approach taken is as follows; As reported in the earlier paper,[14] the following formulae[15] should hold:

$$\Delta\mu_B/R\cdot T=\Delta\mu_A/R\cdot T+\Delta\mu_X/R\cdot T \quad (1)$$

$$\ln(\alpha_B/\alpha_A)=\Delta\mu_X/R\cdot T \quad (2)$$

where A and B are members of a homologous series differing by the functional group X; $\Delta\mu_X$ is the difference in chemical potential of the group

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X in the chromatographic system. R is the gas constant; and T is the absolute temperature. α is the partition coefficient. It follows that each functional group in the solute molecule contributes more or less independently to the differences in standard free energy of the solute between the two chromatographic phases. Thus in general, there is a linear relationship between $\ln \alpha$ and the number of functional groups in a homologous series. If we consider α_A as the partition coefficient of the standard molecular species and α_B as the partition coefficient of each molecular species, α_B/α_A will correspond to the relative retention value, the relative retention time (RRT) of each molecular species. So from equation (2),

$$\ln(\alpha_B/\alpha_A) = \ln(\mathrm{RRT}) = \Delta\mu_X/\mathrm{R}\cdot\mathrm{T}$$

or

$$\log(\alpha_B/\alpha_A) = \log(\mathrm{RRT}) = \Delta\mu_X/2.303\cdot\mathrm{R}\cdot\mathrm{T} \quad (3)$$

should hold. Under most of the chromatographic system, T is constant. Therefore, $1/2.303\cdot\mathrm{R}\cdot\mathrm{T}$ will also be constant.

$$\therefore \log(\mathrm{RRT}) \propto \Delta\mu_X \quad (4)$$

From the aforementioned aspect, then equation (1) can be rewritten as follows:

$$\Delta\mu_B = \Delta\mu_A + \Delta\mu_X \quad (5)$$

This equation can be expanded to cover,

$$\Delta\mu_{TG} = \Delta\mu_{acyl_1} + \Delta\mu_{acyl_2} + \Delta\mu_{acyl_3} \quad (6)$$

where TG is the triglyceride and acyls are the fatty acid residues (functional groups) of TG molecule. From equations (4) and (6),

$$\log(\mathrm{RRT})_{TG} = \log(\mathrm{RRT})_{acyl_1} + \log(\mathrm{RRT})_{acyl_2} + \log(\mathrm{RRT})_{acyl_3} \quad (7)$$

can be obtained. This equation (7) alludes that the logarithm of RRT, we will define this value as relative retention potential index (RPI),[16] can be separated into three pieces of RPIs which are the RPIs of the three fatty acid residues in the TG molecule. So by obtaining the various RPIs of the composing fatty acid residues, the RPI of the individual TG molecular species can be calculated easily by the addition theorem of RPIs of the composed three fatty acid residues. This idea was demonstrated by comparing the theoretically predicted retention time (t_R) of the individual molecular species of TG with the empirically determined one.

Materials and Methods

Triglyceride Standards for the Calculation of Relative Retention Potential Index

Standard TG molecular species and previously known TG molecular species shown in Table 1 were used in order to calculate the RPIs of the fatty acid residues present in these TG. The RPI of each fatty acid residue was calculated by altering equation (7) as follows; From the monoacid TG standards,

$$\log(\mathrm{RRT})_{acyl} = (\log(\mathrm{RRT})_{TG})/3 \quad (8)$$

was used. And from the previously known diacid or triacid TG molecular species,

$$\underset{\text{(unknown)}}{\log(\mathrm{RRT})_{acyl_1}} = \log(\mathrm{RRT})_{TG} - \underset{\text{(known)}}{\log(\mathrm{RRT})_{acyl_2}} - \underset{\text{(known)}}{\log(\mathrm{RRT})_{acyl_3}} \quad (7')$$

$$\underset{\text{(unknown)}}{\log(\mathrm{RRT})_{acyl_2}} = \log(\mathrm{RRT})_{TG} - \underset{\text{(known)}}{\log(\mathrm{RRT})_{acyl_1}} - \underset{\text{(known)}}{\log(\mathrm{RRT})_{acyl_3}} \quad (7'')$$

Table 1. Standard triglyceride molecular species and previously known triglyceride molecular species used for the calculation of relative retention potential indexes of the fatty acid residues

