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Quantification Method for Triglyceride Molecular Species in Fish Oil with High Performance Liquid Chromatography-Ultraviolet Detector

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Abstract: A new quantification method for triglyceride (TG) molecular species contained in fish oil was developed using high performance liquid chromatography (HPLC)-ultraviolet detector (UV) system. In this experiment, triacontyl silane column and a mixture of alcohol and acetonitrile were used for column and mobile phase, respectively. Fifteen kinds of TG molecular species exist in fish oil were collected and the calibration curves monitored at 210nm were acquired for each TG molecular species. Also, evaporative light scattering detector (ELSD), widely used for the detection of fish oil TG molecular species, was tandem jointed after UV to compare the calibration curves for each TG molecular species. As the results, the calibration curves by UV with isocratic elution system were linear lines, on the contrary, those by ELSD were not linear. The slope of each calibration curve by UV was not the same and there was a tendency that TG molecular species having big partition number indicates a small slope calibration curve. The HPLC-UV with gradient system was also examined, but a few of standard TGs did not provide linear calibration curve. Consequently, we concluded that isocratic HPLC-UV system would be an available method for the quantification of TG molecular species in fish oil.

Key words: quantification, triglyceride molecular species, fish oil, HPLC-UV, triacontyl silane column

1 Introduction

Triglyceride (TG) is a principal component of fats and oils and consists of three fatty acids and one glycerol. Many kinds of fatty acids exist in natural fats and oils and partake in TG molecular construction. Consequently, in the case of the combinations of fatty acids are different between two TG molecular, these two TG molecular are distinguished as different kinds of TG molecular species (1). The analysis of TG molecular species is usually conducted with high performance liquid chromatography (HPLC), and silver ion column or reverse phase (RP) column is employed for the separation of TG molecular species (2,3). Silver ion column separates TG molecular species in order of the degree of unsaturation (4-6). On the contrary, RP column separates them in order of the polarity (7-9). Both of them have been well hired for the TG molecular species sep-

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aration, but American Oil Chemists' Society (AOCS) official method (10,11) and International Union of Pure and Applied Chemistry (IUPAC) official method (12,13) adopt RP column, in other word RP-HPLC system, for the separation of TG molecular species in plant oils. The separation of TG molecular species by RP-HPLC obeys partition number (PN) (14). PN is also called equivalent carbon number (ECN)(15) and expressed with the equation of PN=TC-2xDB, where TC is total carbon number of acyl group and DB is total number of double bond in TG. In this system, TG molecular species are eluted in order of PN and this rule can be adapted for every kind of oils and fats such as plant oil, animal fat, milk fat and fish oil (2,3). However, the separation of TG molecular species in milk fat and/or fish oil is not enough compared to those in plant oil or animal fat, because milk fat and/or fish oil consists of more kinds of TG molecular species than plant oil and animal fat (2,3). Therefore, some kinds of efforts, such as gradient elution system, are made in order to obtain fine separation of TG molecular species in milk fat or fish oil (16-18).

The selection of the detector is also very important factor for the TG molecular species analysis. For example, AOCS official method (10) adopts refractive detector (RI), ultraviolet detector (UV) and mass detector usually called evaporative light scattering detector (ELSD). Although the detection is performed for the identification and quantification of TG molecular species, these detectors have merit and demerit for these purpose. For example, RI is not affected by mobile phase nature, is available for the fractionation of the peaks and can be used for the quantification analysis. Fractionation is an important method in order to carry out peak identification with gas liquid chromatography (GC) (14). However, RI is unsuitable for a gradient elution system and is unstable to need much time for the stabilization. In contrast, UV is a stable detector, moreover, is available for the fractionation of the peaks and can be used for the quantification analysis, but it is fairly affected by the mobile phase character. The mobile phase well used for the RP-HPLC system is the mixture of acetonitrile and acetone or the mixture of acetonitrile and dichloromethane (19,20). The detection of TG molecular species is carried out using 200-220 nm originating in carbonyl group (21), but these mobile phases have a big absorption around the wave length. In that case, tetrahydrofurane (THF), 2-propanol, ethanol or methyl-*tert*-butyl ether has been used instead of acetone or dichloromethane (22-25) because these organic solvents have absorption less than 215 nm. AOCS official method (10) hires 220 nm for the detection of TG molecular species and the mixture of acetonitrile and THF as mobile phase in RP-HPLC-UV system. However, the separation is not so clear and the sensitivity is fairly low. ELSD is also a stable detector, furthermore, is not affected by the mobile phase nature and is available gradient elution. Therefore, this detector is commonly used for the TG molecular species detection as a universal detector, but it is not suitable to the quantitative analysis and the fractionation of the peaks. Because the calibration curves give by this detector are not linear equations (26). These ten years, HPLC-mass spectrometry detector (LC-MS) system is getting attention as a new method for the TG molecular species analysis (27-29). LC-MS is a universal detector for the TG molecular analysis, since it can identify the structure of each peak from the fragmentation pattern and quantify the amount of each TG molecular species. Furthermore, good separation of TG molecular species can be acquired by gradient elution system. However, this machine is very expensive (30) and is not handy.

