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Technical Report

Optimizing Analytical Conditions for Lipid Analysis by SFC and Its Application for Precise Preparative Chromatography – Case Study of Diglyceride Isomers –

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Abstract:

Supercritical fluid chromatography (SFC) provides a unique separation behavior for relatively hydrophobic components. Components and columns exhibit complex interactions in supercritical carbon dioxide, which has a high diffusion coefficient. Another key benefit of supercritical carbon dioxide is that it can significantly reduce the volume of fractions by quickly evaporating during preparative chromatography. That means SFC enables both precision separation and efficient preparative fractionation at the same time. This report describes the use of SFC for the analysis of lipids in the presence of multiple structural isomers. SFC could also find applicability in lipidomic research and other applications.

Keywords: Supercritical fluid chromatography (SFC), precision preparative chromatography, preparative SFC, FRC-40SF, lipid, diglyceride, carryover

1. Background

SFC can be used to analyze a wide range of either hydrophobic or hydrophilic compounds. In this reports glycerides were used as model compounds (typical hydrophobic components which have many analogs and isomers) for providing an example of hydrophobic components analysis in food. In contrast to GC and LC, SFC enables separating all glycerides simultaneously, including structural isomers, without derivatization.

2. Diglyceride Isomers

Glyceride structures are either triglycerides (TG), diglycerides (DG), or monoglycerides (MG).

DGs are composed of two fatty acids bound to a glycerol. However, glycerol includes three positions where fatty acids can be bound forming two isomers (1,3-DG or 1,2-DG).

In this report, Distearin (C18:0/18:0) and Diolein (C18:1/18:1) were used as examples for investigating isomer separation. The structural formulas of DG isomers are shown in Fig. 1.

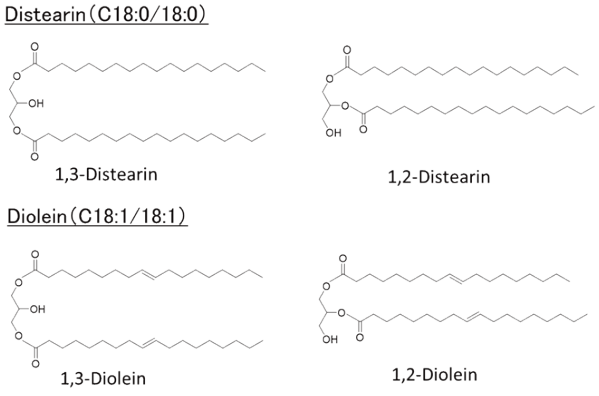

Fig. 1 Structural Formulas of DG Isomers

3. System Configuration

The system configuration used is shown in Fig. 2.

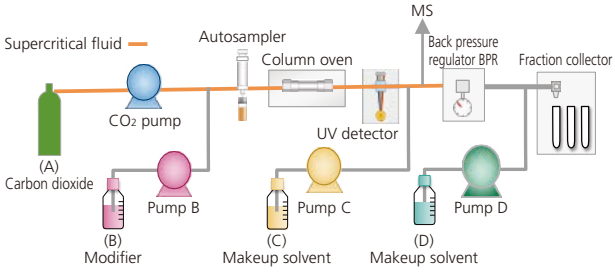

Fig. 2 System Configuration

4. Analytical Condition Settings

SFC is especially useful for analyzing components with relatively high hydrophobicity, such as DG. It enables isomer analysis with better separation than HPLC without using derivatization. An evaluation of various column types indicated that the Shim-pack™ UC-Diol (normal phase column) provides the best separation and confirmed that the column separates all DG isomers. Analytical conditions are listed in Table 1, with the resulting chromatograms shown in Fig. 3. The Diol column showed good separation presumably because of an interaction between the OH group on the glycerol portion of DG and the silanol in the column packing material. In terms of the isomer elution order, the 1,3-DG eluted first and the 1,2-DG isomer eluted second, presumably because the 1,3-DG OH group is in position 2, sandwiched between hydrophobic fatty acids, where it interacts more weakly with silanol than the 1,2-DG isomer with the OH group in position 3. Furthermore, interaction between the fatty acids portions of DG and the diol presumably contributed to the separation of Distearin and Diolein.

