Original Paper

Separation of Triglycerides in Oils and Fats by Comprehensive Two-Dimensional Liquid Chromatography and the Determination of the Fatty Acid Composition in Gas Chromatography

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Abstract

An on-line coupled comprehensive two-dimensional liquid chromatography (2D-LC) system was developed for the separation of triglycerides (TGs) in this work. Taking advantage of two-dimensional separation on the basis of two separation columns with different selectivities, where an silver-ion column and an octylsilica column were employed as the columns in the 1st and 2nd dimensions, respectively, all the TGs in perilla and tuna oils were well separated. Introducing the systematic qualitative analysis in gas chromatography (GC), it was possible to make the assignments for all the fatty acid compositions in the TGs that were fractionated after the comprehensive 2D-LC separations. In the subsequent GC analysis, 23 and 76 types of TGs were determined for perilla and tuna oils, respectively. The results clearly showed an excellent performance of the two-dimensional separation system for the separation of TGs in oils and fats, suggesting a future applications to the high performance separation of other types of fish oils with the on-line coupled comprehensive 2D-LC system developed in this work.

Keywords: Two-dimensional liquid chromatography; Comprehensive separation; Triglycerides; Perilla oil; Tuna oil

1. Introduction

Lipids are extremely important energy sources in natural world and one of the three major nutrients. Triglycerides (TGs), the main component of oils and fats, are esters formed by three fatty acids (FAs) bonded to a glycerin molecule [1-3]. Oils and fats contain essential FAs that cannot be synthesized in human body. On the other hand, it is known to be a causative compound for cardiovascular diseases and obesity [4,5]. Therefore, analyzing oils and fats is important in the food and other related industries.

For the analysis of oils and fats, gas chromatography (GC) and liquid chromatography (LC) are commonly used [6-11]. However, in GC it is very difficult to separate nonvolatile compounds and thermally labile compounds such as TGs. On the other hand, LC can separate a wide variety of samples including nonvolatile compounds,

mainly on the basis of the molecular shape and size, as long as it could be dissolved in the mobile phase solvent [12-22].

In LC, silver-ion liquid chromatography (Ag-LC) and reversed-phase liquid chromatography (RP-LC) are most commonly used for the analysis of oils and fats [23]. Ag-LC uses a column (Ag column), where silver ions are immobilized on the surface of the support material packed into the column. In this method, separation is performed by the difference in the stability of the charge-transfer complex formed by the silver ions and the double bond portion of the sample molecule. In RP-LC, a polar mobile phase such as a mixture of methanol and water and a nonpolar stationary phase such as an octadecylsilica phase are typically employed [24] for the separation on the basis of polarity and other structural parameters of the analytes. TG is reported to be separated according to each effective

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carbon number (ECN) in RP-LC [25,26], where ECN is calculated with the carbon number (CN) and double bond number (DB) as: $ECN = CN - 2 \times DB$. However, when analyzing oils and fats having a complex composition, it is somewhat difficult to get a sufficient separation with one of these analytical methods.

Two-dimensional liquid chromatography (2D-LC) has been widely employed for the analysis of complex mixtures [24,27–33]. In the method, the solutes separated in the first column are introduced to the second column, and then, re-separated therein on the basis of a different selectivity from that of the first column. It has a large peak capacity and is quite suitable for the separation of various complex sample mixtures. 2D-LC can be performed with a mode either off-line or on-line [34]. The former, although it is easier, is time consuming, difficult to automate and reproduce, and easy to have a sample loss and contamination. The on-line method is faster and more reproducible, but needs an instrument having specially-designed interface for an effective hyphenation. 2D-LC is divided into heart-cut two-dimensional liquid chromatography (heart-cutting 2D-LC) and comprehensive two-dimensional liquid chromatography (comprehensive 2D-LC) [35–37]. The former method is employed to precisely separate only the target components of interest in the sample. In contrast, two-dimensional separation of the whole sample is obtained in the latter technique.

In this work, an on-line coupled comprehensive 2D-LC system using Ag-LC for the first dimension and RP-LC for the second dimension was developed, and a comprehensive two-dimensional separation of TGs in oils and fats was studied. After the 2D-LC separation, the obtained TGs fractions were separated again for the assignment of the FA compositions.

2. Experimental

2.1. Chemicals

All of the reagents and solvents, including *n*-hexane, ethanol, acetonitrile (ACN), methyl-*tert*-butyl ether (MTBE), 2-propanol (IPA), were of analytical reagent grade, and obtained from either Kishida Chemical (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan) or Wako Pure Chemical (Osaka, Japan). Water was purified by a Milli-Q Water Purification System (Millipore, Darmstadt, Germany). As the column in the 1st dimension (1D-column), an Ag ion column was prepared with an ion-exchange stationary phase, and the packing material for the column in the 2nd dimension (2D-column) an octylsilica stationary phase (4.6 mm i.d., 150 mm length, 5 µm particle size, GL Science, Tokyo, Japan) was employed. Detail of the Ag column preparation and chromatographic conditions are described below. The perilla oil was obtained from a local grocery store, Toyohashi, Japan, and the tuna oil was kindly donated

from Tuna Advanced Functional Food, Shizuoka, Japan.

