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Technical Report

Study of Effective Parameters for Separation of Isomers of Fluorescent Substance by Supercritical Fluid Chromatography

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Abstract:

In order to detect the sites of trace amounts of cancers within the bodies of human patients in a short time, research and development is being performed with respect to fluorescent probes, molecules that emit light only after the reactions with biomarker enzymes selective for cancer sites.*1 With rhodamine and fluorescein, which are commonly used as the nuclei for fluorescent probes, when molecules with substituents introduced by typical methods are synthesized, isomers are created that differ with respect to the site of substitution on the benzene ring region. In order to be used as the nuclei for fluorescent probes, it is important that these isomers be separated. Separation of isomers of the fluorescent substance carboxytetramethylrhodamine was optimized using the Nexera UC supercritical fluid chromatograph. This article will discuss the factors with an impact on the separation of the isomers.

Keywords: SFC, Supercritical fluid chromatography, method scouting

1. Separating the Isomers of Fluorescent Substances

In order to detect the sites of trace amounts of cancers within the bodies of human patients in a short time, research and development is being performed with respect to fluorescent probes, molecules that emit light only after the reactions with biomarker enzymes selective for cancer sites. With rhodamine and fluorescein, which are commonly used as the nuclei for fluorescent probes, as shown in Fig. 1, molecules consist of a benzene ring region and a xanthene ring region. If molecules are synthesized with substituents introduced by typical methods, isomers are created that differ with respect to the site of substitution on the benzene ring region. (In the case of the substance in Fig. 1, the carboxyl group is bound at either the 5th or 6th positions in the isomers.) In order to be used as the nuclei for fluorescent probes, it is important that these isomers be separated.

To separate the isomers, high-performance liquid chromatography (HPLC) is utilized in reverse phase mode and normal phase mode. However, supercritical fluid chromatography (SFC) is gaining attention as a new separation method. In SFC, the supercritical fluid carbon dioxide, the main mobile phase, has the following two characteristics: 1) a polarity close to that of hexane, and 2) a relatively low viscosity and high diffusibility for a liquid. A separation selectivity different from that of UHPLC is created, which can be expected to improve the separation of components and samples that have been hard to separate to date using HPLC. However, the factors having an impact on separation have not been fully analyzed. In order to obtain better separation, it is important to understand the parameters with an impact on separation.

2. Fluorescent Substances

The fluorescent substance utilized in this article is carboxytetramethylrhodamine, the chemical structure of which is shown in Fig. 1. In this article, we investigate the separation of carboxytetramethylrhodamine, and demethylated isomers created simultaneously during synthesis.

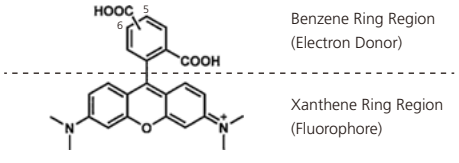

Fig. 1 Chemical Structure of 5 (6) Carboxytetramethylrhodamine

3. Column Scouting

The column scouting conditions and the stationary phase used are shown in Table 1 and 2. A comprehensive investigation was performed utilizing a variety of columns including those with typical octadecyl group and diol group stationary phases.

Table 1 Column Scouting Conditions

Column	:	See Table 2. (250 mm L. × 4.6 mm I.D., 5 µm)
Mobile phase	:	A; CO_2
		B; Modifier: 0.1%TFA Methanol
Gradient	:	10% (0min) -50% (5-8min) -10% (8.01-11min)
Flow rate	:	3 mL/min
BPR pressure	:	10 MPa
BPR temp.	:	50 °C
Column temp.	:	40 °C
Detection	:	Photo diode array detector (wave length = 190-600 nm)
		PDA Chromatogram 550 nm
		LCMS-8060 (ESI, scan mode *m/z*: 400-500)