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Table 1 Analytical Conditions

System	:	Nexera™ UC
Column	:	Shim-pack UC-Diol (250 mm L.×4.6 mm I.D., 5 µm)
Mobile phase	:	A) CO_2
		B) Modifier: 0.1% Ammonium formate / Methanol
Make up	:	C) 0.1% Ammonium formate / Methanol solution
	:	D) –
Modifier conc.	:	5%
Flow rate	:	2.0 mL/min
	:	C) 0.5 mL/min
	:	D) –
Column temp.	:	40 °C
BPR temp.	:	50 °C
BPR pressure	:	10.0 MPa
Detection	:	LCMS-8040 (ESI, Positive mode, *m/z* 603.3, 607.3)

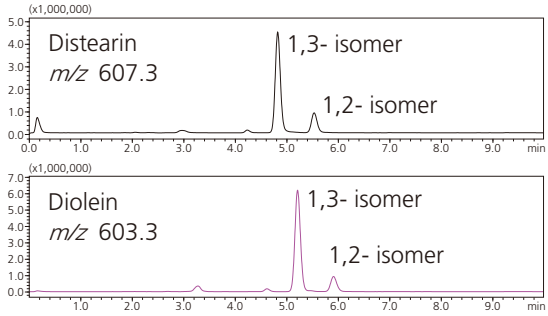

Fig. 3 Mass Chromatograms of Distearin and Diolein Isomers

5. The Method to Reduce Carryover

As a side note, hydrophobic compounds are prone to adsorbing as residues inside instruments, which can cause carryover. Carryover can prevent obtaining accurate quantitative values, which is a major issue for systems used for quantitative analysis. For precision preparative chromatography, carryover can cause serious problems from non-target components contaminating samples. Therefore, methods to prevent carryover were considered.

In this example, methods for preventing carry over at the rotary seal of the autosampler high pressure valve are described. The program below was specified to flush component residues away from the rotor seal by repeatedly switching the rotor between the load position and injection position. The program indicated in Fig. 4 was added to the standard pretreatment program in LabSolutions™ ([Instrument Parameters]-[Autosampler]-[Pretreatment]).

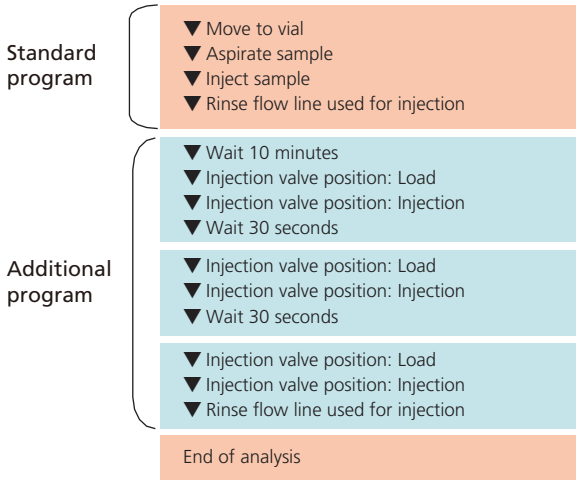

Fig. 4 Autosampler Pretreatment Program

Moreover, to increase rinsing efficiency, a gradient program (Table 2) was added to maintain the modifier concentration at 5% for 2 minutes after the elution of all target components (10 minutes after injection). During this time, the rotor seal was switched for wash. That resulted in reducing carryover levels (Fig. 5). As a precaution for this additional program, be sure to specify a wait time between the injection valve "Injection" and "Load" positions, such as the "Wait 30 seconds" step set in the pretreatment program. The pressure inside the flow lines returns to equilibrium by this wait time, and problems prevent the following analysis.

Table 2 Timing for Executing Pretreatment Program

Gradient Program [B.Conc.]	
0-5%(10 min)	Additional gradient program Executed for switching and rinsing the rotor seal.
5%(2 min)	
5-40%(0.01 min)	
40%(3 min)	