2.2. 2D-LC system

The configuration of the online coupled comprehensive 2D-LC system developed in this study is illustrated in Fig. 1. A 10-port valve (LabPRO, Rheodyne, Cotati, CA, USA) with a laboratory-made valve switching system was used to connect these two columns, where two loops for fraction collection during the 2D separations were also installed in the valve. The 10-port valve was operated as follows. First, TGs eluted from the 1D-column were collected in a sample loop, Loop A. Switching the valve position, the TGs collected in Loop A were introduced to the 2D-column. At the same time, the TGs eluted from the 1D-column were collected in Loop B. When switching the valve again, the TGs collected in Loop B were introduced to the 2D-column, and at the same time, the TGs eluted from the 1D-column were collected in Loop A. By repeating these steps, a comprehensive 2D separation of all the TGs was carried out. Introducing a precise control of the valve operation by a specially-designed laboratory-made software, a more accurate valve operation was possible, allowing more reliable and accurate chromatographic results.

Fig. 1. On-line coupled comprehensive 2D-LC System developed in this work. 1, 1D-pump; 2, 2D-pump; 3, 1D-UV/Vis detector; 4, 2D-UV/Vis detector; 5, 1D-column; 6, 2D-column; 7, Loop A; 8, Loop B.

2.3. Experimental conditions

For the preparation of Ag column, a methanol solution of silver nitrate (1M) was passed through a cation exchange column packed with a silica-based stationary phase having benzenesulfonic acid groups (Capcell Pak SCX-UG-80, 4.6 mm i.d., 250 mm length, 5 µm particle size, Shiseido, Tokyo, Japan). The column was then washed with ethanol. In the resulting Ag column, where Ag ions were immobilized on the stationary phase, the TGs are eluted with a order of increasing the number of double bonds in the molecule, *i.e.* DB [34]. This is because the larger DB,

the higher stability of the charge-transfer complex formed by the silver ions and the double bond portion of analyte. In a typical Ag column, conjugated double bonds in the analyte molecule are weakly retained, and a larger retention is observed for the analyte molecules having a longer distance between two double bonds in their structure. Furthermore, in the 2D-LC system, consisted of Ag-LC and RP-LC, the retention in the second dimension decreases as the retention in the first dimension increases. Therefore, a reverse gradient was used, where the solvent strength of the second dimension decreases with time. During the reversed gradient separation, a baseline correction was carried out.

2.4. FA analysis

Table 1 summarizes all the FAs contained in the TGs of perilla and tuna oils, where their CN, DB are listed along with the abbreviations used in this work. All the TGs are expressed as a set of three symbols of FAs, *e.g.* LnLnLn. For the qualitative analysis, TGs were transesterified to obtain the FA methyl esters [3]. Qualitative analysis of TGs was performed by analyzing the methyl esterified FAs in GC, allowing the assignment of FAs.

Table 1. List of FAs.

Name	CN	DB	ECN	Symbol
Myristic acid	14	0	14	M
Palmitic acid	16	0	16	P
Palmitoleic acid	16	1	14	Po
Stearic acid	18	0	18	S
Oleic acid	18	1	16	O
Linoleic acid	18	2	14	L
Linolenic acid	18	3	12	Ln
Eicosenoic acid	20	1	18	Eb
Eicosadienoic acid	20	2	16	Ec
Eicosatrienoic acid	20	3	14	Ed
Eicosapentaenoic acid	20	5	10	E
Eicosahexaenoic acid	20	6	8	Eg
Docosenoic acid	22	1	20	Db
Docosapentaenoic acid	22	5	12	Df
Docosahexaenoic acid	22	6	10	D
Tetracosenoic acid	24	1	22	Tb

Most of the fractions collected by the 2D-LC separation were subjected to a drying process with a flow of N₂, and then dissolved in 3 mL of hexane. Next, 1 mL of 1M NaOH methanol solution was added and stirred for 2 minutes. The transesterification of TGs with methanol was carried out with NaOH as a catalyst. The resulting FA methyl esters are extracted into hexane. Then, 2 mL of H₂O was added and stirred for 2 minutes. The hexane phase and H₂O-methanol phase were separated, and the hexane phase was concentrated to obtain a hexane solution of 100 µL for the subsequent GC analysis.