In fact, almost all the works on TG molecular species analyses have focused on the identification of their structures and does not pay much attention for their quantification. As mentioned above, the analysis of TG molecular species in fish oil is very hard task. The separation and identification of them has been conducted by RI, UV, ELSD and MS with RP column or silver ion column till now (16,18, 31-36). However, there is no work taking into consideration about the quantification of TG molecular species in fish oil. In this study, the development of quantification method for each TG molecular species contained in fish oil was carried out with HPLC-UV system. Although UV is easily influenced by the mobile phase nature, good analysis conditions were able to be acquired by using triacontyl silane (TAS, C-30) column (37) and the mixture of alcohol and acetonitrile for mobile phase.

2 Materials and Methods

2·1 Chemicals and Materials

Eicosapentaenoic acid (EPA, E) and docosahexaenoic acid (DHA, D) were purchased from SIGMA-ALDRICH JAPAN (Tokyo, Japan). Other reagents were

obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fixed lipase (NOVOZYM 435) was kind present from Novo Vordisk A/S, Bagsvaerd, Denmark.

2·2 Separation of Triglyceride from Fish Oil

The fish oil extracted from bigeye tuna by Bligh and Dyer (38) procedure was separated by thin layer chromatography (TLC) to obtain TG. The oil was dissolved in hexane and spotted on silica gel plates (20 × 20 cm, 0.25 mm thickness, Silica gel 60 F254, Merck, Darmstadt, Germany). The spotted sample on TLC plates were developed using the solvent system, petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v). The separated TG was recovered by scrapping off the appropriate band and extracted using a mixture of chloroform/methanol (20:1, v/v). The solvent used for the TG extraction was removed with rotary evaporator under vacuum.

2·3 Comparison of the Separation Ability between Octadesyl Silane (ODS) and TAS Column

The separation of TG molecular species was carried out using reverse phase HPLC system comprising pump (LC-6A, Shimadzu Co., Kyoto, Japan), UV (SPD-10A, Shimadzu Co., Kyoto, Japan) and chromatopac integrater (C-R6A, Shimadzu Co., Kyoto, Japan). The mixture of 2-propanol/acetonitrile (40:60, v/v) was used as a mobile phase. The flow rate, detection wavelength and column temperature were 0.8 mL, 210 nm and 20℃, respectively. Tandem jointed TAS (DEVELOSIL RPAQUEOUS C-30, i.d. 5 μm, 4.6 × 250 mm, Nomura Chemical Co., Tokyo, Japan) or ODS column (SUPELCOSIL LC-18, 5 μm, 4.6 × 250 mm, SIGMA-ALDRICH JAPAN, Tokyo, Japan) was used for this HPLC system and the separation ability was compared between them.

2·4 Fractionation of Triglyceride Molecular Species in Fish Oil

The separation of TG molecular species in bigeye tuna was done using reverse phase HPLC system comprising pump (LC-6A), UV (SPD-10A) equipped with flow cell, tandem jointed TAS column and chromatopac integrater (C-R6A). The mixture of 2-promanol/acetonitrile (40:60, v/v) was used as a mobile phase. The flow rate, detection wave length and column temperature were 3.0 mL, 210 nm and 20℃, respectively.

2·5 Synthesis of EEE and DDD

One mol of glycerin was mixed with 3 mol of free fatty acid, EPA or DHA, and fixed lipase (NOVOZYM 435, Novo Vordisk A/S, Bagsvaerd, Denmark) in 50 ml round-bottomed glass flask. The mixture was stirred at 40℃ under vacuum for 2 hours (39). After finished the reaction, TG was purified from the mixture by the same way used for the purification of TG in fish oil.

2·6 GC Analyses

Tricosanoic acid methyl ester was used as internal standard of fatty acids analysis with GC. The tricosanoic acid methyl ester was weighted accurately and added to the TG sample by the ratio of about 1/20. Methyl esterification of the TG sample was conducted by following procedure. About 10 mg of TG purified from bigeye tuna and 1ml of 0.5 M sodium hydroxide methanol solution (NaOH-MeOH) were placed in 10 ml screw capped tube and mixed. Two ml of 14 % boron trifluoride methanol solution (BF_3-MeOH) was added to the mixture and heated at 100℃ for 20 sec. The heated tube was cooled to 40℃ under air and 1 ml hexane was put into the solution. Additionally, 3 ml of saturated sodium chloride solution was added to the mixture and mixed vigorously. The tube was left for a few minutes to separate hexane layer and water layer. The hexane layer was subjected to GC- flame ionization detector (FID) system (GC14B, Shimadzu, Tokyo, Japan) equipped with capillary column (Omegawax320, 30 m × 0.25 mm ID, Supelco, Bellefonte, PA) and chromatopac integrater (C-R6A). The temperature of injection port and detector was 250℃ and 260℃, respectively. The initial column temperature was 175℃ and was increased to 225℃ at the rate of 1℃/min. Helium was used as carrier gas and the flow rate was 32 cm/s. The identification of fatty acid species was done using retention time of fatty acid methyl ester standard solution (Supelco 37 Component FAME Mix, SIGMA-ALDRICH JAPAN, Tokyo, Japan).

2·7 Confirming the Purity of TG by LC-MS

The purity of synthesized TG (EEE and DDD), purified TG from bigeye tuna oil with HPLC and purchased TG were verified with LC-MS. The purified TGs were DDE, DDPo, DEO, DDO, DEP, DDP, DOO and DOP.