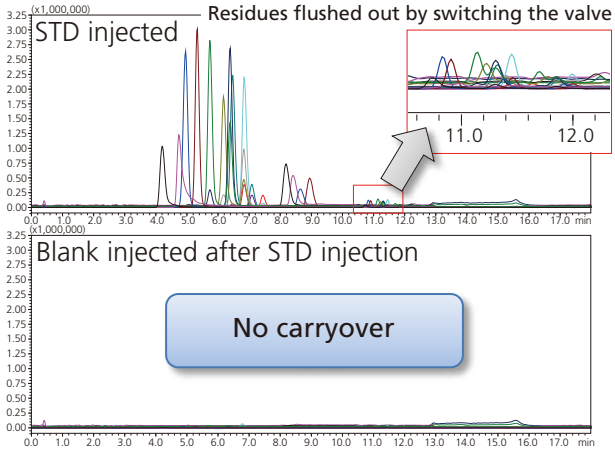

Fig. 5 Mass Chromatograms that Show Effect of Carryover Reduction

6. How to Set Analytical Conditions for Precision Preparative Chromatography

After determining the method to reduce carryover, preparative chromatography conditions were examined. Though a model experiment is described in this article, actual experiments can sometimes involve determining structures by accurate mass spectrometry or NMR analysis after preparative separation of unknown components. Carbon dioxide evaporates during preparative chromatography, so a makeup solvent C or D needs to be added. First, when makeup solvent C was methanol which is used as a modifier, it became evident that there was a problem with isomers contaminating each fraction. The preparative conditions are indicated in Table 3, with the resulting chromatograms for Distearin and Diolein fractions shown in Fig. 6 and 7.

Thus, if fractions are directly separated using those analytical conditions, carryover could prevent precise preparative separation. With SFC, the supercritical carbon dioxide evaporates from the fluid after passing through the BPR. That means the separated components must be completely dissolved in the modifier or makeup solvent. Because the DG has relatively high hydrophobicity, the methanol used as a modifier and makeup solvent presumably had difficulty dissolving the DG. Given that structural analysis by NMR requires a purity of at least 95 %, it is important to consider the solvent used for the makeup to reduce carryover.

Table 3 Analytical Conditions

System	:	Nexera UC
Column	:	Shim-pack UC-Diol (250 mm L.×4.6 mm I.D., 5 µm)
Mobile phase	:	A) CO_2
		B) Modifier: 0.1% Ammonium formate / Methanol
Make up	:	C) Methanol
	:	D) –
Modifier conc.	:	5%
Flow rate	:	2.0 mL/min
	:	C) 1.5 mL/min
	:	D) –
Column temp.	:	40 °C
BPR temp.	:	50 °C
BPR pressure	:	10.0 MPa
Detection	:	UV210 nm

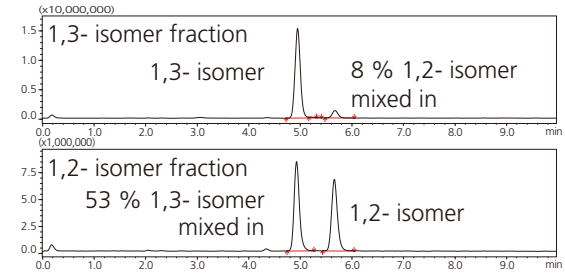

Fig. 6 Mass Chromatograms of Each Distearin Isomer Fraction

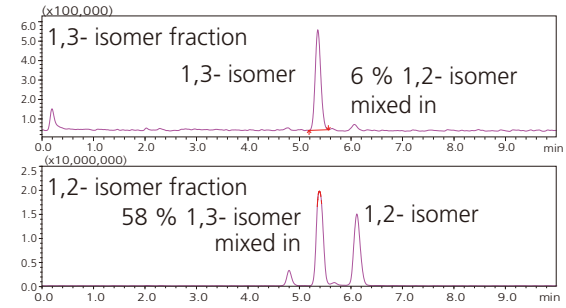

Fig. 7 Mass Chromatograms of Each Diolein Isomer Fraction

6-1. Cause of Carryover

The following discusses the causes of the carryover in more detail.

The first cause is inadequate dissolution of the DG, as mentioned above. That should be improved by switching the methanol used for the makeup solvents to a solvent that dissolves DG more readily.

The second cause is coelution. To verify that phenomenon, the fractions immediately before and after DG elution were analyzed. The preparative fractions for diolein are illustrated in Fig. 8, with the corresponding mass chromatograms shown in Fig. 9. They show that the 1,2- isomer was eluted in fraction 5, whereas the 1,2- isomer eluted mixed into fraction 2 (1,3- isomer). If 1,2- isomer residues were remaining in the flow lines, then the 1,2- isomer should also appear mixed in fractions 1 and 3, but it was detected in neither fraction.

In contrast, the 1,3- isomer eluted during fraction 2 was also detected in other factions. That presumably indicates that 1,3- isomer residues in the flow lines were mixing into the other fractions. In that case, 1,3- isomer peaks should be successively smaller in fractions 3 to 5, but a large peak was detected in fraction 5. In other words, a larger quantity mixed into fraction 5, the fraction that elutes 1,2- isomer elution.

These results indicate that isomer elution was selectively timed to mix with the other isomer. At that point, the tentative explanation for that phenomenon was that DG residues (adsorbed) in the flow lines were being flushed out and coeluted by subsequent DG. That process is illustrated in Fig. 10. The flow line where contamination presumably occurred, between the six position valve (downstream from pump D) and the fraction collector, is made of a fluoropolymer material that is thought to adsorb hydrophobic compounds more easily than stainless steel.