Table 2 Analysis Column

	Column Name	Stationary Phase
(1)	Shim-pack UC-Sil	Silica
(2)	Shim-pack UC-RP	Octadecyl Group+Polar Functional Group
(3)	Shim-pack UC-Choles	Cholesteryl Group
(4)	Shim-pack UC-PyE	Pyrenylethyl Group
(5)	Shim-pack UC-PBr	Pentabromobenzyl Group
(6)	Shim-pack UC-Diol	Diol Group

The separation patterns for six analysis columns are shown in Fig. 2. The reverse phase columns ((2) UC-RP and (3) UC-Choles), in which a hydrophobic interaction with either the octadecyl group or the cholesteryl group was likely, could not perform the separation. Additionally, with the pyrenyl ethyl group ((4) US-PyE), in which a π-π interaction was expected, no peaks were detected, as there was no elution as the compounds were strongly retained. In contrast, with the pentabromobenzyl group ((5) UC-PBr), in which a dispersion force other than a hydrophobic interaction was likely between the bromine and the analyte, four peaks were detected. Two isomers of carboxytetramethylrhodamine, and their respective demethylated compounds were separated.

1 Analytical & Measuring Instruments Division

With normal phase columns, a total of four peaks were detected with both the diol column ((6) UC-Diol) and the silica column ((1) UC-Sil). In particular, in the case of the Shim-pack UC-Sil, the four peaks were clearly separated. This is surmised to be due to the interaction between the hydroxyl group, a stationary phase often present in silica columns, and the carboxytetramethylrhodamine amine group. Additionally, a distinction in the strength of the above-mentioned interaction was created due to the binding position of the carboxyl group at either the 5th or 6th carboxytetramethylrhodamine position, which likely enabled the isomers to be separated.

The separation pattern for the isomers differed significantly depending on the type of stationary phase. In the early stages of method development, column selection becomes important.

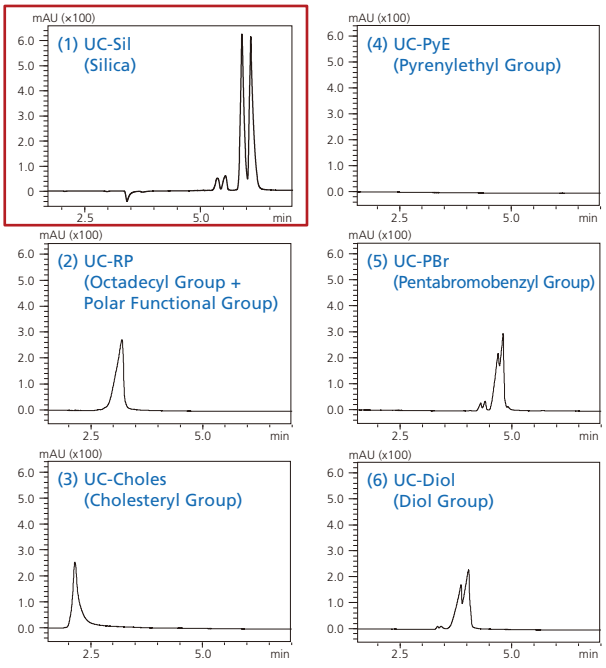

Fig. 2 Comparison of the Separation of Carboxytetramethylrhodamine and Demethylated Compounds by Six Analysis Columns

The MS spectra and PDA spectra for the peaks separated by the Shim-pack UC-Sil shown in the chromatogram in Fig. 3 are shown in Fig. 4. In order to identify the peaks, a mass spectrometer was connected after the PDA detector, and a scan analysis was performed. According to the MS spectrum, the respective protonated compounds were detected. The first two peaks (Peak 1 and Peak 2) were evidently demethylated carboxytetramethylrhodamine compounds, while the latter two peaks were evidently carboxytetramethylrhodamine.

Additionally, it was confirmed that λmax for both the demethylated compounds was 534 nm, and that λmax for carboxytetramethylrhodamine was 546 nm.