For the GC analysis, a 6890N Gas Chromatograph (Agilent, Santa Clara, CA, USA) with a flame ionization

detector was used, where a DB-WAX capillary column, 0.25 mm i.d., 15 m length, 0.25 film thickness, was employed for the separation of FAs. All the separation conditions were determined in preliminary experiments.

3. Results and discussion

3.1. Two-dimensional separation of perilla TG

Typical perilla oil is mainly consisted of TGs, however, other minor components such as diglycerides and monoglycerides are also contained in the oil. Therefore, prior to the 2D-LC separation, TGs in a perilla oil were fractionated with a silica column in normal phase LC (Crest Sil, 10 mm i.d., 150 mm length, 5 µm particle size, Jasco, Tokyo, Japan). The chromatogram obtained for the separation of the perilla oil in LC is shown in Fig. 2a. For tuna oil analysis, a similar fractionation was also carried out. The chromatogram for separating a tuna oil is depicted in Fig. 2b.

Fig. 2. Preparative separations for the fractionation of (A) perilla and (B) tuna oils. Conditions: mobile phase, *n*-hexane containing 1% of ethanol; flowrate, 4.0 mL/min.; detection, UV at 220 nm; injection volume, 400 µL. Other conditions are in the text.

Fig. 3. 1D separation of a perilla oil in Ag-LC. Gradient conditions: solvent A, *n*-hexane; solvent B, ACN/MTBE/*n*-hexane (5:20:100), solvent gradient program, from A/B (70/30) to (5/95) over 1000 min.; flowrate, 20 μ L/min. Detection, UV at 210 nm. Injection volume 10 μ L.

Fig. 3 shows a chromatogram in the 1st dimension, and the 2D-LC separation is shown in Fig. 4. Since the TG in a perilla oil is consisted of three FAs having a DB between 0 and 3, the resulting TGs have a DB from 0 to 9. In Ag-LC, a TG with a larger DB retained longer. Therefore, the last detected peak in Fig. 3 is assigned as a TG with DB of 9. The peak at the retention time about 830 min is assigned as a TG with DB of 8. All the TGs with DB of 6 have an ECN of either 40 or 42. In RP-LC, TG with a smaller ECN is eluted faster. Therefore, among two peak groups that seems to have DB of 6, the group detected earlier in the 2nd dimension was estimated as the TGs having ECN of 40. In a similar way, all the peaks in the 2D-LC separation of the perilla TGs have been assigned as

shown in Fig. 4, where the FAs were expressed as a set of three abbreviations defined in Table 1. In Fig. 4, the peaks estimated to have the same DB were linked with a straight line, and the peaks estimated to have the same ECN were linked by a dotted line.

Fig. 4. Typical Comprehensive 2D separation of the perilla oil. Gradient conditions (1D): solvent A, *n*-hexane; solvent B, ACN/MTBE/*n*-hexane (5:20:100), solvent gradient program, from A/B (70/30) to (5/95) over 1000 min.; flowrate, 20 μ L/min. Gradient conditions (2D): solvent A, ACN/IPA (90/10); solvent B, ACN/IPA (10:90), solvent gradient program, from A/B (55/45) to (80/20) over 1000 min.; flowrate, 2 mL/min. Injection volume 10 μ L. Starting time of 2D gradient elution was 140 min. after the start of 1D separation. Magnification of the 2D chromatogram is shown to the right of the figure, where the 2D chromatogram was divided into 5 sections to clearly show the peaks of the minor components. Other conditions are in the text.

Fig. 5. FAs composition of perilla oil determined in this work. The assignments are begin with the corresponding fractions in Fig. 4, followed by a set of the three FAs, where the order of the FAs is in the reverse order as in Table 1. TGs consisted of one kind of FA are illustrated as one blue bar, whereas TGs consisted of two or three different FAs are illustrated in two or three color bars, with red and green as the second and third colors, respectively.

3.2. Assignment of TGs in perilla oil

Qualitative analysis of the perilla TGs in GC was performed to confirm the assignments in Fig. 4. For the GC analysis, most of the fractions in Fig. 4 were collected, where the peaks having a DB of 2 or less were not able to be analyzed due to a poor sensitivity of these minor components, although an improved sensitivity can be expected with increasing the amount of the perilla oil sample. Since one TG molecule contains three FA molecules, three peaks are observed for the TGs consisted of three components. In this case, the ideal analytical result is that the ratio of the molar fractions of FAs is 1:1:1.

Fig. 6. 1D separation of a tuna oil in Ag-LC. Gradient conditions: solvent A, *n*-hexane; solvent B, ACN/MTBE/*n*-hexane (5:20:100), solvent gradient program, from A/B (60/40) to (5/95) over 300 min.; flowrate, 200 μ L/min. Detection, UV at 210 nm. Injection volume 100 μ L.