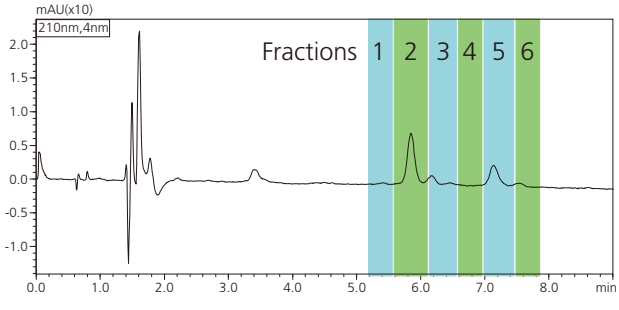

Fig. 8 Illustration of Diolein Isomer Preparative Separation Points

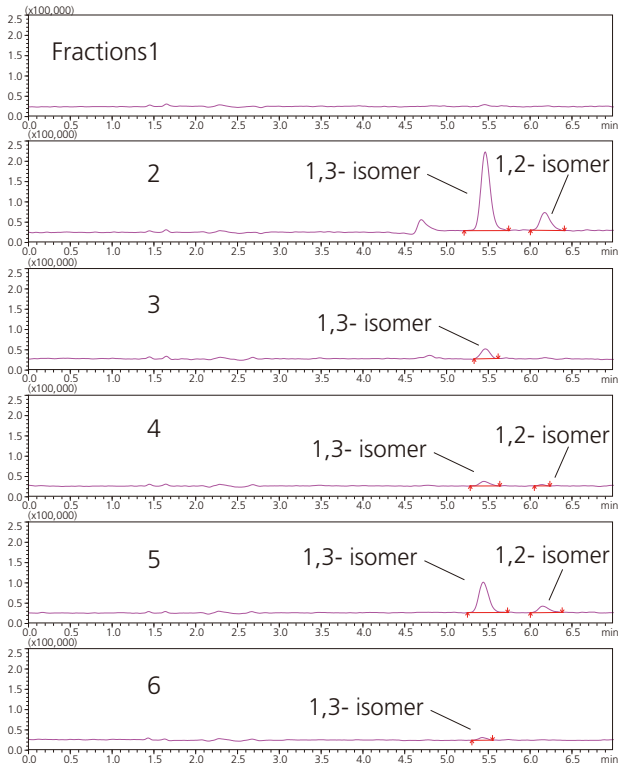

Fig. 9 Mass Chromatograms of Diolein Isomer Fractions

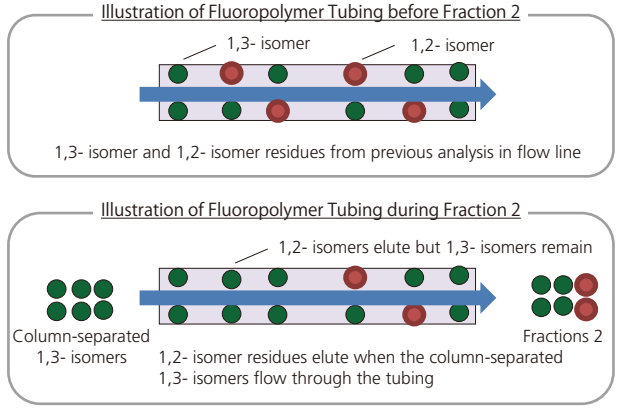

Fig. 10 Illustration of Coelution

6-2. Study of Makeup Solvent C

To verify the first cause, makeup solvent C was changed to a hexane/acetone (2:1) mixture with higher dissolving capacity. The preparative conditions are indicated in Table 4, with the resulting chromatograms shown in Fig. 11 and 12. Changing the makeup solvent to hexane/acetone (2:1), same as for the sample solvent, significantly reduced the amount of isomer carryover.