The purchased TGs were PoPoPo, OOO, LLL, LnLnLn and LLO (where D, E, Ln, L, Po, O and P are DHA (22:6n-3), EPA (20:5n-3), α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), palmitoleic acid (16:1n-9), oleic acid (18:1n-9) and palmitic acid (16:0), respectively). Furthermore, order of the abbreviations, e.g. DOP, does not mean the binding position of each fatty acid, but it just indicates the combination of those fatty acids.

The samples were injected to the LC-MS system consists of pump (Waters alliance 2560, Waters Co., Tokyo, Japan), photodiode array detector (PDA) (Waters 996, Waters Co., Tokyo, Japan), column oven (CA-202, Lab-Quatec Co., Tokyo, Japan) and MS (Micromass ZMD, Waters Co., Tokyo, Japan) fitted with an atmospheric pressure chemical ionization (APCI) source by full scan acquisition. Data acquisition, processing and instrument control were performed using MassLynx software (Waters Co., Tokyo, Japan). The conditions of column, mobile phase, flow rate and column temperature were the same as the conditions used for the separation of TG molecular species from fish oil. The range of the detection wavelength of PDA was 200 nm-250 nm and scan time was 0.5 s. Existence of hydroperoxide was evaluated by the absorbance around 234 nm originating from conjugated diene formed together with hydorperoxide. The APCI conditions on capillary voltage, positive mode cone voltage, negative mode cone voltage, heater temperature, gas pressure and corona voltage were 4.0 kV, 80 V, 40 V, 400℃, 500 L/h and 15 V, respectively. The analyses were carried out by both positive and negative ion mode. Spectra for positive ion mode and negative ion mode were obtained over the range *m/z* 540-1200 and 220-390 with a scan time 0.5 s, respectively.

2·8 Quantitative Analyses by HPLC-UV-ELSD with Isocratic System

Fifteen kinds of TG standard samples shown above were diluted with ethanol in appropriate concentrations. The HPLC comprising pump (LC-10A, Shimadzu Co., Tokyo, Japan), TAS column, UV (SPD-10A), ELSD (PL-ELS 1000, Polymer Laboratories LTD, Church Stretton, UK) and chromatopac integrater (C-R6A) was used for the analyses. The mixture of ethanol/acetonitrile (90:10, v/v) was hired as a eluent and the flow rate and column temperature were 0.8 mL and 20℃, respectively. The detection wavelength of UV detector was 210 nm. The evaporator temperature, nebrizer temperature and gas flow rate of ELSD detector were 90℃, 40℃ and 1.0 ml/min, respectively.

2·9 Quantitative Analyses by HPLC-UV-ELSD with Gradient System

The HPLC comprising pump (LC-10A), tandem jointed TAS column, UV (SPD-10A), ELSD (PL-ELS 1000) and chromatopac integrater (C-R6A). The gradient elution with a mixture of 2-propanol and acetonitrile was hired and the gradient program is shown in **Table 1**. The flow rate and column temperature were 0.8 mL and 20℃, respectively. The detection wavelength was 210 nm. The evaporator temperature, nebrizer temperature and gas flow rate of ELSD detector were 110℃, 90℃ and 1.0 ml/min, respectively.

3 Results and Discussions

TG molecular species are eluted in order of PN in RP-HPLC (14,15). However, many kinds of TG molecular species which have the same PN exist in oil, for example, both triolein (OOO) and tripalmitin (PPP) have the same PN in 42 and exist in plant oils. Furthermore, PN of TG molecular species consisting of two oleic acids and one palmitic acid (OOP) and one oleic acid and two palmitic acids (OPP) are also 42. In the case, these peaks appear at very near retention times. Fish oil is constructed a lot of kinds of fatty acids and also has a lot of kinds of TG molecular species expressing the same PN that does not exist in usual plant oil. For example, all the TG molecular species consisting of three EPAs (EEE), two EPAs and one DHA (EED), one EPA and two DHAs (EDD) and three DHAs (DDD) have the same PN in 30. These TG molecular species elute at early time and have almost the same retention

Table 1 Gradient Condition for TG Molecular Species Separation. (ratio of volume)

Time (min)	2-Propanol	Acetonitrile
0	40	60
50	50	50
80	60	40
100	70	30
120	70	30

Flow rate: 1.0 mL/min. Column temperature: 25℃.
Column: tandem of TAS (250 × 4.6 mm) column

time. The column used in RP-HPLC for TG molecular analysis is mainly an ODS column and the mixture of acetone and acetonitrile or the mixture of dichloromethane and acetonitrile is well used as mobile phase (19,20). These conditions can separate OOO, OOP, OPP and PPP clearly, however, cannot separate EEE, EED, EDD and DDD finely, because the PN of EEE group is smaller than that of OOO group and EEE group elute earlier than OOO group before fine separation is accomplished in the column. These facts indicate that the separation of TG molecular species in fish oil is very difficult. To separate TG molecular species in fish oil, many kinds of conditions have been explored and some of them attained good separation (16,18, 31-36). However, none of them does not taking into account of the quantification of TG molecular species contained in fish oil.