Molecular species*1	Source	Purity
(14: 0, 14: 0, 14: 0)	Sigma Chemical Company	Approx. 99%
(16: 0, 16: 0, 16: 0)	〃	〃
(16: 1, 16: 1, 16: 1)	Nu-Chek-Prep, Inc.	90%
(18: 1, 18: 1, 18: 1)	Sigma Chemical Company	Approx. 99%
(18: 2, 18: 2, 18: 2)	〃	〃
(18: 3, 18: 3, 18: 3)	〃	〃
(20: 3, 20: 3, 20: 3)	Nu-Chek-Prep, Inc.	90%
(20: 4, 20: 4, 20: 4)	From "Ogonori" (*Gracilaria verrucosa*)	Approx. 90%
(20: 5, 20: 5, 20: 5)	From sand flounder (*Linanda punctatissima*) muscle TG	Approx. 80%
(18: 0, 18: 1, 18: 0)	From cacao butter	Approx. 90%
(22: 6, 20: 5, 20: 5)*2	From sand flounder (*Linanda punctatissima*) muscle TG	Approx. 75%

*1 16: 1 and 18: 1 are ω9, 18: 2 and 20: 4 are ω6, 20: 3, 20: 5 and 22: 6 are ω3.
*2 The binding position of the fatty acid residues are not discriminated here.

$$\log(\mathrm{RRT})_{acyl_3} = \log(\mathrm{RRT})_{TG} - \log(\mathrm{RRT})_{acyl_1} - \log(\mathrm{RRT})_{acyl_2} \qquad (7''')$$

(unknown) (known) (known)

were used. The RPIs of the various fatty acid residues that have been obtained from the above equations were employed in order to predict the RPI of the individual TG molecular species. And from these predicted RPI of the individual TG molecular species, the RRT and the t_R on HPLC were calculated by obtaining the antilogarithm (≡RRT) of the RPI and by multiplying 1/100 of the reference peaks' t_R to the RRT. These theoretically predicted t_R values were compared with the t_Rs' of the peaks of the individual TG molecular species observed in fish muscle HPLC chromatogram.

Analytical Condition of Reverse Phase High Performance Liquid Chromatography

In order to obtain a thorough resolution of the individual TG molecular species on HPLC, a column packed with 3 μm particles was used. Supersphere RP-18 cartridge that was inserted into LiChroCART® 250×4 mm column holder (Merck, Darmstadt, F. R. Germany) was empolyed for this purpose. Sample loop of Hitachi 638–50 liquid chromatograph (Hitachi, Ltd., Tokyo, Japan) was shortened to approximately 6–7 μl. The pipes that connect the injector, the column and the RI detector (Shodex SE-11, Showa Denko K. K., Tokyo, Japan) were shortened to avoid solute dispersion. The mobile phase used was 1: 1 acetone/acetonitrile at a flow rate of 0.8 ml/min. Column temperature was ambient, though the whole chromatographic system was equipped at a room that has less fluctuation in temperature. Standard TGs (Those shown in Table 1) were dissolved in small amount of chloroform, and 6–7 μl of this solution was applied to HPLC. Though the peaks appearing on the HPLC chromatogram could be identified by elution sequence, all of them were collected and monitored by inference of results obtained by GLC*1 in order to ascertain the identification of the peaks. The RRTs of the peaks were calculated by using a Hitachi D-2000 Chromato-Integrator. And the RPIs of the standard TG molecular species were calculated by obtaining the logarithm of RRTs. Then the value of the RPI of the standard TG molecular species was separated into three parts which are the RPIs of the three fatty acid residues. The RPI of each fatty acid residues was calculated using equations (8), (7′), (7″) and (7‴). On the other hand, muscle TG from sand flounder (*Linanda punctatissima*) was prepared using the method of Bligh and Dyer[17] followed by preparative thin layer chromatography (Merck Kieselgel 60, 20×20 cm, 0.5 mm thickness, 7: 3 n-hexane/ethylether), and 3–4 μl of it were applied to HPLC under the same condition with the TG standards. Samples were collected near the top of the sharp and dominant peaks of TG molecular species from sand flounder and analyzed by GLC after methyl esterification, as was done with the TG standards, in order to know the fatty acid combination. The t_R of the samples taken were compared with the theoretically predicted values determined by the RPIs of the composed fatty acid residues that had been obtained from the TG standards.