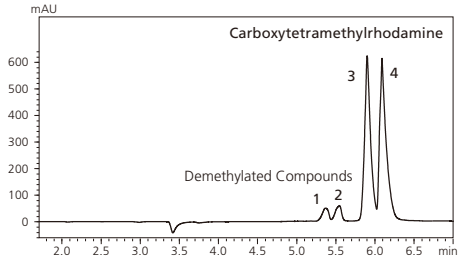

Fig. 3 Chromatogram from the Shim-pack UC-Sil

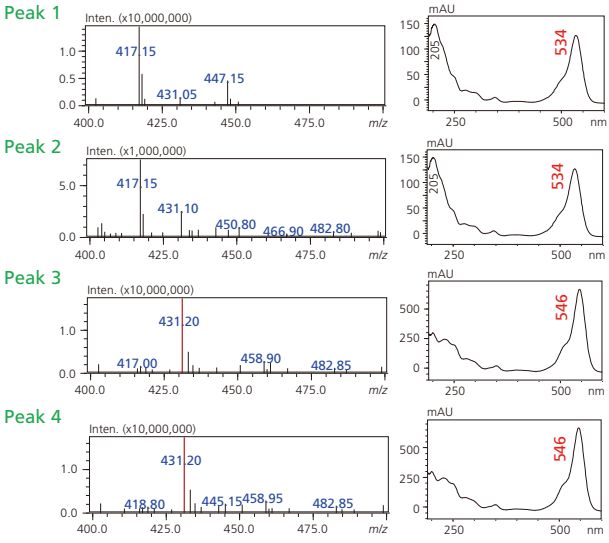

Fig. 4 MS Spectra and PDA Spectra for the Peaks

4. Effects of Modifier Solvents and Additive Salts

Using the Shim-pack UC-Sil, the optimal silica column for separating the carboxytetramethylrhodamine, a comparison was performed of solvents and additive salts used as modifiers. The analysis conditions are shown in Table 3.

Table 3 Analysis Conditions

Column	:	Shim-pack UC-Sil (250 mm L. × 4.6 mm I.D., 5 µm)
Mobile phase	:	A; CO_2 B; Modifier: 0.1%TFA Acetonitrile Alternatively 0.1 % TFA Methanol Alternatively 0.1 % Formic Acid Methanol
Gradient	:	10% (0min) -50% (5-8min) -10% (8.01-11min)
Flow rate	:	3 mL/min
BPR pressure	:	10 MPa
BPR temp.	:	50 °C
Column temp.	:	40 °C
Detection	:	Photo diode array detector (wave length = 190-600 nm) PDA Chromatogram 550 nm

Fig. 5 shows the chromatograms when acetonitrile or methanol was used as an organic solvent. In the case of acetonitrile, very broad peaks were obtained, unlike when methanol was used. Acetonitrile is a solvent with a lower elution capability than methanol for SFC, so it was surmised that the analyte components were strongly retained by the column, and that elution was insufficient.

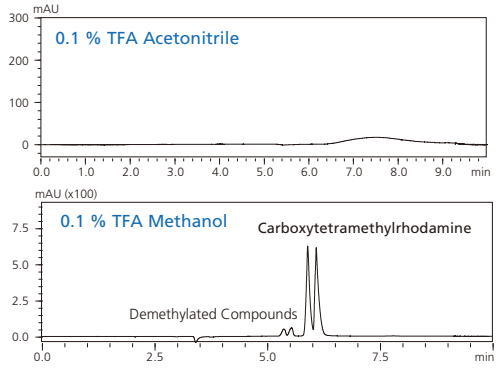

Fig. 5 Comparison of Organic Solvents Used in Each Modifier

When trifluoroacetic acid (TFA) was used as an additive salt, the isomers could be separated. However, as shown in Fig. 6, in the case of formic acid, the peaks were not separated, and it was confirmed that the elution order was different from the PDA spectrum. It was evident that the salt additive had an impact on elution.

