C190-E270

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Supercritical Fluid Chromatograph

SFC Basic Guide

CO_2 cylinders used for supercritical fluid chromatography (SFC) are filled with supercritical carbon dioxide generated by industrial plants that manufacture ammonia, refine petroleum, and so on. In other words, no new carbon dioxide is generated for operating SFC. On the other hand, we generate new carbon dioxide in environment while treating the organic solvent waste from HPLC. Therefore, switching the separation method from HPLC to SFC reduces the quantity of CO_2 greenhouse gas emitted, achieving one of the sustainable development goals (SDGs) proposed by the United Nations, "13. Take urgent action to combat climate change and its impacts."

In this book, let's discuss about

- Basics of SFC
- SFC set-up and workflows
- Method development and settings
- Variety of SFC applications
- Frequently asked questions

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Chapter 1 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is a separation method that uses supercritical carbon dioxide as the mobile phase. Though SFC technology has existed for a long time, but due to recent advancements in SFC instrument and column technologies, it is now beginning to be used for regular lab use and measurements.

By using supercritical carbon dioxide, which can be mixed with water-soluble solvents despite its low polarity, SFC offers potential advanced separation methods that are different from conventional GC and HPLC separation. This chapter describes what is supercritical carbon dioxide with an overview about new separation methods which are made possible using supercritical carbon dioxide.

1-1 Properties of Supercritical Fluids

1-1-1 Physical Properties of Supercritical Fluids

Substances can change their state between solid, liquid, and gas states depending on the temperature and pressure. Vaporization and sublimation are phenomena that occur when heat activates the thermal motion of solid or liquid molecules in a substance, to an extent that the molecules break free from the intermolecular forces keeping the substance in its current state. For example, solid CO_2 (dry ice) in a sealed container sublimates to reach a supercritical state under high-temperature high-pressure conditions (Fig. 1). Supercritical state is when substance have properties of both gas and liquid and they are thus called as Supercritical Fluid. This point in phase diagram with corresponding conditions of temperature and pressure is called Critical Point (Table 1). Supercritical fluids have a higher coefficient of diffusion with lower density and viscosity than liquids. Consequently, higher diffusivity results in better distribution into the mobile phase and better separation, and lower viscosity makes SFC a faster method than HPLC.

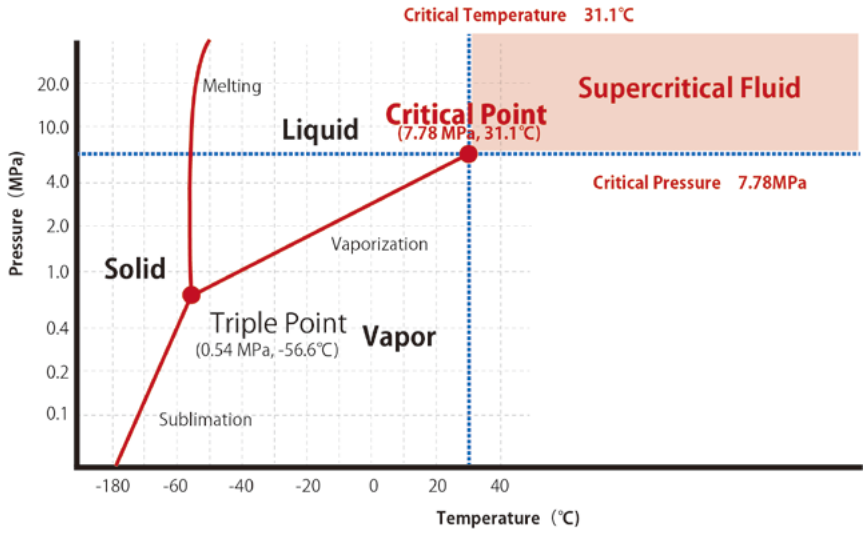


Fig. 1 Phase Diagram for Carbon Dioxide

Table 1 Physical Properties of Supercritical Fluids

	Diffusion Coefficient (m^2/s)	Density (g/cm^3)	Viscosity ($g/cm \cdot s$)
Liquids	10^{-6}	1	10^{-2}
Supercritical Fluids	10^{-3}	0.2-0.8	10^{-3}
Gases	10^{-1}	10^{-3}	10^{-4}

1-1-2 Supercritical Carbon Dioxide

Critical pressures and critical temperatures for generating a variety of supercritical fluids are shown in Fig. 2. Water is commonly used for high-performance liquid chromatography, but due to its critical pressure of 22.1 MPa and critical temperature of 374 °C, it cannot be easily kept under conditions necessary for transitioning to a supercritical state. In contrast, supercritical carbon dioxide has a critical pressure of 7.4 MPa and a critical temperature of 31.1 °C, hence allowing transition to the supercritical state under relatively modest conditions.

Supercritical carbon dioxide offers the following advantages.

(1) Handling

With critical points at 31 °C and 22 MPa, CO_2 becomes a supercritical fluid under modest conditions.

(2) Safety

CO_2 is not flammable and has minimal risk of explosion even if used compressed. CO_2 offers intrinsically low toxicity, when compared to other organic solvents.

(3) Cost

CO_2 is mainly sold as a liquid stored in high-pressure cylinders and in most cases can be acquired at a low cost.

(4) Environmental Friendliness

Other organic solvents are incinerated for disposal, which emits large amounts of energy and CO_2. However, liquified carbon dioxide gas is collected as a byproduct of fermentation processes or chemical plants. That means using liquified CO_2 causes minimal environmental impact.

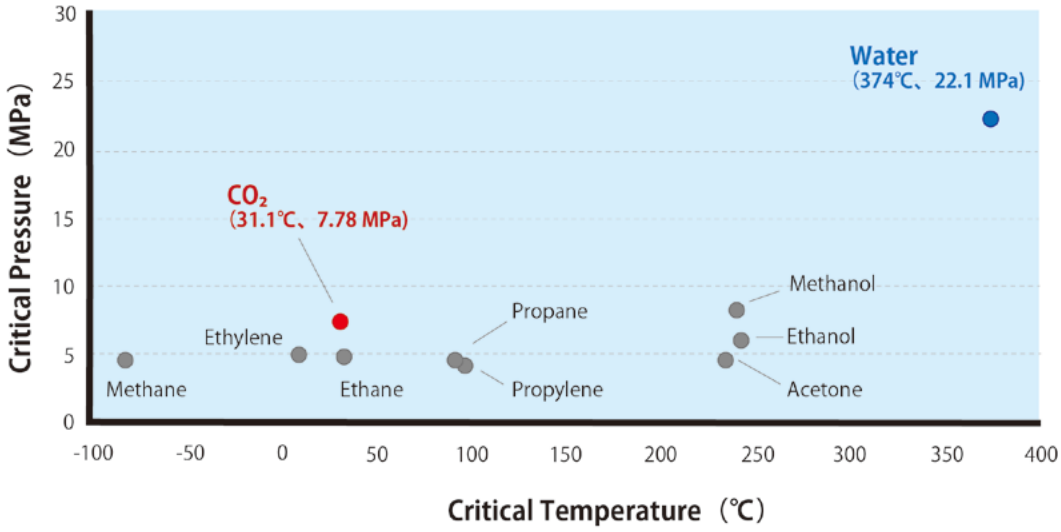


Fig. 2 Conditions for Generating Supercritical Fluids

1-2 Supercritical Fluid Chromatography

1-2-1 Chromatography in General

Chromatography is the collective term for various techniques used to separate target components in mixture samples, either chemically or physically, so that they can be qualitatively or quantitatively analyzed, collected, or for other purposes. In chromatography techniques, components are separated within two phases, a mobile phase and a stationary phase. If a gas is used as the mobile phase, the technique is referred to as gas chromatography (GC). If a liquid is used, it is referred to as liquid chromatography (LC). Both GC and LC have become essential techniques for analyzing organic compounds.

LC systems that include a pump for pumping the mobile phase, an autosampler for automatically loading samples, and other devices for increasing performance are referred to as high-performance liquid chromatograph (HPLC) systems. In recent years, there has been increasingly widespread use of ultra high performance liquid chromatograph (UHPLC) systems. These systems can sustain very high back pressures generated by use of sub 3u particles as stationary phase. However, low particle size results in ultra fast analysis with and sharper peak shapes.

1-2-2 Supercritical Fluid Chromatography

Research on supercritical fluid chromatography (SFC) as an analytical technique began in the 1960s. Typically, SFC refers to chromatographic technique involving using supercritical carbon dioxide as the mobile phase. Since SFC uses CO_2 like a liquid, it is also referred to as "LC using CO_2."

Before commercialization of SFC, measuring compounds with a wide range of properties required using multiple analytical techniques, however using a combination of SFC and LC for "unified chromatography" has now enabled simultaneous analysis of heterogeneous components repeating[*1]

Fig. 3 shows an simultaneous analysis of 510 standard pesticides in single injection using SFC. Table 2 shows the octanol-water partition coefficient, repeatability, and linearity results for few target pesticides. The technique enables accurate simultaneous analysis of compounds with a wide range of polarities. For more details about the analysis in this example, refer to Application News No. L497 "Using the Nexera UC Online SFE-SFC-MS System to Analyze Residual Pesticides in Agricultural Products"

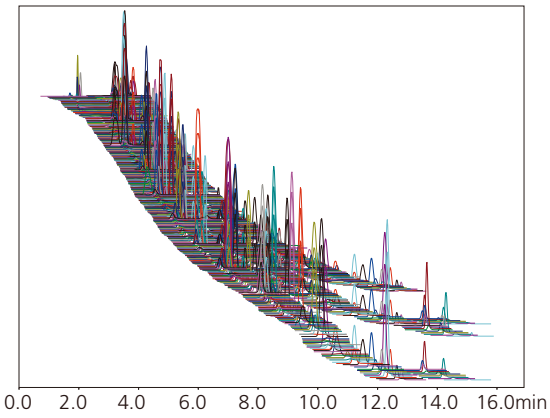


Fig. 3 Mass Chromatogram of Standard Pesticide Mixture Solution

Table 2 Repeatability and Linearity for Pesticide Analysis

Compounds	LogPow	Repeatability (%RSD, n=5)	Range (ng/g)	R^2
Ethofenprox	6.9	6.1	1-100	0.9991
Hexaflumuron	5.68	6.8	1-100	0.9992
Benzofenap	4.69	1.4	2-200	0.9990
Mepronil	3.66	4.6	1-100	0.9993
Prometryn	3.34	2.7	1-100	0.9994
Fenamidone	2.8	3.0	2-200	0.9991
Ethylchlozate	2.5	3.0	1-100	0.9996
Imazosulfuron	1.6	6.2	1-100	0.9998
Bensulfuron methyl	0.79	8.1	1-100	0.9996
Primisulfuron methyl	0.2	5.5	1-100	0.9994
Halosulfuron methyl	-0.02	5.5	1-100	0.9996
Azimsulfuron	-1.4	4.2	1-100	0.9998

1-2-3 Instrumentation for Supercritical Fluid Chromatography

SFC systems involve roughly the same configuration of instruments as HPLC systems. Fig. 4 shows a comparison of flow diagrams for SFC and HPLC systems. The four major differences between SFC and HPLC configurations are described below.