In this study, RP-HPLC-UV was employed to develop a quantification method for the TG molecular species in fish oil. ELSD was tandem jointed after UV to compare the calibration curves between them. In fact, RP-HPLC-UV was used for the separation of TG molecular species in plant oil containing γ-linolenic acid (22), in fish oil (18) and etc. so far. Fine separation of the plant oil TG molecular species was obtained with ODS column monitored at 210 nm by isocratic elution system using the mixture of acetonitrile, 2-propanol and hexane as a mobile phase (22). However, it was a level which can separate TG molecular species somehow in this condition. On the contrary, the separation of TG molecular species in fish oil was attained by the gradient elution system in RP-HPLC-UV system. This condition used 225 nm for the detection (18), therefore, the detection sensitivity was not high.

To decrease the polarity of the column is one of the alternatives to improve the peak separation in RP-HPLC system. Recently, TAS column possessing low polarity compared to ODS was developed (37). **Figure 1** shows the comparison of the separation patterns of tuna TG molecular species between ODS and TAS columns eluted by the mixture of acetonitrile and 2-propanol monitored at 210 nm. As shown in **Table 2**, bigeye tuna is consisted of many kinds of highly unsaturated fatty acids such as EPA and DHA. The TG

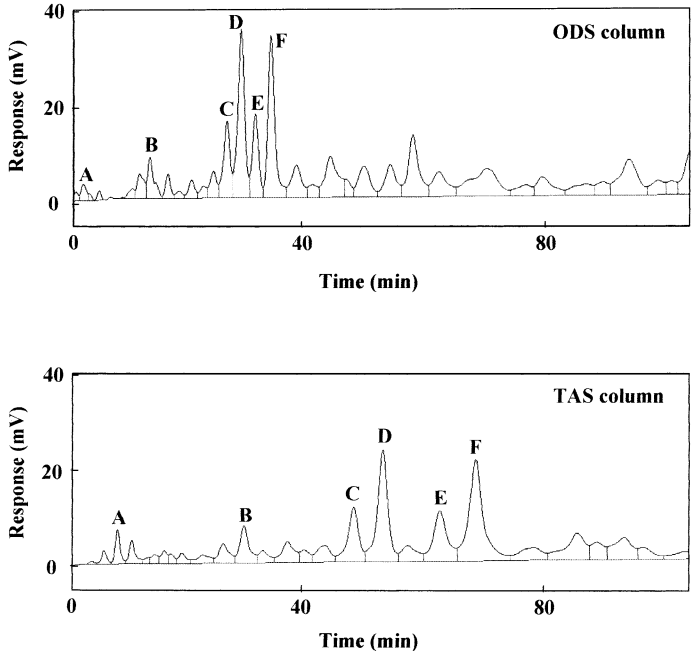

Fig. 1 The Comparison of Chromatogram of Bigeye Tuna TG Molecular Species between ODS (Upper) and TAS (Lower) Columns. Mobile phase: acetonitrile/2-propanol (60:40, v/v) UV: 210 nm, Flow rate: 1.0 mL/min, Column: TAS column × 2, A: DDE, B: DDPo, C: DEO, D: DDO, E: DEP, F: DDP.

Table 2 Fatty Acid Compositions of TG Extracted from Bigeye Tuna.

Fatty acids	Relative area percent with GC chromatogram (%)
14:0	2.96
15:0	0.75
16:0	18.82
16:1n-9	4.87
17:0	1.35
17:1	0.78
18:0	5.10
18:1n-9	20.34
18:1n-7	2.20
18:2n-6	0.89
18:3n-3	0.21
20:0	0.25
20:1n-9	2.96
20:5n-3 (EPA)	6.52
22:1	0.33
22:5n-3 (DPA)	1.43
22:6n-3 (DHA)	23.99
others	6.24
SFA*	29.23
MUFA**	31.50
PUFA***	33.04

*) SFA: saturated fatty acid **) MUFA: monounsaturated fatty acid ***) PUFA: polyunsaturated fatty acid

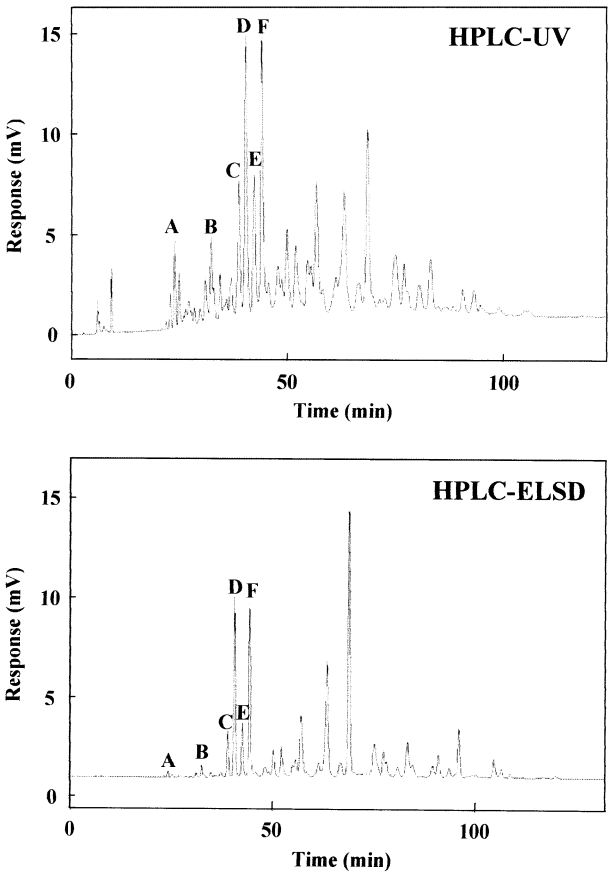

Fig. 2 The Comparison of Chromatogram of Bigeye Tuna TG Molecular Species between Tandem Jointed UV (Upper) and ELSD (Lower). acetonitrile/2-propanol (60:40, v/v) UV: 210 nm, Flow rate: 1.0 mL/min, Column: TAS column × 2, A: DDE, B: DDPo, C: DEO, D: DDO, E: DEP, F: DDP.