Table 4 Analytical Conditions

System	:	Nexera UC
Column	:	Shim-pack UC-Diol (250 mm L.×4.6 mm I.D., 5 µm)
Mobile phase	:	A) CO_2
		B) Modifier: 0.1% Ammonium formate / Methanol
Make up	:	C) Hexane / Acetone (2:1)
	:	D) –
Modifier conc.	:	5%
Flow rate	:	2.0 mL/min
	:	C) 2.5 mL/min
	:	D) –
Column temp.	:	40 °C
BPR temp.	:	50 °C
BPR pressure	:	10.0 MPa
Detection	:	UV210 nm

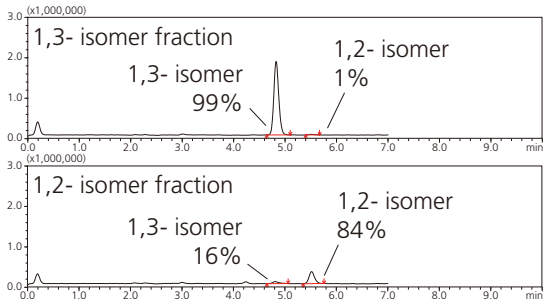

Fig. 11 Mass Chromatograms of Distearin Isomer Fractions

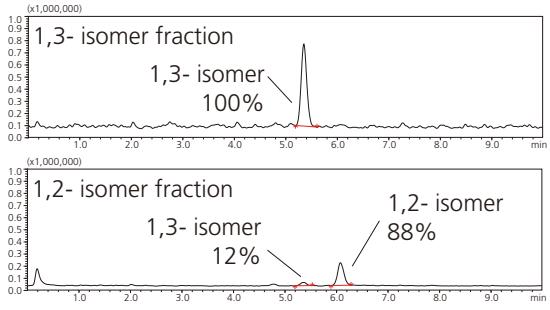

Fig. 12 Mass Chromatograms of Diolein Isomer Fractions

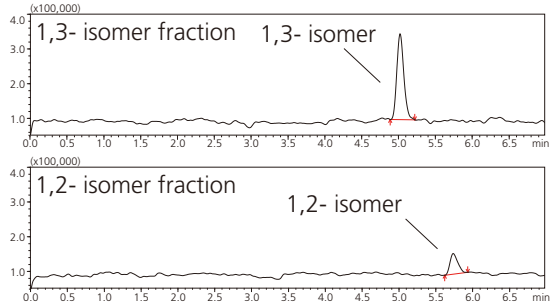

Fig. 13 Mass Chromatograms of Distearin Isomer Fractions

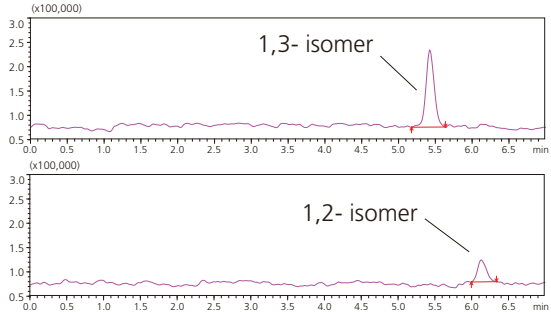

Fig. 14 Mass Chromatograms of Distearin Isomer Fractions

6-3. Study of Makeup Solvent D

Next, the second cause was verified. To prevent DG from adsorbing to the fluoropolymer tubing, makeup solvent D was changed to chloroform, which has higher solubility than the sample solvent. The preparative conditions are indicated in Table 5, with the resulting chromatograms shown in Fig. 13 and 14. The results showed that carryover was eliminated from respective fractions and established the preparative conditions for obtaining high-purity DG isomer fractions. Note that, though similar effects could be expected using chloroform for makeup solvent C, the chloroform in makeup solvent D provided higher rinsing capacity than the hexane/acetone (2:1) mixture.

Table 5 Analytical Conditions

System	:	Nexera UC
Column	:	Shim-pack UC-Diol (250 mm L.×4.6 mm I.D., 5 µm)
Mobile phase	:	A) CO_2
		B) Modifier: Hexane / Acetone (2:1)
Make up	:	C) Hexane / Acetone (2:1)
	:	D) Chloroform
Modifier conc.	:	5%
Flow rate	:	2.0 mL/min
	:	C) 0.5 mL/min
	:	D) 2.0 mL/min
Column temp.	:	40 °C
BPR temp.	:	50 °C
BPR pressure	:	10.0 MPa
Detection	:	UV210 nm

7. Conclusions

In this report, optimal conditions for analysis of diglyceride isomers and preparative chromatography by SFC were established. The process of examining analytical conditions and precision preparative chromatography parameters revealed that carryover occurred in two locations.

The first location was at the rotor seal of the autosampler high pressure valve. The second location was in flow lines downstream from the BPR, where carbon dioxide is vaporized. These carryover levels were successfully reduced by modifying the autosampler pretreatment program and pumping more optimal makeup solvents.

In this report, the analysis points specific to SFC were introduced Hopefully, by sharing similar examples among more people, SFC will be used effectively in more fields.

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