- **Pumps Designed Specifically for Pumping Supercritical Carbon Dioxide**

  The carbon dioxide used for SFC is liquified by cooling. Therefore, the delivery pump must have built-in cooling functionality.

- **Back Pressure Regulator (BPR)**

  A unit is required to keep the CO_2 in a solvent state (liquid or supercritical fluid) within the system and prevent it from vaporizing by maintaining the pressure level within flow channels. In an SFC system, the BPR unit is positioned downstream from a UV, PDA, or other detector or upstream from an evaporative light scattering detector (ELSD) or mass spectrometer (MS).*2 The BPR unit detects pressure in the flow channels and then rapidly opens and closes valves to maintain a constant pressure within the flow channels.

- **Modifier Delivery Pump**

  For SFC analysis, an organic solvent such as methanol or acetonitrile (modifier) is pumped for mixing with the supercritical carbon dioxide. That means separate pumps are required for pumping the supercritical carbon dioxide and modifier.

- **Make-up Delivery Pump**

  If an ELSD or MS unit is used for detection or if the system is used for preparative separation, then a solvent (make-up solvent) is pumped to prevent precipitation in the flow channels or to improve the recovery rate of components in separated fractions. Make-up solvent is also pumped to improve sensitivity during MS detection, because supercritical carbon dioxide does not promote ionization.

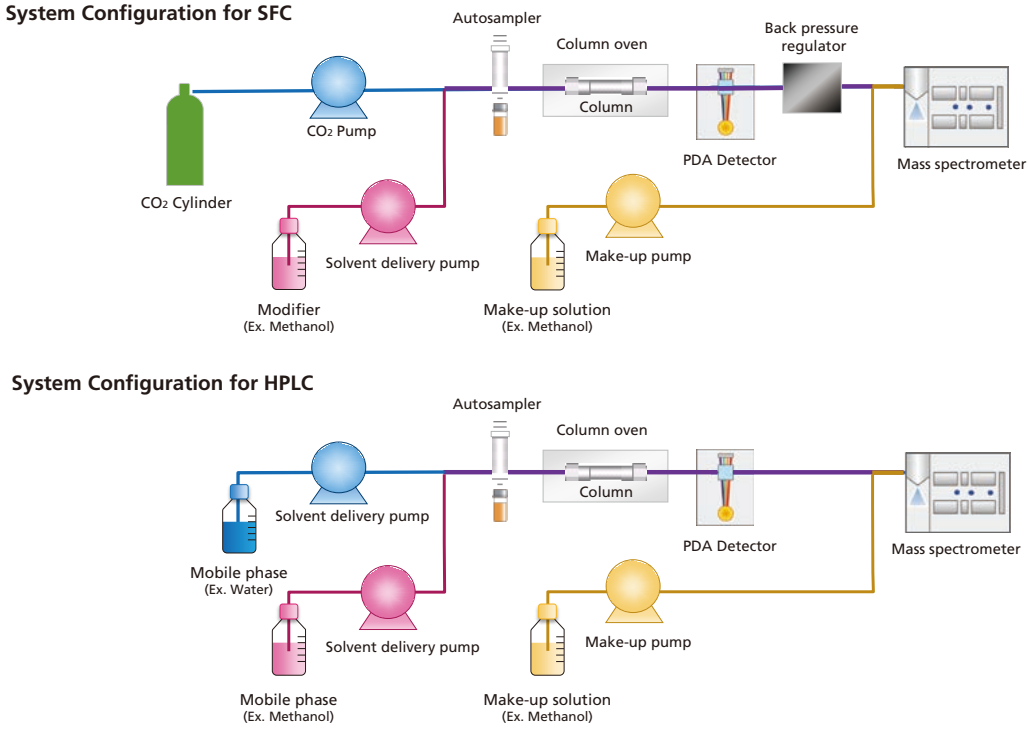


Fig. 4 Comparison of HPLC and SFC System Configurations

1-3 Analytical Advantages of SFC

The supercritical carbon dioxide used as a mobile phase for SFC offers unique advantages. Five of the advantages of using supercritical carbon dioxide for SFC analysis are described below.

(1) Low Polarity

The polarity of supercritical carbon dioxide is similar to n-hexane.

(2) Miscibility with Other Organic Solvents

Supercritical carbon dioxide can be mixed with methanol, which is a water-soluble solvent. In contrast, n-hexane, which offers low polarity similar to supercritical carbon dioxide, cannot be mixed with methanol, due to its relatively high polarity.

(3) Low Viscosity

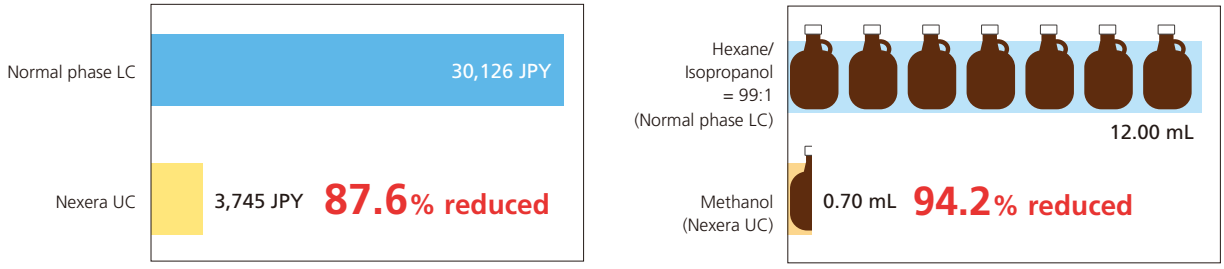
Supercritical carbon dioxide has lower viscosity than solvents such as water. That results in lower column back pressure, assuming the column has the same internal diameter and length, is packed with the same size particles, and supercritical carbon dioxide is pumped through the column at the same rate.

(4) Vaporization

Supercritical carbon dioxide evaporates at a temperature and pressure lower than the critical point.

(5) Solvent Cost

Similar polarity of of super critical CO_2 is and of n-hexane, makes former appropriate to be used for normal phase separations. Normal-phase chromatography consumes large amounts of solvent, however SFC consumes less solvent and involves treating lesser liquid waste, the overall solvent cost is lower than for HPLC (Fig. 5).



Comparison of costs　　Comparison of organic solvent consumption

Fig. 5 Comparison of Analytical Costs and Solvent Consumption Quantities for HPLC and SFC

The following sections describe the analytical advantages of SFC provided by the supercritical carbon dioxide advantages (1) to (4) indicated above.

1-3-1 Low Polarity — Better Structural Identification

Normal-phase chromatography is a separation method that uses a solvent with low-polarity, such as n-hexane, as the mobile phase and a substance with high-polarity, such as silica, as the stationary phase. One advantage of normal-phase chromatography is it offers better structure recognition ability than reversed-phase chromatography.

SFC uses supercritical carbon dioxide, which has similar properties as n-hexane, and also offers excellent structural recognition ability similar to normal-phase chromatography (Fig. 6). By using a column designed for chiral chromatography, which is packed with a stationary phase with optically active functional groups, SFC suitable for separation of chiral compounds.

1-3-2 Miscibility with Organic Solvents — Separations Covering Larger Chemical Space

Supercritical carbon dioxide can be mixed with certain organic solvents, such as methanol, ethanol, isopropyl alcohol (IPA), acetonitrile, and tetrahydrofuran (THF). By mixing the supercritical carbon dioxide with such modfiers, SFC can achieve a wide variety of separation patterns. This allows comprehensive analysis for a wide range of low-to-high polarity compounds not previously possible with HPLC. For example, earlier GC was required for analyzing fatty acids and HPLC for glycerides. With SFC, both fatty acids and glycerides can now be analyzed simultaneously due to the hexane-like properties of the supercritical carbon dioxide in combination with a column designed for reversed-phase chromatography (Fig. 7).

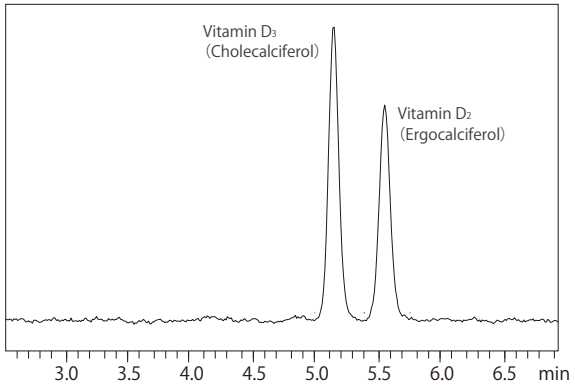


Fig. 6 Separating Vitamins D_2 and D_3 (UC-PyE Column)

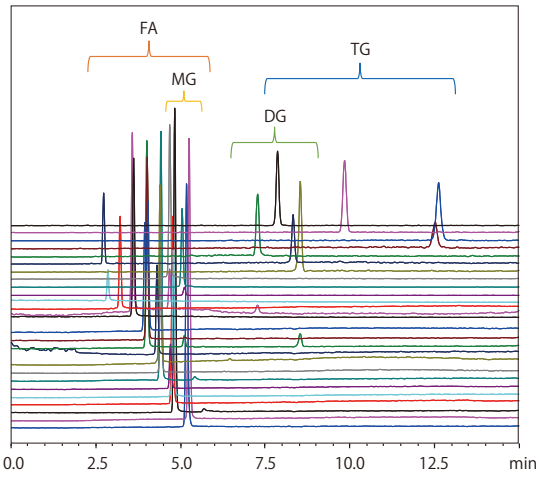


Fig. 7 Example of Simultaneously Analyzing Fatty Acids and Triglycerides by SFC

1-3-3 Low Viscosity — Achieving Ultra-Fast Analysis

Supercritical carbon dioxide has both low viscosity and a high diffusion coefficient. That enables analysis with a high linear velocity (flow speed with respect to the column cross-section). Fig. 8 shows a comparison of linear velocity and column pressure plots for HPLC and SFC.

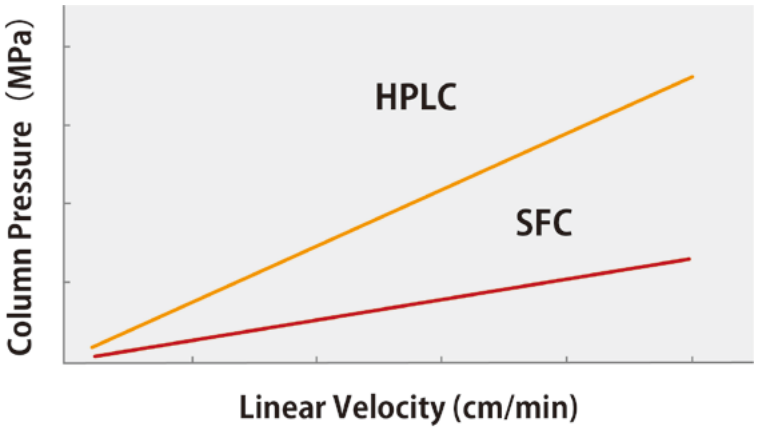


Fig. 8 Linear Velocity vs. Column Back Pressure

Though higher linear velocity can shorten HPLC analysis time, but it results in increased column pressure. Upper range of column pressure is limited by instrument design and column capability. CO_2 in the supercritical state has lower viscosity than water and other organic solvents typically used for HPLC. That means shorter analysis time are possible for SFC than HPLC, without loss of resolution and separation pattern.