molecular containing EPA and/or DHA appears at very early time in RP-HPLC system as mentioned above. As shown in **Fig. 1**, ODS column can separate these peaks, however, TAS column can separate them more clearly. According to these results, using TAS column would be better than using ODS column for the detection and quantification of TG molecular species in fish oil. Furthermore, this condition with TAS column is suitable for the fractionation of TG molecular species because each peak separate very clearly. The best combination of alcohol and acetonitrile for TAS column in HPLC-UV was also examined. As the results, 2-propanol/acetonitrile (40:60, v/v) for tandem jointed TAS columns and ethanol/acetonitrile (90:10, v/v) for TAS column were determined as the best eluents for the separation of TG molecular species in fish oil with isocratic elution HPLC-UV system (detailed data not shown).

Figure 2 shows the comparison of two chromatograms on TG molecular species of bigeye tuna detected with tandem jointed UV and ELSD. The comparison reveals that many peaks appear in the UV chromatogram compared to the ELSD chromatogram. These results mean that UV is more sensitive detector than ELSD for the detection of low level of TG molecular species.

ELSD is handy detector and have been commonly used for the detection of TG molecular species in fish oils. The detection of TG molecular species with ELSD conducts by the means of the light scattering (26). The TG molecules are sprayed together with mobile phase solvent in ELSD and the solvent is evaporated. The TG molecular removed the mobile phase solvent forms particle and the particle passes through a focused high intensity light beam. This passing causes light scatter-

ing. This scattered light is detected photodiode and expresses as the TG molecule peak in chromatogram. As the result, the relation between the concentration of the solution and peak area of chromatograph by ELSD does not become linear function. It is known that the ELSD response follows the equation of $A=a \times C^b$, where A is the peak area of chromatogram, C is the sample concentration and both a and b are numerical constant (26). The plot between logarithmic C and logarithmic A gives the first order proportion with slope b. Therefore, ELSD is not suitable detector for the detection and quantification of low concentration of TG. On the other hand, the calibration curve with HPLC-UV gives first order calibration curves because this detection way obeys Lambert-Beer law. To reconfirm this matter for TG molecular species, fifteen kinds of TG standard molecules as shown in **Table 4** were obtained by several ways indicated in Material and Methods. The purity of them was confirmed with LC-APCI-MS and the existence of hydroperoxide was checked with PDA. As the results, standard samples used in this experiment were highly purified and were not oxidized. The calibration curves for DDPo, DDE, DEP, DOP, DOO, LLL and OOO were drawn with peak area of chromatogram by UV detector and ELSD. The results were shown in **Fig. 3** and **4**. The calibration curves plotted logarithmic TG amount versus logarithmic area of chromatogram by ELSD was presented in **Fig. 5**. As mentioned above, the calibration curves by ELSD were not straight line (**Fig. 4**). The plots for logarithmic concentration versus logarithmic area are drawn in straight line calibration curves (**Fig. 5**). The calibration curves by UV detector gives the straight line can be expressed as linear equation. Accordingly, it can be said that HPLC-UV is a suitable method for the detection and quantification of TG molecular species contained in fish oil. However, the slops differs each TG molecular species. Because each fatty acid has different absorbance coefficient at 210 nm and TG molecular species consists of the combination with three of these fatty acids. The magnitude of absorbance coefficient is affected by the numbers of double bond in fatty acid. Generally, the fatty acids having many double bonds possess a high absorbance coefficient because the electrons comprising double bonds can affect the carbonyl group and increase the absorbance coefficient. **Table 3** summarized the correlation factors of calibration curves detected by UV and ELSD for each TG molecular species. These data show

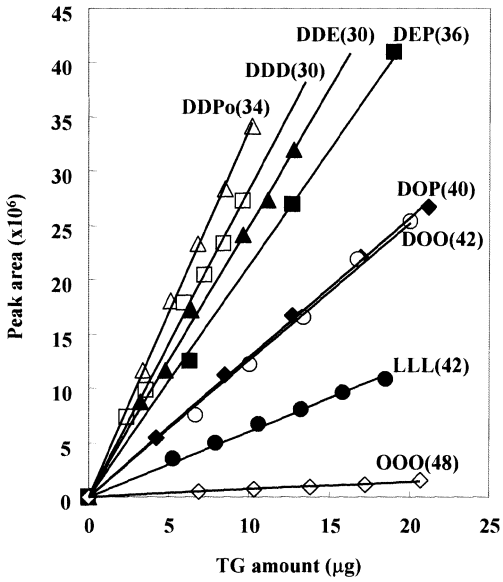

Fig. 3 The Calibration Curves of TG Molecular Species Contained in Fish Oil by UV Monitored at 210 nm. Plots are given TG amounts versus area of chromatogram. The numbers in the bracket which are indicated beside the abbreviation of TG molecular species mean PN of each TG molecular species.