Results and Discussion

Table 2 shows the theoretically determined RPI of each fatty acid residue of the standard TG molecular species. Each value shown in this table was calculated as follows; For example,

Table 2. Relative retention potential indexes of the fatty acid residues of triglyceride on reverse phase high performence liquid chromatography*1,2

Fatty acid residue	Relative retention potential index	Fatty acid residue	Relative retention potential index
14:0	0.874	18:2 ω6	0.861
16:0	1.033	18:3 ω3	0.740
18:0	1.178	20:3 ω3	0.834
		20:4 ω6	0.781
16:1 ω9	0.861	20:5 ω3	0.667
18:1 ω9	0.993	22:6 ω3	0.692

*1 The "Q" value examination proposed by Dean & Dixon[18] was used in order to minimize the error after four replicates of HPLC runs.

*2 Relative retention time of (20: 5, 20: 5, 20: 5) is regarded as 100.0, namely, the relative retention potential index of this standard molecular species is log 100.0=2.000.

*1 Gas chromatographic conditions: instrument, Hitachi 063 gas chromatograph; column, Unisole 3000 (Gasukuro Kogyo, Tokyo, Japan) 3 m×3 mm glass column; column temp., 225°C; injection temp., 270°C; detector, FID; carrier gas, N_2 40 ml/min. Methylesters were prepared by the method of Christopher & Glass as described by Prevot & Mordret.[19]

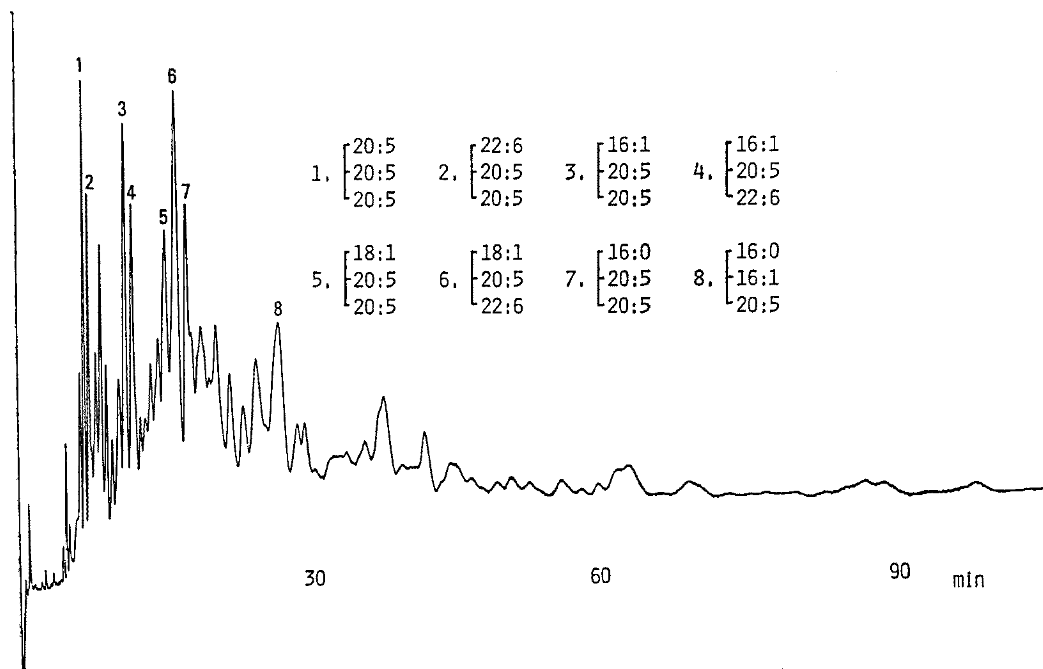

Fig. 1. Reverse phase high performance liquid chromatogram of sand flounder muscle triglyceride by high theoretical plate and high resolution column.

The retention time of the numbered peaks were compared with the theoretically predicted retention time (Table 3).

from the RRT datum of (20: 5, 20: 5, 20: 5) that is 100.0, the RPI of 20: 5 fatty acid residue was calculated as (log 100.0)/3=0.667. In the same way, the RPI of 18: 1 fatty acid residue was calculated as (log 952.8)/3=0.993. The HPLC chromatogram of sand flounder muscle TG is illustrated in Fig. 1. Outstanding peaks are numbered in sequence of elution. Peak number 2 in Fig. 1 was composed of two molar 20: 5 and one molar 22: 6 and these two fatty acids were absolutely dominant by infering the results obtained by GLC, therefore, this peak was identified as (22: 6, 20: 5, 20: 5).*1 The t_R of this molecular species was 7.49 on the HPLC employed. And the RPI of 22: 6 fatty acid residue shown in Table 2 was calculated as follows;

Step 1. The RRT of (22: 6, 20: 5, 20: 5) was calculated as $100 \times 7.49/7.06$*2 =106.1

Step 2. The RPI of (22: 6, 20: 5, 20: 5) was calculated as log 106.1=2.026.