Supercritical carbon dioxide also has a higher coefficient of diffusion than solvents used for LC. The diffusion coefficient contributes to determining how quickly compounds reach equilibrium between stationary and mobile phases during chromatography and affects resolution. The van-Deemter equation below indicates the relationship between physical parameters that determine the height equivalent to a theoretical plate (HETP) value, which is used to indicate column resolution.

$$\mathrm{HETP}=A\cdot dp+\frac{B}{V}+C\cdot dp^2\cdot V$$

A: Diffusion coefficient for multiple flow channels
B: Diffusion coefficient in the axial direction
C: Resistance to mass transfer coefficient
V: Linear velocity
dp: Packing material particle diameter

Plots of the van-Deemter equation for HPLC and SFC are shown in Fig. 9.[*3] Term A is based on the multi-flow diffusion generated by the packing material in the column and is proportional to the packing material particle size. Term B is determined by the molecular diffusion in the axial direction of the column. This diffusion phenomenon occurs while compounds stagnate in the stationary or mobile phases and is inversely proportional to linear velocity, regardless of particle size. Term C is derived from how easily compounds can penetrate pores in the packing material and the mass transfer diffusion that contributes to linear velocity. The mass transfer diffusion value is proportional to linear velocity and the square of the particle diameter, which results in higher HETP values for small particles with low linear velocities. In the case of HPLC, mobile phases with a high linear velocity will result in larger mass transfer diffusion values, but supercritical carbon dioxide has a higher diffusion coefficient than HPLC solvents and can penetrate packing material pores more easily. That results in a lower mass transfer diffusion values even for high linear velocities.

That makes SFC an analytical technique that can achieve ultra-fast analysis without sacrificing resolution even at high linear velocities.

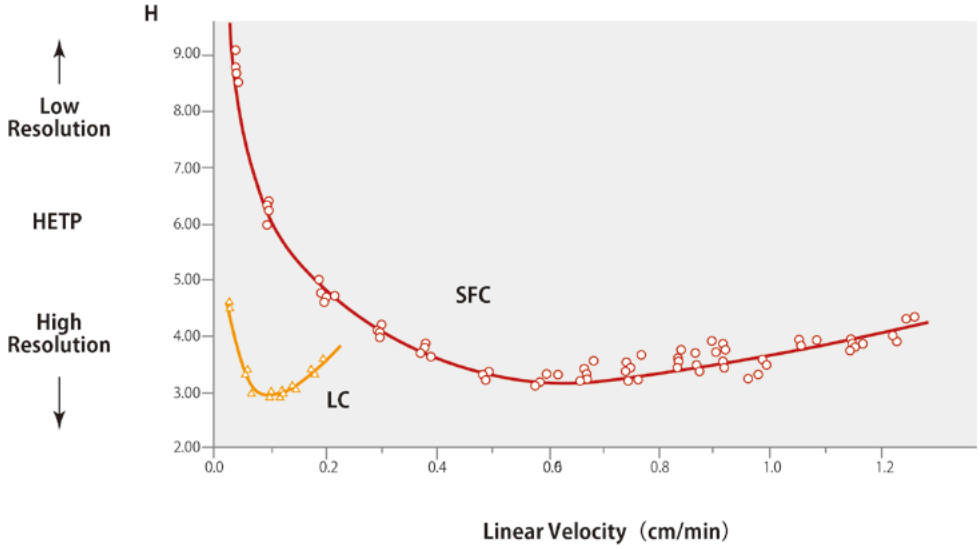


Fig. 9 Van-Deemter Plot Comparison for LC and SFC

These plots indicate the regions where the optimal linear velocity for SFC is higher than for HPLC. An example comparing the analysis of tocopherols by HPLC and SFC is shown in Fig. 10.[*4]

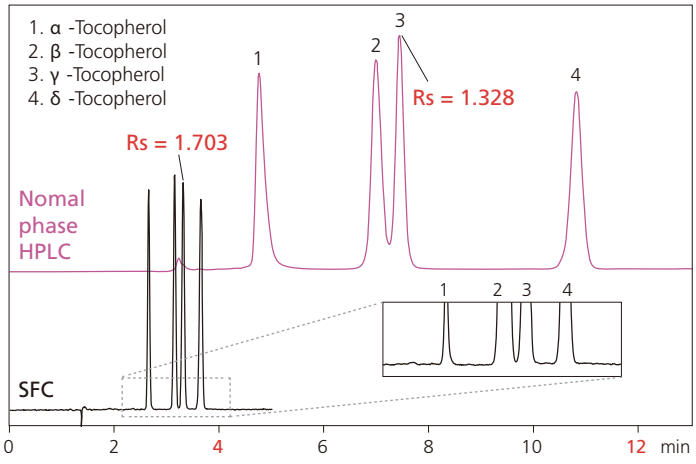


Fig. 10 Comparison Example of Analyzing Tocopherols by HPLC and SFC

1-3-4 Vaporization — Improved LC-MS Detection Sensitivity

Under certain temperature and pressure conditions, CO_2 will become a solvent, which is used as a mobile phase for SFC. If an MS unit is used as a detector, then a back pressure regulator is installed just before the MS unit, so that only the modifier or make-up solvent reaches the MS unit. Consequently, unlike conventional LC-MS analysis, this reduces the diluting effects of the mobile phase, consequently potentially increasing the sensitivity whilst using SFC-MS. This can be seen from below mentioned example where 400 components were analysed with 90% of these showing higher sensitivity with SFC-MS (Fig 11). Fig. 12 shows a comparison of results from using LC-MS and SFC-MS to measure samples prepared to 10 ppb.

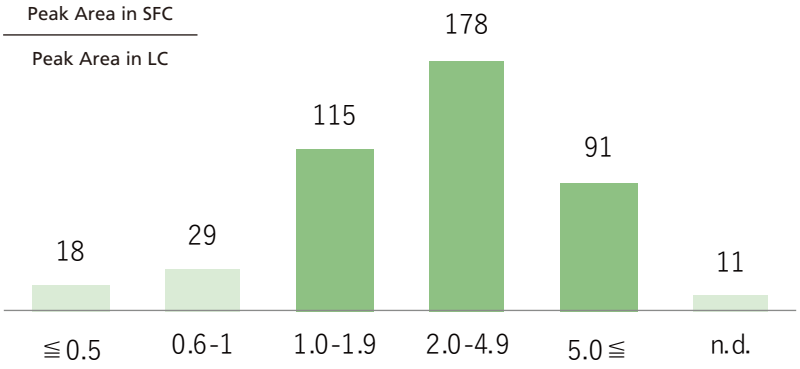


Fig. 11 Area Comparison for LC-MS and SFC-MS

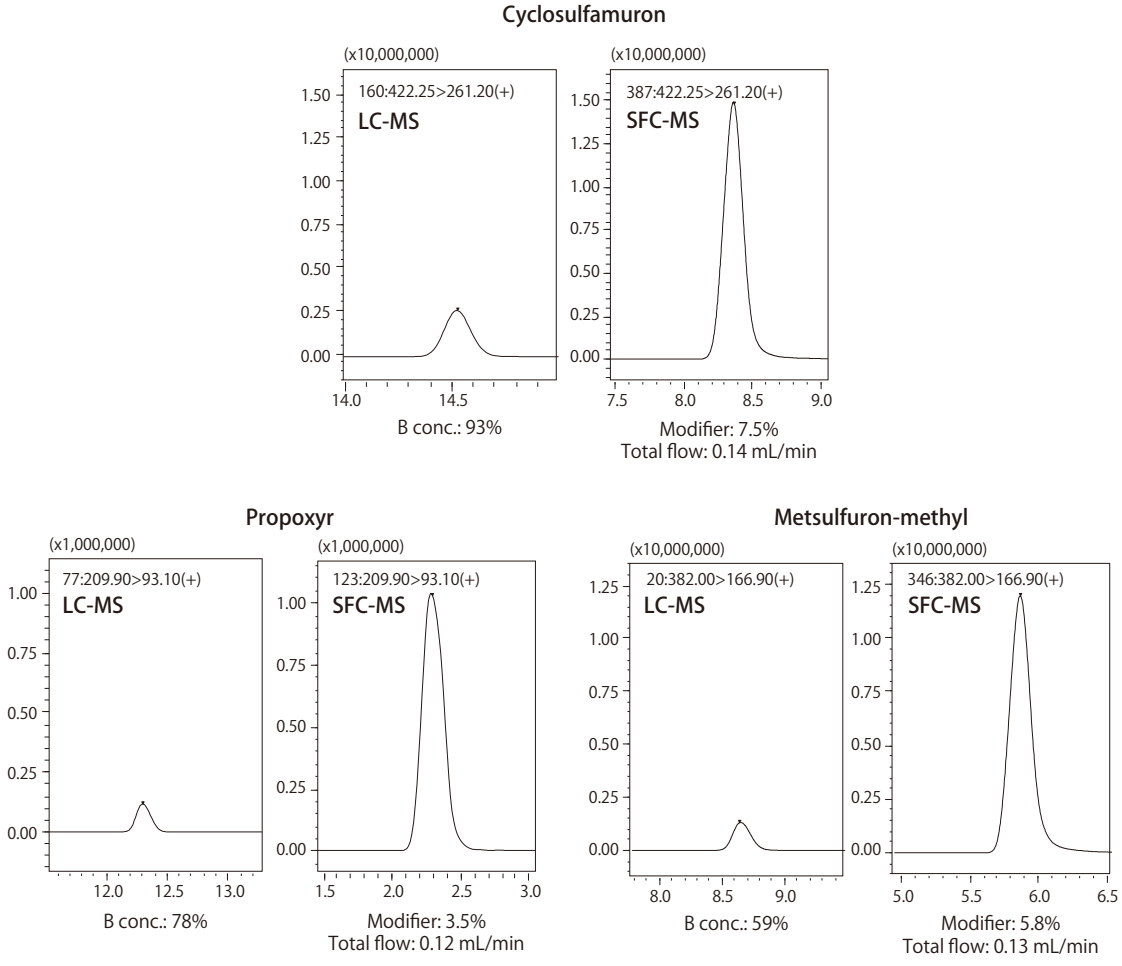


Fig. 12 Comparison of Mass Chromatograms of Standard Pesticide Mixture Solution Measured by LC-MS and SFC-MS

1-4 SFC at Preparative Range

GC and HPLC are commonly used to pre-separate samples, but SFC can be used in the same way as well. Preparative separation by SFC offers the following advantages.