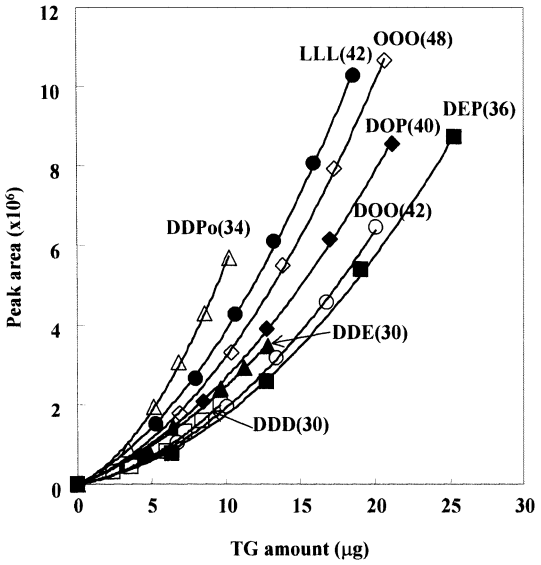

Fig. 4 The Calibration Curves of TG Molecular Species Contained in Fish Oil by ELSD. Plots are given TG amounts versus area of chromatogram. The numbers in the bracket which are indicated beside the abbreviation of TG molecular species mean PN of each TG molecular species.

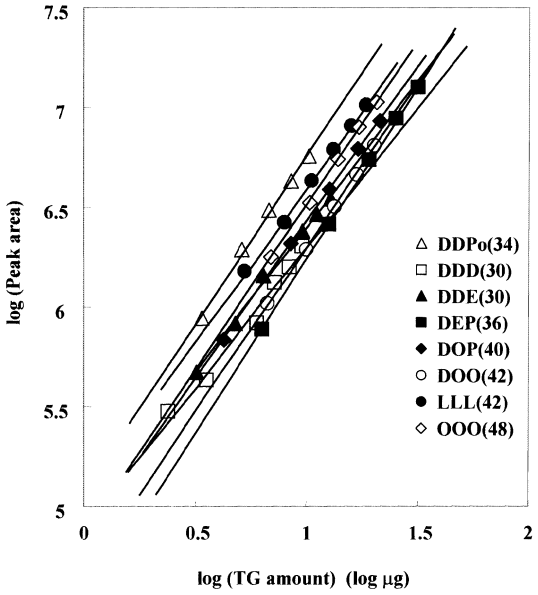

Fig. 5 The Calibration Curves of TG Molecular Species Contained in Fish Oil by ELSD. The plots are given logarithmic TG amount versus logarithmic area of chromatogram. The numbers in the bracket which are indicated beside the abbreviation of TG molecular species mean PN of each TG molecular species.

Table 3 Correlation Factor for the Calibration Curve of Each TG Molecular Species Detected by UV and ELSD.

TG molecular species	PN	UV	ELSD (log)
EEE	30	0.9975	0.9992
DDE	30	0.9967	0.9926
DDD	30	0.9979	0.9967
DDPo	34	0.9963	0.9962
DEO	36	0.9949	0.9953
DDO	36	0.9936	0.9962
DEP	36	0.9997	0.9997
DDP	36	0.9980	0.9999
LnLnLn	36	0.9935	0.9942
DOP	40	0.9989	0.9999
DOO	42	0.9968	0.9962
PoPoPo	42	0.9970	0.9991
LLL	42	0.9956	0.9992
LLO	44	0.9942	0.9965
OOO	48	0.9949	0.9995

that the calibration curves of UV detector and the curves plotted for logarithmic concentration versus logarithmic area of ELSD chromatogram give very good correlation factors. **Table 4** represents the slope of each calibration curve monitored at 210nm. These results are classified with PN and the slope differed among them. There was a big difference on the slope, for instance, the slope of DDPo is 46 times bigger than that of OOO. The TG molecular species having high PN (42, 44, 48) exhibit a small slope because these consist of one or two unsaturated fatty acids. As the results, the detection limit of this method for each TG molecular species differ each other (data not shown).

The HPLC-UV system with isocratic elution gave a straight line calibration curve for each TG molecular species. However, isocratic elution spends much time to detect all TG molecular species in fish oils. HPLC-UV with gradient elution system was already reported for the detection of TG molecular species (18). Consequently, the gradient condition was examined to shorten the analysis time with RP-HPLC-UV-ELSD system. The calibration curves acquired with UV is shown in **Fig. 6**. In this condition, some of the calibration curves

Table 4 Slope of the Calibration Curve of Each TG Molecular Species Detected by UV.