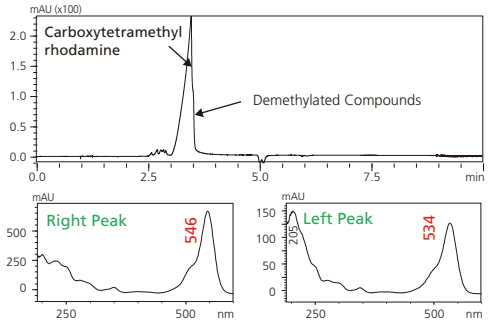

Fig. 6 Chromatogram and PDA Spectra when Using 0.1 % Formic Acid Methanol

A small amount of water was used, as it is the modifier with the greatest elution capability in SFC, and its impact on peak shape and separation patterns was investigated. The chromatograms when water was added and when it was not are shown in Fig. 7. Sharper peaks were obtained by adding water to the modifier, an effect that was striking for the demethylated compounds with quick elution times. It was evident that adding a small amount of water improved the separation of the isomers.

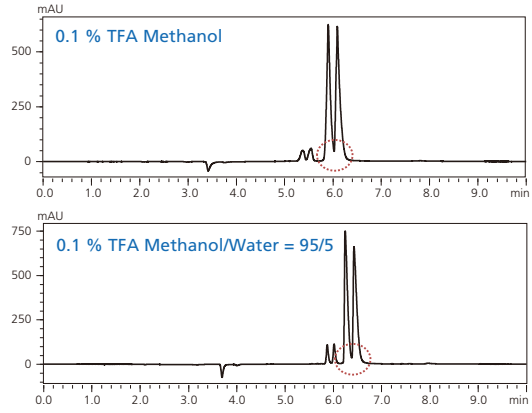

Fig. 7 Chromatograms with Water Added/Not Added to the Modifier

5. Impact of Column Temperature

The impact of the column oven temperature setting investigated utilizing the analysis conditions in Table 3 is shown in Fig. 8. The separation was better when the temperature setting was 20 °C. It was confirmed that lowering the column temperature improved the separation of the isomers.

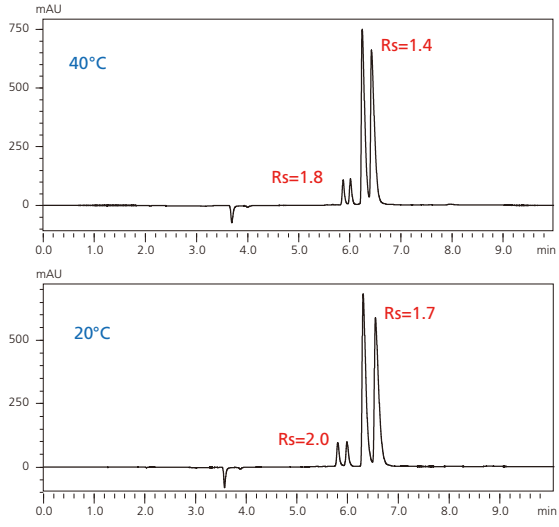

Fig. 8 Chromatograms for Two Column Oven Temperature Settings
(The USP method was used to calculate the degree of separation Rs.)

6. Impact of the Mobile Phase Flowrate

Due to the low viscosity and high diffusivity of supercritical fluid carbon dioxide, the SFC column load pressure is low even at high flow speeds, which enables the analysis speed to be increased without losing column efficiency. The impact of mobile phase flowrate was investigated utilizing the analysis conditions shown in Table 4. The chromatograms at each flowrate are shown in Fig. 9. The best degree of separation was obtained at 2 mL/min. It was evident that when the flowrate was further increased, peaks were sharpened without losing the degree of separation.