(1) Posttreatment after Preparative Separation

Supercritical carbon dioxide evaporates at ambient temperature and pressure conditions, which eliminates the need for posttreatment.

(2) Solvent Cost for Large Preparative Separation Quantities

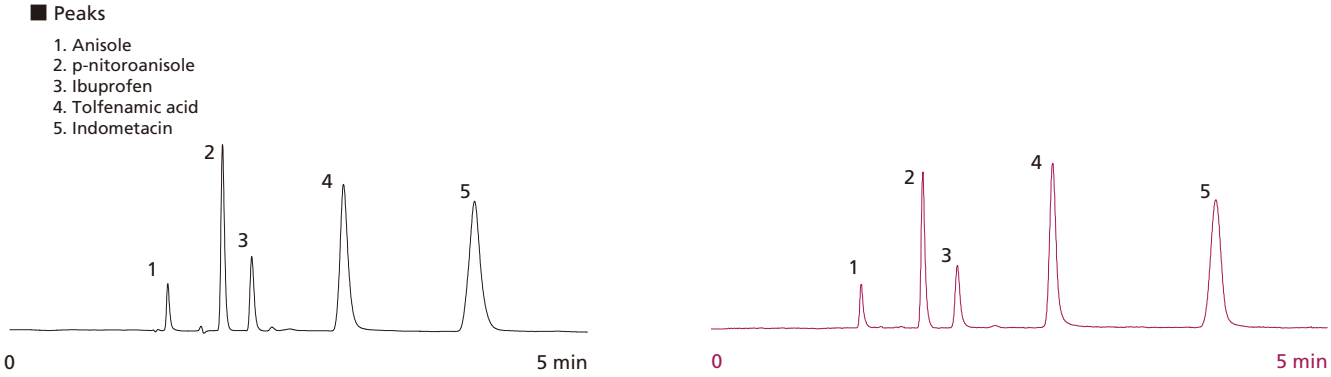
Using inexpensive and environmentally-friendly CO_2 can reduce the cost of purchasing and disposing of solvents.

(3) Preparative Separation Recovery Rates

After preparative separation, HPLC requires posttreatment steps, such as solvent evaporation or concentration. In contrast, SFC solvents evaporate easily, which minimizes the need for posttreatment and prevents component fragmentation or decomposition during posttreatment.

If SFC is used for preparative separation, an organic solvent that can dissolve target components is sometimes added after column separation to prevent precipitation of target components within flow channels, and gas liquid separators can be used to remove these solvents. A variety of gas-liquid separators are currently being developed by various companies.

Just as with preparative separation by HPLC, SFC involves reviewing results from the analysis scale and then switching to a preparative size column, changing the mobile phase flowrate, and changing the sample injection volume. Equivalent separation can be achieved by increasing the flowrate and injection volume in proportion to the column cross-sectional area. Fig. 13 shows an example of changing from an analysis scale to a preparative scale. By using a column with the same stationary phase, separation equivalent to the analysis scale can be achieved.



Analytical Scale

Flow rate	:	mL/min
Mobile phase	:	CO_2 : MeOH=90:10
Column	:	Shim-pack UC-Sil (250 mmL. x 4.6 mm, 5 µm)
Column Temp.	:	40 ˚C
Pressure	:	10 MPa
Injection Vol.	:	0.1 µL

Preparatory Scale

Flow rate	:	60 mL/min
Mobile phase	:	CO_2 : MeOH=90:10
Column	:	Shim-pack UC-Sil (250 mmL. x 20 mm, 5 µm)
Column Temp.	:	40 ˚C
Pressure	:	10 MPa
Injection Vol.	:	50 µL

Fig. 13 Migrating from Analysis Scale to Preparative Scale with SFC

Column Manufacturing Decaffeinated Coffee — Supercritical Fluid Extraction

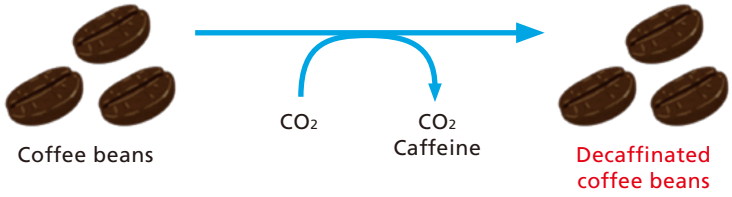
A representative example of using supercritical carbon dioxide for supercritical fluid chromatography is from manufacturing decaffeinated coffee. Previously, a solvent was used to remove the caffeine from coffee beans, but there were concerns about the health risks from the residual solvent. Since supercritical carbon dioxide is a safe substance that does not involve any health or environmental concerns, it is now increasingly used for manufacturing decaffeinated coffee.

The following lists some of the advantages of using supercritical carbon dioxide for manufacturing decaffeinated coffee.

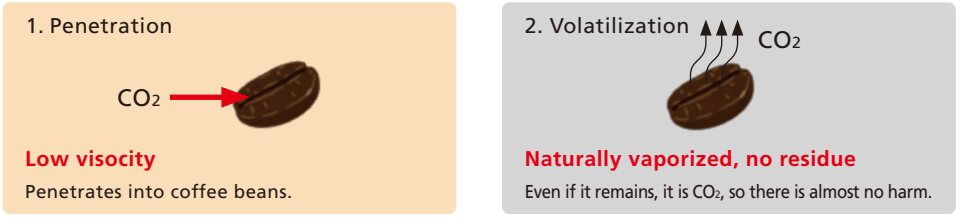
- **Advantage 1: High penetration**
  With higher penetration ability than water, it can easily pass through coffee beans. Supercritical carbon dioxide can penetrate the interior of coffee beans without having to grind the beans to increase their surface area.
- **Advantage 2: High solubility**
  Substances that contact supercritical carbon dioxide are quickly dissolved and extracted from the coffee bean.
- **Advantage 3: Evaporation at ambient temperatures/pressures**
  CO_2 residues are unlikely to remain in coffee beans.
- **Advantage 4: Low toxicity**
  Even if residues remained in coffee beans, they would not be toxic.

In this way, the properties of supercritical carbon dioxide are used beneficially for manufacturing decaffeinated coffee.

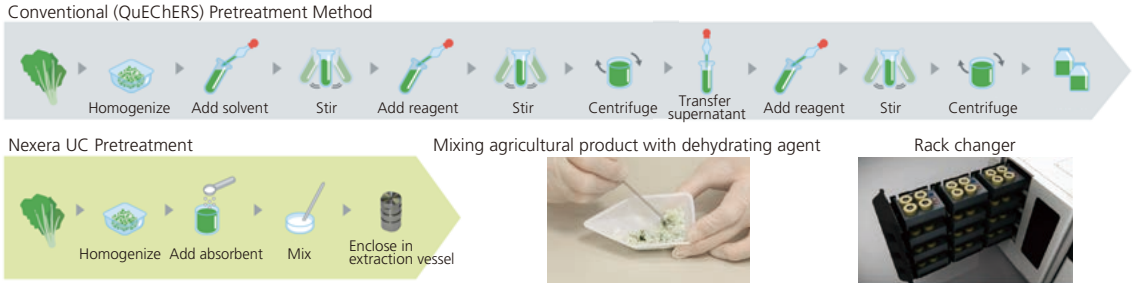
Decaffeinated coffee manufactuaring process using supercritical fluid CO_2



The properties of supercritical fluid CO_2 from the process of caffeine removal



The method of using supercritical carbon dioxide to remove caffeine from coffee beans is based on supercritical fluid extraction technology used to extract target components from the interior of samples. Previous technologies required pretreatment that involved tissue homogenization, dissolution, and extraction (liquid-liquid extraction with a solid phase cartridge), but supercritical fluid extraction can extract target compounds by simply placing the sample in a chamber and filling it with supercritical carbon dioxide. Furthermore, compounds extracted by supercritical carbon dioxide can be injected directly into columns. That enables the automation of all steps from pretreatment and separation to analysis.



Chapter 2 Nexera UC Supercritical Fluid Chromatograph

The Nexera UC supercritical fluid chromatograph product line includes 1-SFC systems, 2-SFC/UHPLC switching systems, 3-preparative SFC systems, 4-online SFE-SFC systems, 5-offline SFE pretreatment systems. Various Nexera UC systems can be configured by adding a newly developed SFC CO_2 solvent delivery unit (LC-30AD$_{SF}$), a back pressure regulator (SFC-30A), and/or an SFE extraction unit (SFE-30A) to a base Nexera ultra high performance liquid chromatograph.

This chapter describes the instruments used in Nexera UC systems and as system extension technologies.

2-1 Components of SFC System

The SFC system product line includes a variety of systems, such as SFC-UV systems that use UV (or PDA) detection, UFMS (SFC-MS) systems that include a mass spectrometer capable of SFC speeds, and screening systems that can automatically switch between multiple columns and modifiers for considering various analytical conditions.

The appearance of a Nexera UC UFMS system is shown in Fig. 14.

To visit the Nexera UC website, read this QR code.

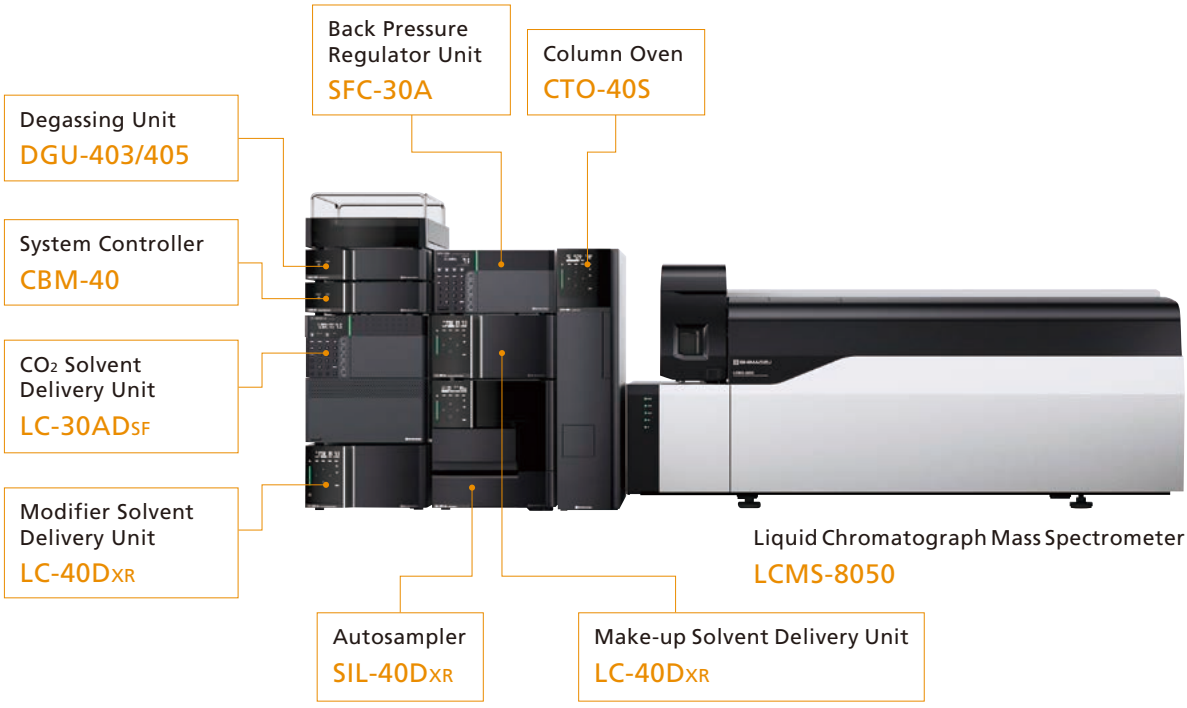


Fig. 14 Nexera UC UFMS System

2-2 Nexera UC/s: SFC/UHPLC Switching System

If both UHPLC and SFC are available during analytical condition development, optimal separation parameters can be selected from a wider range of potential separation parameters. With the Nexera UC/s system, the same system can be used for both UHPLC and SFC analysis modes. Fig. 15 shows the flow diagram of the Nexera UC/s system, which integrates both UHPLC and SFC capability in a single system. The system is configured with LC-30AD$_{SF}$ and SFC-30A units added to a standard UHPLC system. Both UHPLC and SFC analyses can be performed by switching between solvent delivery units and switching ON or OFF the pressure control mode of the back pressure control valve. That can reduce installation space and initial cost and increase the instrument utilization rate. By using the mobile phase solvent switching valve in combination with the column switching valve, mobile phase conditions can be changed automatically and continuously for up to twelve columns, which enables comprehensive measurements that improve method development efficiency.