TG molecular species	PN	Slope
EEE	30	2.8×10^6
DDE	30	2.5×10^6
DDD	30	2.8×10^6
DDPo	34	3.3×10^6
DEO	36	2.0×10^6
DDO	36	2.1×10^6
DEP	36	2.1×10^6
DDP	36	2.3×10^6
LnLnLn	36	1.2×10^6
DOP	40	1.3×10^6
DOO	42	1.3×10^6
PoPoPo	42	6.4×10^4
LLL	42	6.0×10^5
LLO	44	4.1×10^5
OOO	48	7.1×10^4

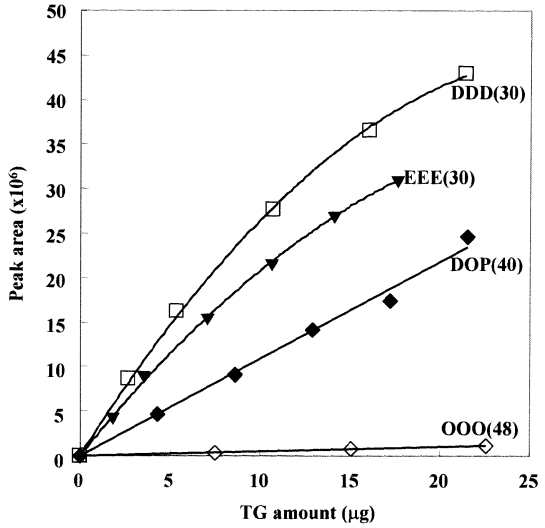

Fig. 6 The Calibration Curves of TG Molecular Species Contained in Fish oil Monitored at 210 nm Eluted by Gradient System. Plots are given TG amounts versus area of chromatogram. The numbers in the bracket which are indicated beside the abbreviation of TG molecular species mean PN of each TG molecular species.

of UV were not straight. This result indicates that gradient system affects the UV detection and some more improvement is required to obtain straight calibration curve like isocratic elution system.

4 Conclusion

The method to separate TG molecular species using TAS column eluted by the mixture of alcohol and acetnitrile made it possible to detect TG molecular species with UV detector and gave a calibration curves expressed as linear equation. This method would be applied to detect and quantify the TG molecular species in fish oils.

Acknowledgement

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References

1. B.F. DAUBERT, The Composition of Fats, *J. Am. Oil Chem. Soc*., Vol. **26**, 556-558 (1949).
2. J.J. MYHER and A. KUKSIS, General Strategies in Chromatographic Analysis of Lipids, *J. Chromatogr. B*, Vol. **671**, 3-33 (1995).
3. V. RUIZ-GUTIERREZ and L.J.R. BARRON, Methods for the Analysis of Triacylglycerols, *J. Chromatogr. B*, Vol. **671**, 133-168 (1995).
4. W.W. CHRISTIE, Separation of Molecular Species of Triacylglycerols by HPLC with a Silver ion Column, *J. Chromatogr*., Vol. **454**, 273-284 (1988).
5. B. NIKOLOVA-DAMYANOVA, W.W. CHRISTIE and B. HERSLOF, Retention Properties of Triacylglycerols on Silver Ion HPLC, *J. Chromatogr. A*, Vol. **694**, 375-380 (1995).
6. R.O. ADLOF, Analysis of Triacylglycerol Positional Isomers by Silver Ion HPLC, *J. High Resolut. Chromatogr*., Vol. **18**, 105-107 (1995).
7. B. NIKOLOVA-DAMYANOVA, Reversed-Phase High-Performance Liquid Chromatography: General Principles and Applications to the Analysis of Fatty Acids and Triacylglycerols, in *Advances in Lipid Methodology - Four*, (W.W. CHRISTIE, ed.), The Oily Press Ltd., Dundee, pp. 193-251 (1997).
8. W.W. CHRISTIE, *High-Performance Liquid Chromatography and Lipids*, Pergamon Books, Oxford (1987).
9. S. HERON, J. BLETON and A. TCHAPLA, in *New Trends in Lipid and Lipoprotein Analyses* (J.L. SEBEDIO and E.G. PERKINS, ed.), AOCS Press, Champaign, pp. 205-231 (1995).
10. AOCS, Triglycerides in Vegetable Oils by HPLC, *AOCS Official Method*, Ce 5b-89.
11. AOCS, Individual Triglycerides in Oils and Fats by HPLC, *AOCS Official Method*, Ce 5c-93.
12. IUPAC, Determination of the Composition of Triglycerides in Vegetqable Oils in Terms of Their Partition Number by High Performance Liquid Chromatography (HPLC), *IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives*, 2.324.
13. IUPAC, Determination of the Individual Triglycerides in Oils and Fats by High Performance Liquid Chromatography, *IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives*, 2.325.
14. S. WADA, C. KOIZUMI and J. NONAKA, Analysis of Triglycerides of Soybean Oil by High-performance Liquid Chromatography in Combination with Gas Liquid Chromatography, *Yukagaku*, Vol. **26**, 95-99 (1977).
15. R.D. PLATTNER, G.F. SPENCER and R. KLEIMAN, Triglyceride Separation by Reverse Phase High Performance Liquid Chromatography, *J. Am. Oil Chem. Soc*., Vol. **54**, 511-515 (1977).
16. C. JAYASINGHE, N. GOTOH, S. TOKAIRIN, H. EHARA and S. WADA, Inter Species Changes of Lipid Compositions in Liver of Shallow-Water Sharks from the Indian Ocean, *Fish. Sci*., Vol. **69**, 644-653 (2003).
17. J.L. ROBINSON and R. MACRAE, Comparison of Detection Systems for the High-Performance Liquid Chromatographic Analysis of Complex Triglyceride Mixtures, *J. Chromatogr*., Vol. **303**, 386-390 (1984).