Fig. 7. 2D separation of a tuna oil. Sample: (A) F1; (B) F2 and (C) F3 in Fig. 6. Solvents for gradient elution (1D): solvent A, *n*-hexane; solvent B, ACN/MTBE/*n*-hexane (5:20:100). Solvents for gradient elution (2D): solvent A, ACN/IPA (90/10); solvent B, ACN/IPA (10:90). Gradient program for F1 (1D): from A/B (70/30) to (10/90) over 1000 min.; flowrate, 20 μ L/min. Gradient program for F1 (2D): from A/B (60/40) to (78/22) over 1000 min.; flowrate, 2 mL/min. Injection volume 10 μ L. For F1 starting time of 2D gradient elution was 145 min. after the start of 1D separation. Gradient program for F2 (1D): from A/B (50/50) to (5/95) over 1000 min.; flowrate, 20 μ L/min. Gradient program for F2 (2D): from A/B (75/25) to (80/20) over 1000 min.; flowrate, 2 mL/min. Injection volume 10 μ L. For F2 starting time of 2D gradient elution was 270 min. after the start of 1D separation. Gradient program for F3 (1D): from A/B (40/60) to (5/95) over 1000 min.; flowrate, 20 μ L/min. Gradient program for F3 (2D): from A/B (80/20) to (84/16) over 400 min.; flowrate, 2 mL/min. Injection volume 10 μ L. For F3 starting time of 2D gradient elution was 600 min. after the start of 1D separation. Other conditions are in the text.

Fig. 8. FAs composition of the tuna oil sample determined in this work. The assignments are begin with the corresponding fractions in Fig. 7. Drawing rule is the same as in Fig. 5.

Introducing the GC analysis, all the FAs were separated, and the area ratios of the FA peaks are obtained. In order to confirm the molar fraction from the area ratio of the peaks, it is necessary to correct the molar sensitivity to the peak area. In addition, when a mixture of compounds having different boiling points is injected into a gas chromatograph, a phenomenon called discrimination occurs. Components having a low boiling point are easily introduced into the column, and that with high boiling point are relatively difficult to be introduced. In the case of FA methyl esters for perilla TGs, a smaller peak area for Ln to that of P is observed. Taking into account the molar sensitivity and discrimination, all the FA compositions were estimated in this work.

The results are summarized in Fig. 5. All the FA compositions in perilla TGs were determined. In most of the TGs, the peak areas have a good agreement with the ideal molar fraction ratios. In the GC analysis of FAs, a trace component of FAs were observed, however, these very minor peaks were ignored. This is because the resolution of peaks in Fig. 4 was not satisfactory to the perfect separation of all peaks. In addition, in the case of TGs with DB of 2 or less, it was possible to determine the FA compositions of the TGs, if they were not qualitatively analyzed in GC.

3.3. Two-dimensional separation of tuna TGs and the assignments

Next, two-dimensional separations were performed for tuna TGs having a more complicated composition than that of perilla. The chromatogram of the tuna TGs in Ag-LC is depicted in Fig. 6. Because of a kind of very long analytical time for the separation of the tuna TG sample, it was roughly separated into three peak groups. Each peak groups were fractionated in fractions, F1, F2 and F3 in Fig. 6. It is thought that the tuna TGs are roughly separated in Ag-LC according to the number of eicosapentaenoic acid and docosahexaenoic acid, where the TGs in F1 contain 0 or 1 of these FAs. Therefore, it can be concluded from the above consideration that DBs of the tuna TGs in the fractions F1, F2 and F3 are 0 to 9, 10 to 13 and 14 to 18, respectively.

As similar to the separation of the perilla TGs, all the fractions obtained from the tuna oil were separated with the comprehensive 2D-LC system developed in this work. A typical 2D-LC chromatograms of three fractions are shown in Fig. 7. The assignments of FAs are confirmed with the subsequent GC analysis of all the peak components as a similar manner as described above. The peak area ratios of the FA peaks of the tuna TGs are summarized in Fig. 8. As the same as the perilla TGs, the results of the qualitative analysis were consistent with the estimated FA compositions.

4. Conclusions

An on-line coupled comprehensive 2D-LC system was developed for the separation of TGs. Introducing a 2D-LC separation for the analysis, the TGs in perilla and tuna oils have been comprehensively separated in this work. With a systematic analysis of the GC data, all the peak assignments in the 2D-LC separation were successfully made, suggesting an excellent performance of the developed system for the comprehensive 2D-LC separation of TGs in perilla and tuna oils. The performance of the developed comprehensive 2D separation system can be comparable to that obtained by a 2D separation by supercritical fluid chromatography (SFC) [10] reported earlier, however, the operation of the present 2D separation system is much easier than that of typical SFC instruments.

Further applications of the developed 2D-LC system are expected, especially for a high performance separation of other complex samples such as sardine and salmon oils, and also for a more effective separation when introducing appropriate sample preparation techniques [38-44] before the separations.

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