For more information about Nexera UC/s systems, refer to Technical Report (C190-E212) "Improving Separation and Method Development Efficiency Using the Nexera UC/s UHPLC/SFC Switching System"

A chiral screening system designed specifically for separating chiral compounds is also available. It offers automatic modifier blending functionality for up to 12 columns using only the SFC mode, which eliminates the need to switch between UHPLC and SFC modes. A specific example of using this system to determine separation conditions for chiral compounds is described in Chapter 4.

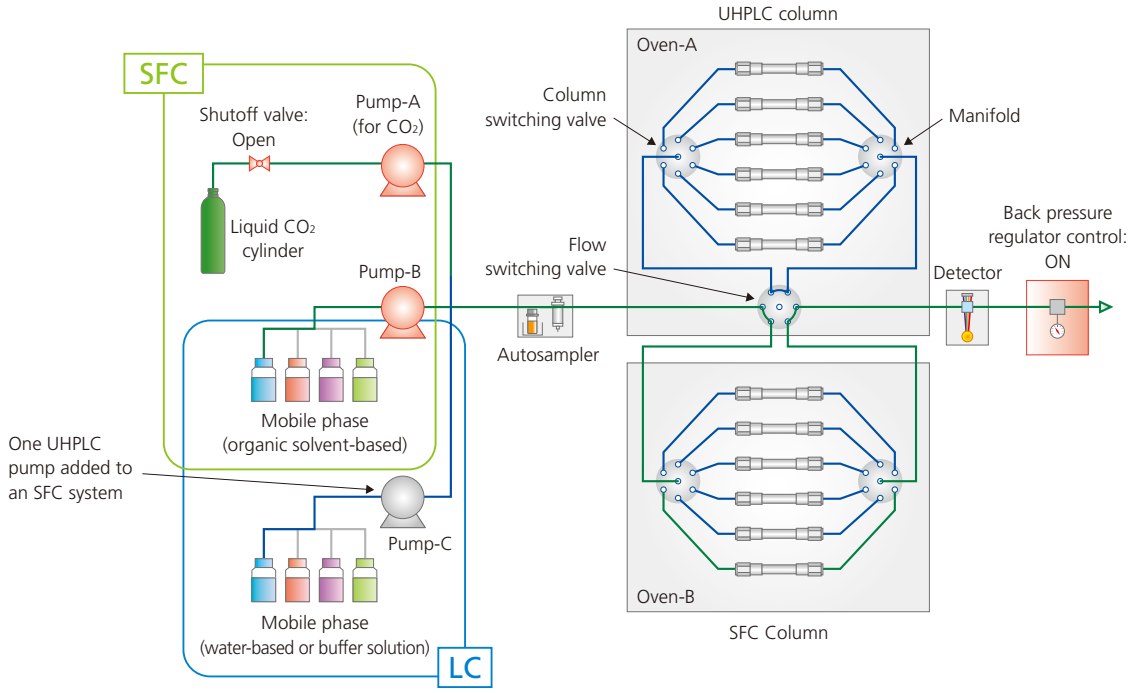


Fig. 15 Flow Diagram for Nexera UC/s: SFC/UHPLC Switching System (Green Line Indicates Section Used during SFC.)

2-3 Preparative SFC Systems

Preparative LC is a technique used to purify samples for specific target components. It is used in a wide range of fields, including chemicals, pharmaceuticals, and foods. Preparative LC serves as a powerful tool for achieving higher purity and recovery rate levels of target components, but it requires drying and powderizing steps. SFC can improve preparative workflow efficiency by significantly reducing the amount of work involved in the powderization process after preparative purification. The Nexera UC product line includes three systems—a stacked fraction system intended for large volume fractionation, a multi-fraction system for separating multiple peaks, and an analytical fraction system intended for analysis-scale fractionation.

These products also feature Shimadzu's unique gas-liquid separator (patented) that inhibits sample dispersion and carryover to obtain high recovery rates. Therefore, excellent recovery rates can be achieved even for highly volatile compounds, such as the fragrance linalool, regardless of the flowrate or modifier concentration. The following sections describe the three product lines and the gas-liquid separator.

To visit the Nexera UC Prep website, read this QR code.

2-3-1 Stacked Fraction System

This system is optimized for large volume fractionation that involves repeatedly injecting compounds with up to several components (Fig. 16). The FRS-40 injection and collection unit can function as both an injector and fraction collector for repeatedly injecting the same sample to collect gram-level preparative fractions. It can inject volumes up to 20 mL* and collect ten fractions. It supports flowrates from 10 to 150 mL/min and connecting columns with an internal diameter from 10 to 30 mm. For more information about stacked fraction systems, refer to Technical Report (C190-E247) "Improved Efficiency of Isomer Preparative Operations by Supercritical Fluid Chromatography with Stacked Injection"

* When using optional parts

2-3-2 Multi-Fraction System

This system is suitable for applications involving fractionation of multiple components in samples with many peaks detected, such as for impurities in pharmaceuticals (Fig. 17). Volumes up to 2 mL* can be injected using an autosampler that holds up to 162 samples (when using 1.5 mL vials). The FRC-40 SF fraction collector can collect up to 540 fractions (using 10 mL vials). It supports flow rates from 10 to 150 mL/min and connecting columns with an internal diameter from 10 to 30 mm.

* When using optional parts

Fig. 16 Nexera UC Prep Stacked Fraction System

Fig. 17 Nexera UC Prep Multi-Fraction System

2-3-3 Analytical Fraction System

This system enables analysis-scale fractionation for applications that only require fraction quantities up to several milligrams, such as for checking synthesis (Fig. 18). By connecting an FRC-40 SF fraction collector to the Nexera UC system, the same system can be used for applications ranging from determining analytical conditions by method scouting to preparative separation of about a few milligrams. With a maximum flowrate of 5 mL/min, it supports using analytical size columns.

Fig. 18 Nexera UC Analytical Fraction System

2-3-4 LotusStream Gas-Liquid Separator

When carbon dioxide transitions from the supercritical fluid state to the gas state during preparative SFC, its volume immediately expands by about 500 times, which can cause eluate to splatter from the column, a factor leading to lower recovery rates.

The newly developed LotusStream (patented) gas-liquid separator uses multiple flow channels to limit the flowrate without increasing the tubing diameter. As a result, the carbon dioxide is discharged externally and the liquid travels along the column and then drips directly below without the eluate splattering (Fig. 19). For more information about LotusStream technology, refer to Technical Report (C190-E250) "Evaluating the Performance of the LotusStream Gas-Liquid Separator for Preparative Supercritical Fluid Chromatography"

To watch a video of the LotusStream separator, read this QR code.

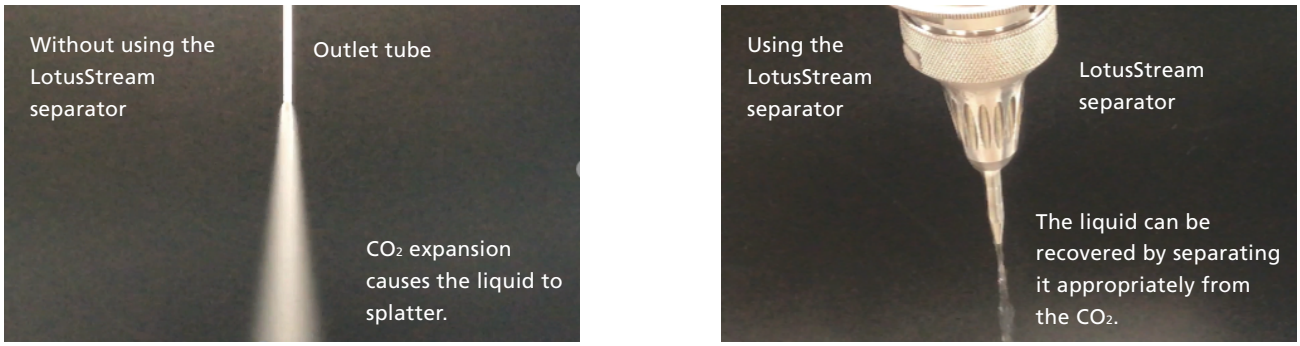


Fig. 19 Gas-Liquid Separation with and without the LotusStream Separator

2-4 Online SFE-SFC System

Online SFE-SFC is extraction and separation technology used to extract components by supercritical fluid extraction (SFE) and then inject the extract directly into a column for separation by SFC. The system flow diagram and the process flow from extraction to analysis are shown in Fig. 20. After using a valve inside the SFE unit to switch flow channels so that supercritical carbon dioxide flows into the extraction vessel, components can be extracted by letting the solution stand without solution flowing through the vessel (static extraction) or with solution flowing through the vessel (dynamic extraction). In the case of online SFE-SFC, the sample is sent to the analytical column during dynamic extraction. For more information about the operating principle of online SFE-SFC systems, refer to Technical Report (C190-E205) "Online Supercritical Fluid Extraction-Supercritical Fluid Chromatography (Online SFE-SFC)"