18. M.J. WOJTUSIK, P.R. BROWN and J.G. TURCOTTE, Semi-preparative HPLC Fractionation of Consumer Fish Oil (Triacylglycerols), *J. Liquid Chromatogr*., Vol. **11**, 2091-2107 (1988).
19. P. LAAKSO, W.W. CHRISTIE and J. PETTERSEN, Analysis of North Atlantic and Baltic Fish Oil Triacylglycerols by High-Performance Liquid Chromatography with a Silver Ion Column, *Lipids*, Vol. **25**, 284-291 (1990).
20. P. LAAKSO, Characterization of α- and γ-linolenic Acid Oils by Reversed-Phase High-Performance Liquid Chromatography-Atomospheric Pressure Chemical Ionization Mass Spectrometry, *J. Am. Oil Chem. Soc*., Vol. **74**, 1291-1300 (1997).
21. E. ANGIONI, G. LERCKER, N.G. FREGA, G. CARTA, M.P. MEILS, E. MURRU, S. SPADA and S. BANNI, UV Spectral Properties of Lipids as a Tool for Their Identification, *Eur. J. Lipid Sci. Technol*., Vol. **104**, 59-64 (2002).
22. K. AITZETMULLER and M. GRONHEIM, Separation of Highly Unsaturated Triglycerols by Reversed Phase HPLC with Short Wavelength UV Detection, *J. High Resolut. Chromatogr*., Vol. **15**, 219-226 (1992).
23. M. HOLCAPEK, P. JANDERA, P. ZDERADICKA and L. HRUBA, Characterization of Triacylglycerol and Diacylglycerol Composition of Plant Oils Using High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry, *J. Chromatogr. A*, Vol. **1010**, 195-215 (2003).
24. B. HERSLOF and A.J.P. KINDMARK, HPLC of Triglycerides with Gradient Elution and Mass Detection, *Lipids*, Vol. **20**, 783-789 (1985).
25. T. REZANKA and P. MARES, Determination of Plant Triacylglycerols Using Capillary Gas Chromatography, High-Performance Liquid Chromatography and Mass Spectrometry, *J. Chromatgr*., Vol. **542**, 145-159 (1991).
26. W.W. CHRISTIE, Evaporative Light-Scattering Detectors in lipid Analysis, *Lipid Technology*, Vol. **5**, 68-70 (1993).
27. W.C. BYRDWELL, Atmospheric Pressure Chemical Ionization Mass Spectrometry for Analysis of Lipids, *Lipids*, Vol. **36**, 327-346 (2001).
28. W.E. NEFF and W.C. BYRDWELL, Soy Bean Oil Triacylglycerol Analysis by Reversed-Phase High-Performance Liquid Chromatography Coupled with Atomospheric Pressure Chemical Ionization Mass Spectrometry, *J. Am. Oil Chem. Soc*., Vol. **72**, 1185-1191 (1995).
29. H.R. Mottram, S.E. Woodbury and R.P. Evershed, Identification of Triacylglycerol Positional Isomers Present in Vegetable Oils by High Performance Liquid Chromatography/Atmospheric Chemical Ionization Mass Spectrometry, *Rapid Commun. Mass Spectrom*., Vol. **11**, 1240-1252 (1997).
30. N.U. OLSSON and N. SALEM Jr., Molecular Species Analysis of Phospholipids, *J. Chromatogr. B*, Vol. **692**, 245-256 (1997).
31. P. LAAKSO and W.W. CHRISTIE, Combination of Silver Ion and Reversed-Phase High-Performance Liquid Chromatography in the Fractionation of Herring Oil Triacylglycerol, *J. Am. Oil Chem. Soc*., Vol. **68**, 213-223 (1991).
32. T. TSUTSUMI, Y. SAKAGUCHI, S. TOHKAIRIN, H. EHARA and S. WADA, Molecular Species Analysis of Naturally Occurring Triacylglycerols by High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry, in *JOCS/AOCS World Congress*, *Kyoto*, Abstracts, p. 225 (2000).
33. E.H. LEE, S. WADA, C. KOIZUMI, T. OHSHIMA and J. NONAKA, Fatty Acid Combination of Major Triglyceride in Hagfish Flesh Lipids, *Bull. Korean Fish. Soc*., Vol. **17**, 291-298 (1984).
34. S. WADA, C. KOIZUMI, A. TAKIGUCHI and J. NONAKA, Triglyceride Composition of Black Cod Lipid-I. Triglyceride Composition Based on Acyl Cazrbon Number and Degree of Unsaturation, *Bull. Jap. Soc. Sci. Fish*., Vol. **45**, 611-614 (1979).
35. S. WADA, C. KOIZUMI, A. TAKIGUCHI and J. NONAKA, Triglyceride Composition of Black Cod Lipid-II. Possible Fatty Acid Combinations in Triglycerides and Their Contents, *Bull. Jap. Soc. Sci. Fish*, Vol. **45**, 615-622 (1979).
36. M.J. WOJTUSIK, P.R. BROWN and J.G. TURCOTTE, UV and RI Detection for the HPLC Analysis of Triglycerides in Fish Oils, *BioChromatography*, Vol. **3**, 76-83 (1988).
37. N. NAGAE, *USA Pat*., 6241891 (2001).
38. E.G. BLIGH and W.J. DYER, A rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol*., Vol. **37**, 911-917 (1959).
39. G.G. HARALDSSON, B.Ö. GUDMUNDSSON and Ö. ALMARSSON, The Synthesis of Homogeneous Triglycerides of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase, *Tetrahedron*, Vol. **51**, 941-952 (1995).