Table 4 Analysis Conditions

Column	:	Shim-pack UC-Sil (250 mm L. × 4.6 mm I.D., 5 µm)
Mobile phase	:	A; CO_2
		B; Modifier: 0.1%TFA Methanol/Water=95/5
Gradient	:	10% (0min) -50% (5-8min) -10% (8.01-11min)
Flow rate	:	2, 2.5, 3, 3.5 mL/min
BPR pressure	:	10 MPa
BPR temp.	:	50 °C
Column temp.	:	20 °C
Detection	:	Photo diode array detector (wave length = 190-600 nm)
		PDA Chromatogram 550 nm

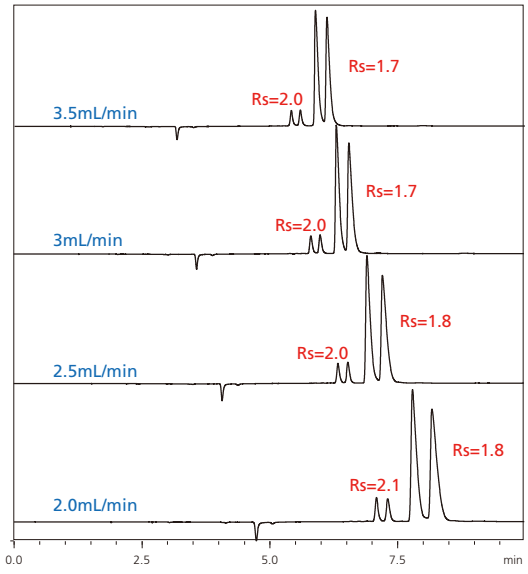

Fig. 9 Chromatograms at Each Mobile Phase Flowrate

7. Impact of Back Pressure

In SFC, the pressure of the supercritical fluid carbon dioxide is controlled with a back pressure valve. The impact of the pressure setting for the back pressure control valve was compared utilizing the analysis conditions in Table 5. As indicated in Fig. 10, there was no noticeable difference in the degree of separation of the isomers at 10 MPa and 15 MPa. However, sharper peaks were obtained at 15 MPa. When the back pressure was increased, the CO_2 concentration also likely increased. As a result, the elution was faster, and sharper peaks were obtained.

Table 5 Analysis Conditions

Column	:	Shim-pack UC-Sil (250 mm L. × 4.6 mm I.D., 5 µm)
Mobile phase	:	A; CO_2
		B; Modifier: 0.1%TFA Methanol/Water=95/5
Gradient	:	10% (0min) -50% (5-8min) -10% (8.01-11min)
Flow rate	:	3 mL/min
BPR pressure	:	10 MPa, 15 MPa
BPR temp.	:	50 °C
Column temp.	:	20 °C
Detection	:	Photo diode array detector (wave length = 190-600 nm)
		PDA Chromatogram 550 nm

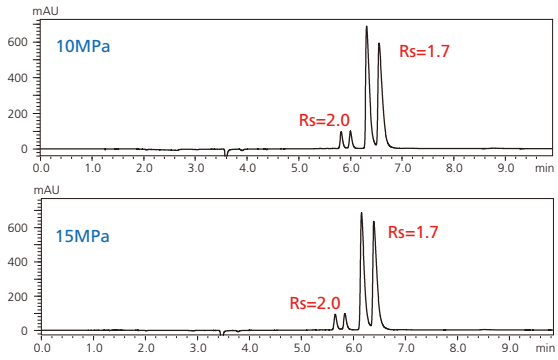

Fig. 10 Chromatograms at Each Back Pressure

8. Improved Separation Using Multiple Solvents as the Modifiers

A comparison performed when using multiple organic solvents as the modifiers utilizing the analysis conditions shown in Table 6 is shown in Fig. 11.

Table 6 Analysis Conditions

Column	:	Shim-pack UC-Sil (250 mm L. × 4.6 mm I.D., 5 µm)
Mobile phase	:	A; CO_2 B; Modifier: 0.1%TFA Methanol/Water=95/5 0.1%TFA Methanol/Ethanol/Water=50/45/5 0.1%TFA Methanol/Isopropanol/Water=50/45/5
Gradient	:	10% (0min) -50% (5-8min) -10% (8.01-11min)
Flow rate	:	2 mL/min
BPR pressure	:	15 MPa
BPR temp.	:	50 °C
Column temp.	:	20 °C
Detection	:	Photo diode array detector (wave length = 190-600 nm) PDA Chromatogram 550 nm