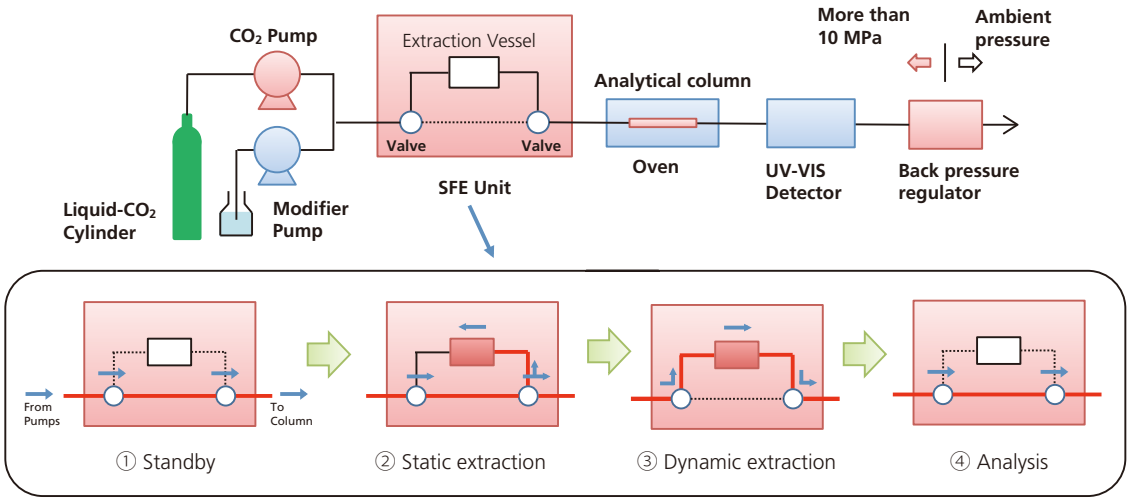


Fig. 20 Nexera UC Online SFE-SFC-MS System Flow Diagram

The entire online SFE-SFC process, from extraction to separation and detection, can be completed within a single system, which eliminates the need for any complicated pretreatment processes and enables automation. This can significantly reduce the time and effort required for the various operations involved in analysis. It also means that the entire process, from extraction to separation and detection, can be performed without exposure to light, without oxidation, and in a moisture-free environment. Therefore, the method is extremely useful for analyzing unstable compounds, such as compounds with components easily decomposed by light, easily oxidized, or easily hydrolyzed. Unlike offline SFE, online SFE-SFC does not involve preparing sample solutions, which means sensitivity can be increased easily because target components are not diluted by the sample solvent. Fig. 21 shows a comparison of analyzing reduced coenzyme Q10 by online SFE-SFC versus solvent extraction-SFC. For more information about this analysis, refer to Application News No. L496 "Analysis of Unstable Compounds Using Online SFE-SFC"

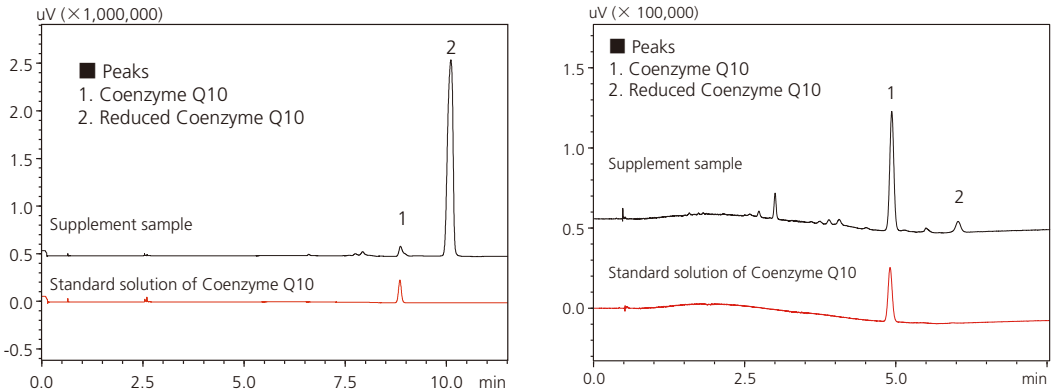


Fig. 21 Example of Analyzing Reduced Coenzyme Q10 (Left: Online SFE-SFC; Right: Solvent Extraction-SFC)

Conventional pretreatment methods by solvent extraction involve adding a solvent to the sample to extract components, which dilutes the extract solution and results in lower concentrations of compounds in the extract solution. Furthermore, for analysis-scale flowrates, only a few microliters of extract can be injected due to the small internal diameter of columns specified. Therefore, if a sample with a low extract concentration is analyzed, extraction, concentration, and other operations must be performed repeatedly due to inadequate sensitivity. In contrast, the Nexera UC online SFE-SFC system can inject extracts directly into the column, which results in injecting a much larger quantity of the sample into the column than when using solvent extraction. A comparison example* is shown in Fig. 22. Direct injection with a Nexera UC system enables high sensitivity analysis because it injects 10,000 times more of the sample into the column than when using 10 mL of solvent to inject 1 µL by solvent extraction.

* Assumes equivalent extraction efficiency and a 1 % concentration of the target compound in the sample.

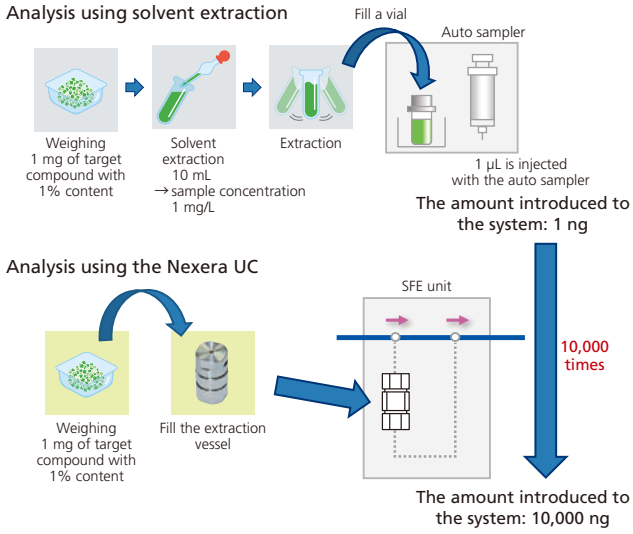


Fig. 22 High Sensitivity Extract Analysis Using an Online SFE-SFC-MS System

Techniques for analyzing residual pesticides in foods must be quick and easy, including for pretreatment, due to the large number of regulated pesticides involved and to shorten inspection times. Using an online SFE-SFC system, the only operations involved are pretreating the sample by mixing it with a dehydrating agent and then filling the extraction vessel. Results from pesticide analysis using the online SFE-SFC-MS system are shown in Table 3. The system can be used for pesticides with a wide range of polarities, from hydrophilic compounds with a -1.4 LogPow value to hydrophobic compounds with a 6.9 LogPow value.

Table 3 Repeatability and Linearity of Pesticide Analysis Using an Online SFE-SFC-MS System

Compounds	$LogP_{ow}$	Repeatablity (%RSD,n=5)	Range (ng/g)	R^2
Ethofenprox	6.9	6.1	1-100	0.9991
Hexaflumuron	5.68	6.8	1-100	0.9992
Benzofenep	4.69	1.4	2-200	0.9990
Mepronil	3.66	4.6	1-100	0.9993
Prometryn	3.34	2.7	1-100	0.9994
Fenamidone	2.8	3.0	2-200	0.9991
Ethlychlozate	2.5	3.0	1-100	0.9996

Compounds	$LogP_{ow}$	Repeatablity (%RSD,n=5)	Range (ng/g)	R^2
Imazosulfuron	1.6	6.2	1-100	0.9998
Bensulfuron methyl	0.79	8.1	1-100	0.9996
Primisulfuron methyl	0.2	5.5	1-100	0.9994
Halosulfuron methyl	-0.02	5.5	1-100	0.9996
Azimsulfuron	-1.4	4.2	1-100	0.9998

2-5 Offline SFE-SFC System

This is an offline SFE pretreatment system designed specifically for extracting target components from solid samples. The extraction process flow is shown in Fig. 20. The extraction vessel is packed with the sample and placed in the SFE unit (Fig. 23A). The extraction vessel is filled with supercritical carbon dioxide for static extraction (Fig. 23B). Then supercritical carbon dioxide is pumped through the extraction vessel for dynamic extraction (Fig. 23C). The extract is trapped in a trap column and the eluate solution that contains the target components is collected in the fraction collector (Fig. 23D) for measurement with the specified analytical instruments, such as by LC-MS, GC-MS, or FTIR. For more information about the operating principle of offline SFE pretreatment systems, refer to Technical Report (C190-E199) "Improved Sample Pretreatment Using Offline Supercritical Fluid Extraction"

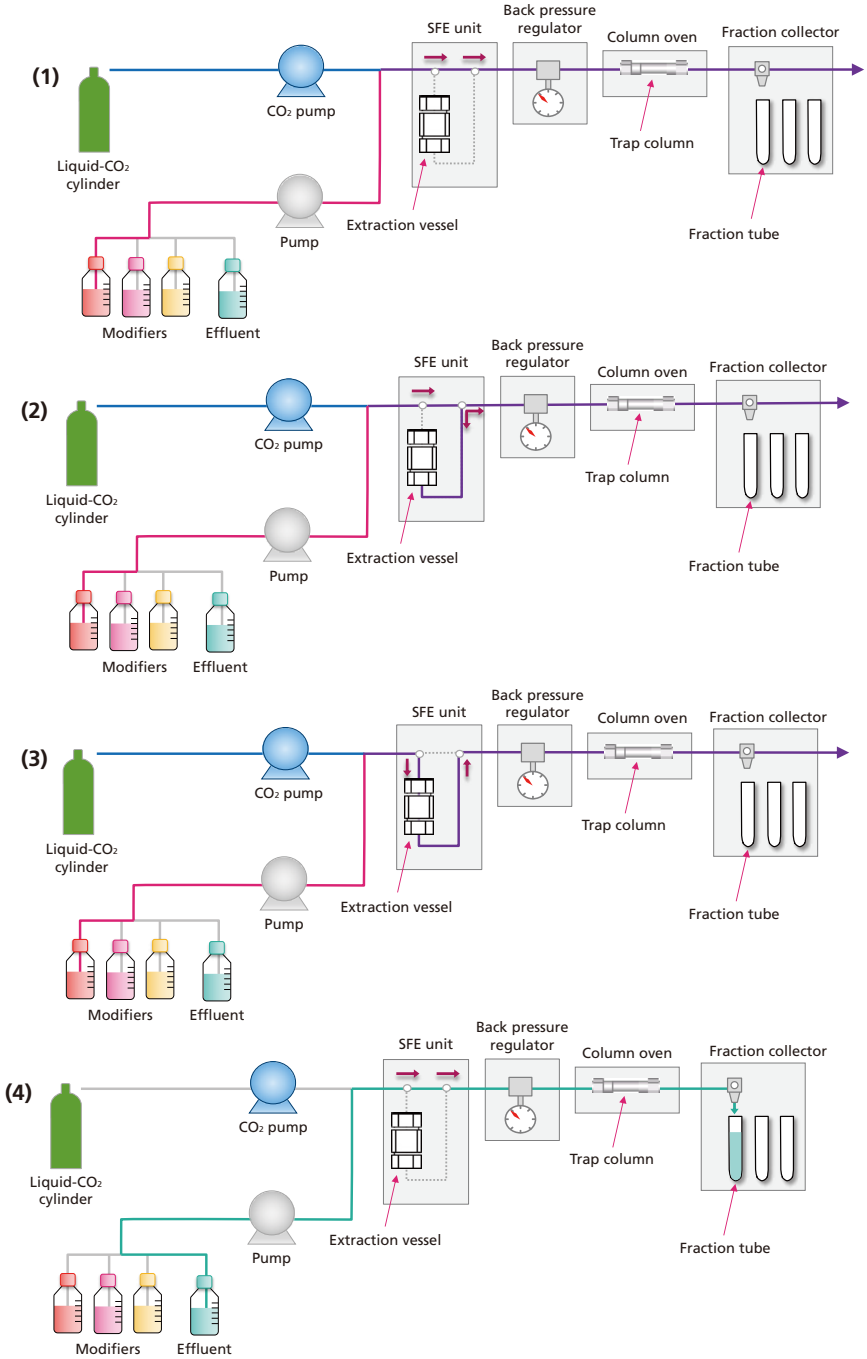


Fig. 23 Extraction Process Flow in Nexera UC Offline SFE Pretreatment System

Chapter 3 SFC Analytical Condition Parameters

The approach for the SFC analytical condition development process is basically the same as for determining analytical conditions for HPLC. HPLC can generally target any component in compounds that can be dissolved in the mobile phase. In contrast, SFC can be used to analyze any compound that is compatible with supercritical carbon dioxide and can be dissolved in an organic solvent.