Table 3. Comparison of the predicted and the empirically determined retention time of the prominent triglyceride molecular species of sand flounder on reverse phase high performance liquid chromatography*1

Peak No.	Molecular species*2	Predicted retention time (min)	Empirically determined retention time (min)	Relative deviation
1	(20: 5, 20: 5, 20: 5)*3	—	7.06	—
2	(22: 6, 20: 5, 20: 5)*3	—	7.49	—
3	(16: 1, 20: 5, 20: 5)	11.06	11.05	0.1%
4	(16: 1, 20: 5, 22: 6)	11.72	11.77	0.4%
5	(18: 1, 20: 5, 20: 5)	14.99	14.89	0.7%
6	(18: 1, 20: 5, 22: 6)	15.88	15.94	0.4%
7	(16: 0, 20: 5, 20: 5)	16.44	16.89	2.7%
8	(16: 0, 16: 1, 20: 5)	25.69	25.70	≒0%

*1 See Fig. 1.
*2 16: 1 and 18: 1 are ω9, 20: 5 and 22: 6 are ω3.
*3 Standard (reference) peak.

*1 The binding position of the fatty acid residues are not discriminated here.

*2 t_R of (20:5, 20:5, 20:5) in the actual HPLC chromatogram.

Step 3. So from equation (7′), the RPI of 22: 6 was calculated as $\log RRT_{22:6}=2.026-0.667-0.667=0.692$.

In these ways, the RPIs of the fatty acid residues in Table 2 were calculated. Then these values shown in Table 2 were used in order to predict the RPI of the individual TG molecular species. And from the predicted RPI of the individual molecular species, the RRT and the t_R were calculated as described in the "Materials and Methods".

Table 3 shows the theoretically predicted t_R values obtained by utilizing the data shown in Table 2. For example in this table, the RPI of (18: 1, 20: 5, 22: 6) was obtained as $0.993+0.667+0.692=2.352$. And the RRT of this molecular species was calculated as $10^{2.352}=224.9$. So the t_R of this molecular species became $224.9\times 7.06^{*1}/100=15.88$ (min) as shown in this table. The actual t_R of (18: 1, 20: 5, 22: 6) that was identified by inference of results obtained by GLC was 15.94 (min) (peak number 6 in Fig. 1) in the HPLC chromatogram of sand flounder as shown in the same table (Table 3). Other prominent peaks in Fig. 1 were identified by inference of results obtained by GLC and the t_Rs of these molecular species were compared with those of the theoretically predicted ones in the same way as above. As shown in Table 3, the theoretically predicted t_Rs that have been calculated by addition theorem of RPIs of the composed fatty acid residues of TG coincide well with the actual values observed in the HPLC chromatogram of TG from sand flounder (Fig. 1).

Though there still remain some difficulties even by HPLC for the perfect quantitative analysis of TG molecular species from marine sources owing to the overlap of the peaks in some degree, we can conclude that the prediction of t_Rs by the proposed theory can be practically used for contemporary HPLC analysis.

Appendixes

Discrimination of ω Isomers on Reverse Phase High Performance Liquid Chromatography

As it is obvious from other workers chromatograms of phospholipid molecular species,[20-23] ω3 elutes earlier than ω6 followed by ω9 on reverse phase HPLC. But the contribution of this factor is relatively small compared with the contributions of carbon number or number of double bonds in the phospholipid molecule. From this aspect, it is considered that the contribution of ω factor is considerably small in TG molecule than in phospholipid molecule, since the molecular size of TG is larger than phospholipid. At present, the data of the differences in RPIs among the TG that contain ω isomers are not forthcoming. But this does not fade the usefulness of the RPI prediction method presented in this study. Because if the monoacid TG standards of the ω isomers are forthcoming, then, it is possible to predict the RPI of the individual TG molecular species that contain ω isomers by using equations (8), (7′) or (7″) or (7‴) and (7). It is obvious that the proposed method, in theory, can discriminate the individual TG molecular species that contain ω isomers. The practicality of the proposed method in discriminating the ω isomers depends on the resolution and precision of the HPLC system employed.

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*1 t_R of (20: 5, 20: 5, 20: 5) in the actual HPLC chromatogram.

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