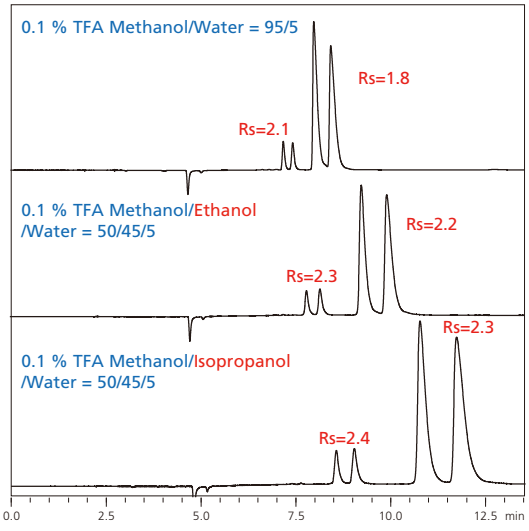

Fig. 11 Chromatograms when Using Multiple Organic Solvents

Separation was improved by utilizing a liquid mixed with solvents with a lower elution capability than methanol. (Elution capability: Methanol > Ethanol > Isopropanol) However, with isopropanol, the peaks were significantly broader. It was evident that the separation could be controlled by using multiple organic solvents.

9. Comparison with HPLC

In the investigation to this point, gradient elution was performed starting from an initial concentration of 10 %. However, the analyte compounds were eluted while the modifier was held at 50 %, so an investigation was performed under isocratic conditions with a 50 % modifier, to confirm that there was no impact on separation. The SFC chromatogram when an isocratic analysis was performed using the optimal values for each parameter (Table 7) is shown in Fig. 12. In addition, the HPLC chromatogram when using the analysis conditions in Table 8 is shown. (The data was provided courtesy of the Urano Laboratory of the Faculty of Pharmaceutical Sciences, the University of Tokyo.)

Table 7 SFC Analysis Conditions

Column	:	Shim-pack UC-Sil (250 mm L. × 4.6 mm I.D., 5 µm)
Mobile phase	:	A; CO_2 B; Modifier: 0.1%TFA Methanol/Ethanol/Water=50/45/5 (Isocratic A/B=1:1)
Flow rate	:	2 mL/min
BPR pressure	:	15 MPa
BPR temp.	:	50 °C
Column temp.	:	20 °C
Detection	:	Photo diode array detector (wave length = 190-600 nm) PDA Chromatogram 550 nm

Table 8 HPLC Analysis Conditions

Column	:	ODS (250 mm L. × 4.6 mm I.D.)
Mobile phase	:	A; 0.1%TFA Water B; 0.1%TFA Acetonitrile
Gradient	:	20% (0min) -100% (25min)
Flow rate	:	1.0 mL/min
Detection	:	wave length 550 nm

With SFC and HPLC, it can be seen that the elution order is different for the analyte compounds. With SFC, the demethylated compounds were eluted first, followed by the two carboxytetramethylrhodamine compounds. In contrast, with HPLC, the demethylated compounds and carboxytetramethylrhodamine compounds were alternately eluted, indicating a difference in the separation behavior.

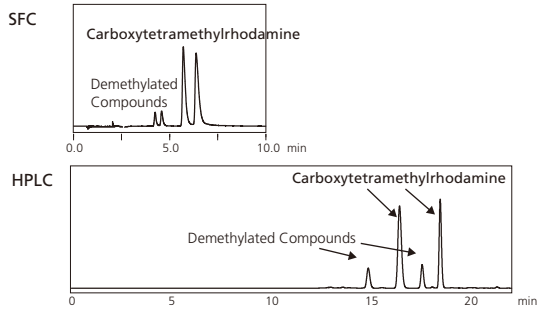

Fig. 12 SFC and HPLC Chromatograms for Carboxytetramethylrhodamine

Acknowledgments

The samples and data were provided courtesy of Professor Yasuteru Urano of the Graduate School of Pharmaceutical Sciences, the University of Tokyo.

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