If chromatography is used for quantitative analysis, then optimal analytical conditions should achieve resolution of 1.5 or more. The basic equation for calculating resolution Rs is indicated below.

$$Rs=\frac{1}{4}(\alpha-1)\cdot\frac{k'}{1+k'}\cdot\sqrt{N}$$

α: Separation factor
k': Retention factor
N: Number of theoretical plates

The α, k, and N values that affect Rs are all independent factors that vary depending on the column and mobile phase used, where N represents separation efficiency and α selectivity. In particular, separation can be improved by increasing N and α values.

Because the supercritical carbon dioxide used for SFC offers different properties than mobile phase solvents used for HPLC, when a column used for HPLC is used for SFC, it can sometimes result in different elution behavior than HPLC (Fig. 24). This chapter describes the characteristics of columns and mobile phases involved in determining analytical conditions for SFC.

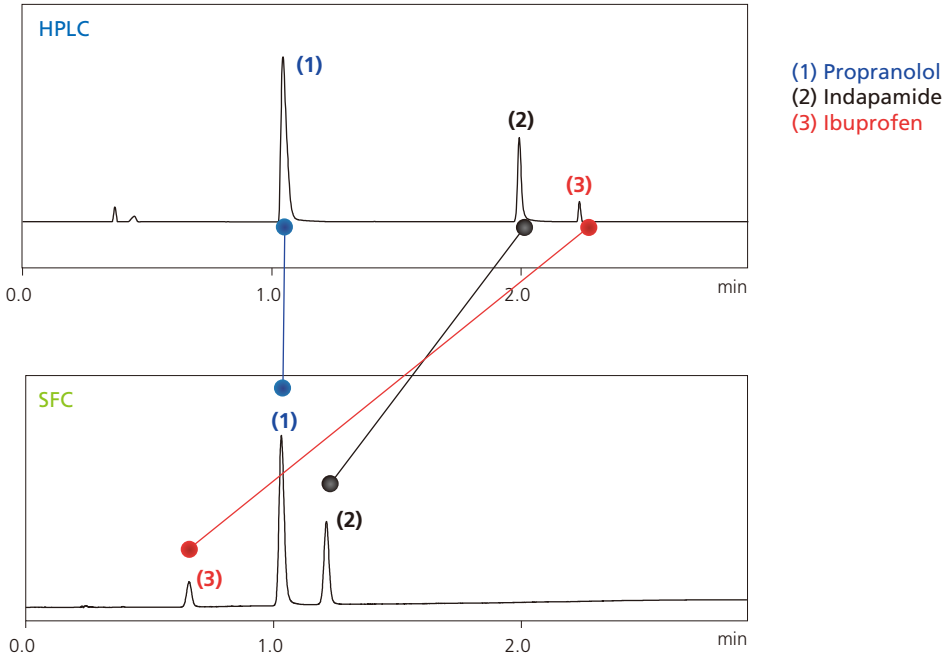


Fig. 24 Separation of Three Types of Pharmaceutical Ingredients (Upper: HPLC; Lower: SFC)

3-1 SFC Columns

In principle, the various columns used for HPLC could also be used for SFC, but in order to ensure the CO_2 is kept in a supercritical fluid state, make sure the column has the pressure capacity necessary for maintaining the required pressure level. Specially, polymer-based columns require particular care because the pressure increases during use as the stationary phase expands.

Since supercritical carbon dioxide has about the same polarity as hexane, the main SFC separation modes will presumably behave similar to normal-phase chromatography, but unlike HPLC, columns with properties of both normal-phase columns (such as silica, diol, and CN) and reversed-phase columns (such as C18, cholesteryl, and phenyl) can be used. Furthermore, the supercritical carbon dioxide used for SFC has high mobile phase diffusivity and low molecular density, making it more prone to secondary interactions than HPLC and resulting in major changes in separation behavior depending on the column stationary phase.*5 Therefore, to determine optimal conditions, it is important to consider analytical conditions using a wide variety of stationary phase types.

Though supercritical carbon dioxide has low polarity similar to hexane, as mentioned above, it can be mixed with highly polar solvents. That means when optimizing the mobile phase and stationary phase for SFC, normal-phase columns can be replaced with a reversed-phase column without changing the type or composition of mobile phase or modifier. Fig. 25 shows a comparison of the elution order for HPLC and SFC. For HPLC, mobile phases are matched to either the reversed-phase or normal-phase separation mode based on mobile phase properties. That involves purging the solvent from inside the system each time the separation mode is changed. For SFC, the same mobile phase conditions can be used for either the reversed-phase or normal-phase separation modes, which means separation results can be checked by simply replacing the column.

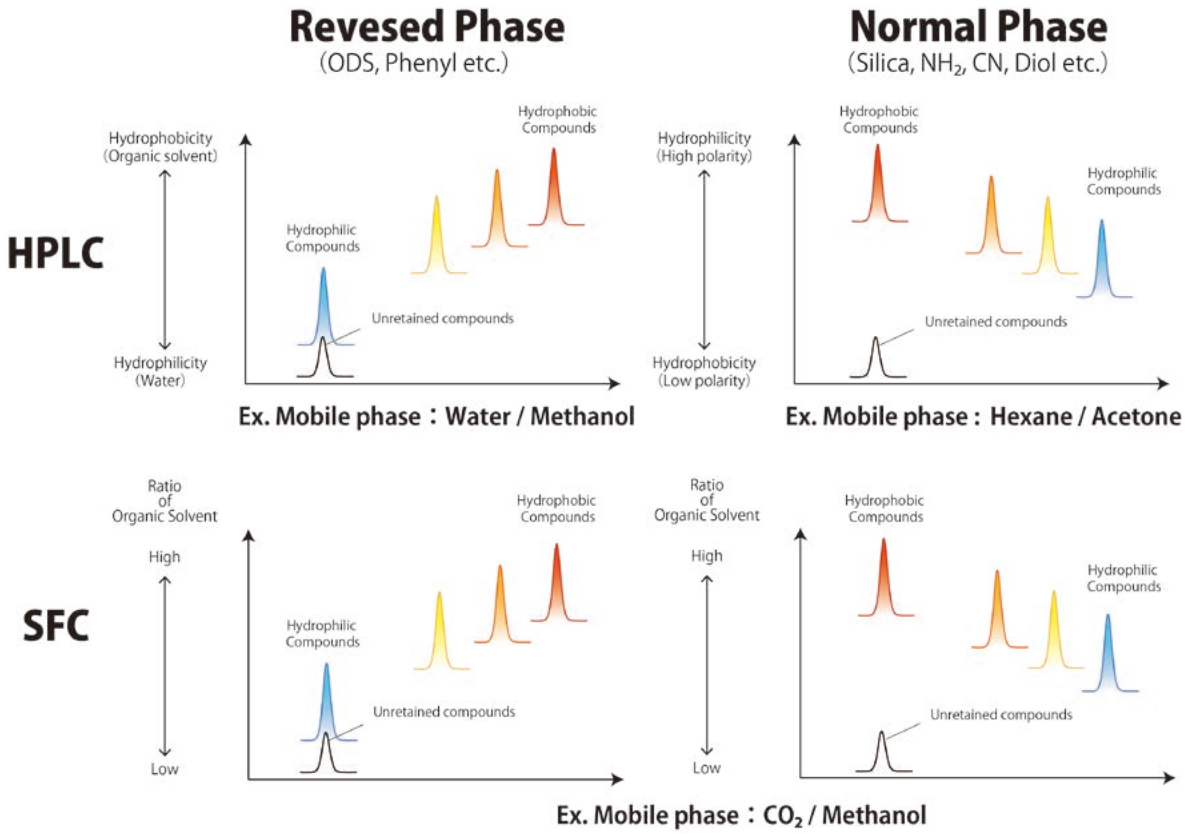


Fig. 25 Comparison of Elution Order for HPLC and SFC

3-1-1 Shim-pack UC Series Columns

Shimadzu offers a Shim-pack UC series of columns designed specifically for SFC analysis. The line of Shim-pack UC series columns is listed in Table 4. All the columns include a certificate certifying that they were tested in accordance with the regulation.

For more details about Shim-pack UC series columns, refer to the corresponding brochure (C190-E251).

Table 4 Shim-pack UC Series Columns

Column	Carbon Loading	Surface Area	Pore Size	Bonded Phase
Shim-pack UC RP	9%	450 m^2/g	10 nm	(Polar Embedded) Octadecyl group
Shim-pack UC GIS II	11%			Octadecyl group
Shim-pack UC Phenyl	9.5%			Phenyl group
Shim-pack UC CN	14%			Cyanopropyl group
Shim-pack UC Diol	20%			Diol group
Shim-pack UC Sil	—			(Unmodified silica)
Shim-pack UC Amide	18%			Carbamoyl group
Shim-pack UC NH2	8%			Aminopropyl group
Shim-pack UC Diol II	ND	3 um：340 m^2/g 5 um：300 m^2/g		Diol group
Shim-pack UC Sil II	—			(Unmodified silica)
Shim-pack UC Choles	20%			Cholesteryl Group
Shim-pack UC PBr	8%			Pentabromobenzyl Group
Shim-pack UC PyE	18%			Pyrenylethyl Group
Shim-pack UC HyP	ND			3-Hydroxyphenyl Group
Shim-pack UC Py	ND			Pyridinyl Group
Shim-pack UC Triazole	ND			Triazolyl group

3-1-2 Column Selection Guide

For SFC, there are no columns available that offer broad applicability, such as the C18 column does for HPLC reversed-phase chromatography. Since supercritical carbon dioxide can penetrate tiny pores more easily, it promotes higher interaction with the stationary phase. That means SFC offers the possibility of separating isomers or other compounds that are difficult to be separated by HPLC. In particular, columns with unique or multiple interactive effects can help improve separation using SFC.

Fig. 26 shows an index of elution order and retention behavior for a variety of columns used to analyze ibuprofen (acidic compound), indapamide (neutral compound), and propranolol (basic compound). Fig. 27 shows chromatograms obtained using diol group, hydroxyphenyl group, and cholesteryl group columns.

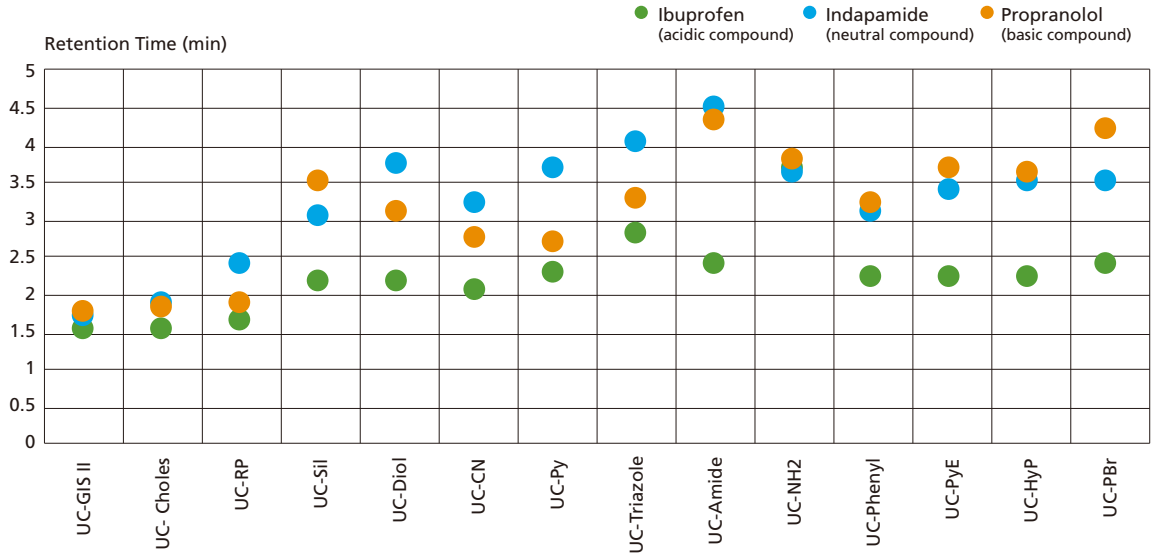


Fig. 26 Retention Behavior of Respective Shim-pack UC Series Products

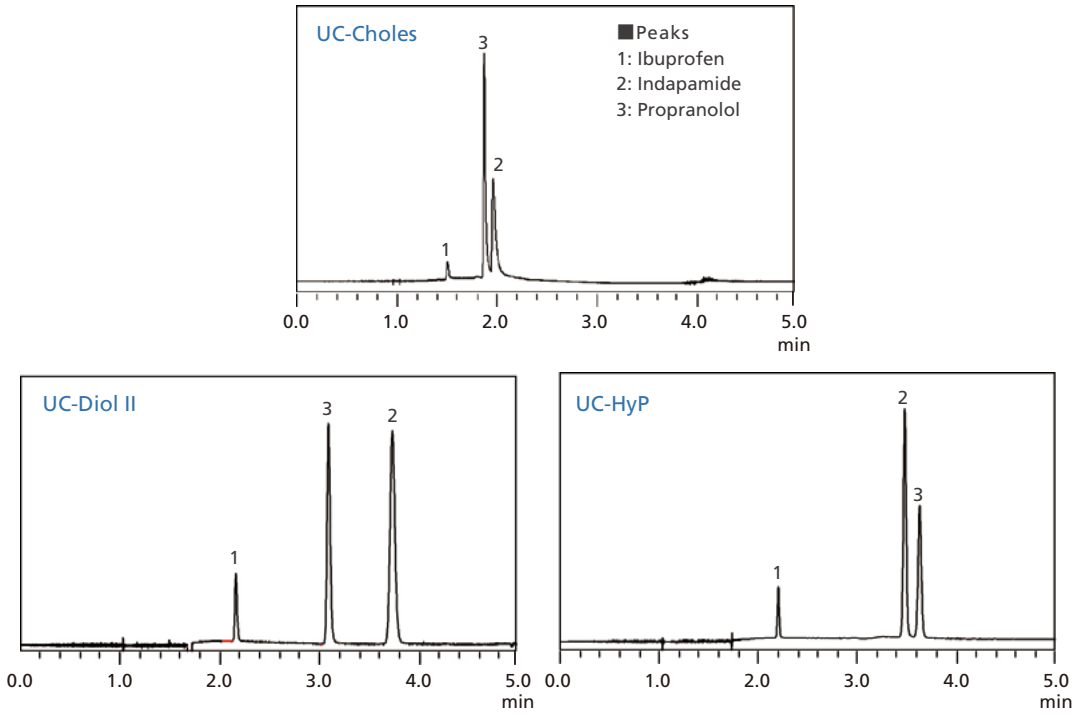


Fig. 27 Differences in Retention Behavior for Respective Stationary Phases (normal-phase for Diol, normal-phase + static electric interaction for HyP, and hydrophobic interaction for Choles)

3-2 SFC Mobile Phases

When determining the mobile phase for HPLC, not only the solvent but also the acid/base level, the addition of ion-pair reagents or other modifiers, and other factors that help separation must be considered. For SFC, separation is affected by the quantity and type of modifier added, the acid or other additives added to the modifier, and the pressure and temperature applied to maintain the CO_2 in a fluid state. CO_2 is typically used as the mobile phase for SFC, but there are also cases of supercritical fluoroform being used.[*6]

3-2-1 Modifiers

Due to the nonpolarity of supercritical carbon dioxide, a modifier such as methanol, ethanol, or acetonitrile can be added to provide polarity to the mobile phase. Increasing the modifier quantity could break down the supercritical fluid state of the CO_2 and potentially change it to a subcritical or liquid state. Nevertheless, it will continue to function as a mobile phase regardless of the state and is not a problem for chromatography. Adding a modifier increases the critical temperature and critical pressure necessary for maintaining supercritical carbon dioxide hence adding high percentages of a modifier is not desirable. For HPLC, reversed-phase chromatography and normal-phase chromatography have symmetric properties that can counteract the ability of the mobile phase solvent to elute components. For SFC, the supercritical carbon dioxide always has a low elution capability and the modifier has a high elution capability for both reserved-phase and normal-phase chromatography. Table 5 shows examples of solvents used as modifiers. Fig. 28 shows a comparison of chromatograms separated using different types of modifiers.

Table 5 Examples of Solvents Used as Modifiers

Organic Solvent	Log P_{ow}	Organic Solvent	Log P_{ow}
Hexane	3.9	Acetone	- 0.24
Toluen	2.69	Dioxane	- 0.27
Tetrahydrofuran	0.47	Ethanol	- 0.32
1-propanol	0.329	Acetonitrile	- 0.34
2-propanol	0.05	Methanol	- 0.82

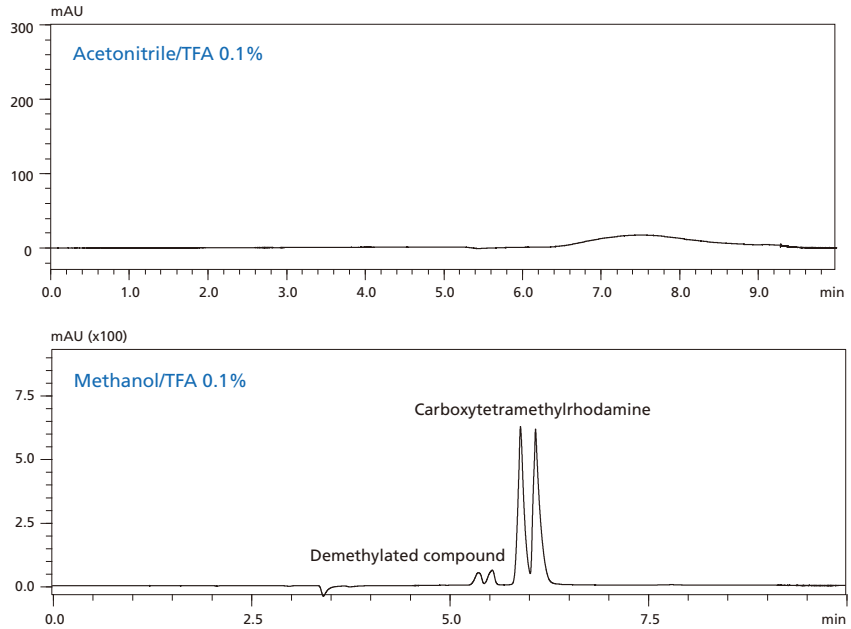


Fig. 28 Comparison of Modifiers

3-2-2 Using Additives to Adjust Separation and/or Improve Peak Shape

For HPLC analysis, buffer solutions, ion-pair reagents, or other additives are used to change separation selectivity or improve peak shape. Similarly, additives are used for the same reasons in SFC as well.

Additives used for SFC are added to the modifier rather than the supercritical carbon dioxide. The main additives used are listed in Table 6. Adding an acid such as acetic acid or a base such as an amine can improve peak shape by inhibiting ionization of target components or by masking secondary functional groups in the stationary phase. For LC-MS detection, a volatile salt such as ammonium formate is often used because ion suppression can occur. It has been reported that adding a small amount of water ionizes part of the supercritical carbon dioxide so that it behaves like an acidic additive.[*7]

Table 6 Examples of Additives and Applicable Compounds

Acid	Acetic acid, Formic acid, Trifluoro acetic acid, Citric acid
Base	Triethylamine
Other	Water

Ion-pair reagent	Alkylbenzoic sulfonate salts Tetrabutyl ammonium salts
Volatile salt	Ammonium formate Ammonium acetate

3-2-3 Other Factors that Affect Separation

For HPLC, the column temperature affects separation only slightly, but for SFC, the temperature and pressure can affect the change in CO_2 state, which changes the density, diffusion coefficient, and other properties of the CO_2. Therefore, the temperature and pressure setting values are factors that can potentially affect separation (Fig. 29).

As explained in 1-3-3 regarding the Van-Deemter equation, the large diffusion coefficient makes it easier for supercritical carbon dioxide to penetrate other substances, which results in a lower C value that is related to the mass transfer diffusion and enables higher separation efficiency. Increasing the temperature increases the diffusion coefficient and decreases the viscosity, which enables using a longer column and measuring samples at higher flowrates. Temperature and pressure mainly contribute to the number of theoretical plates, but the number of theoretical plates contributes to resolution by a factor equivalent to its square root. That means it does not have a large effect compared to modifiers, stationary phases, or other parameters that contribute to the selectivity of separation.

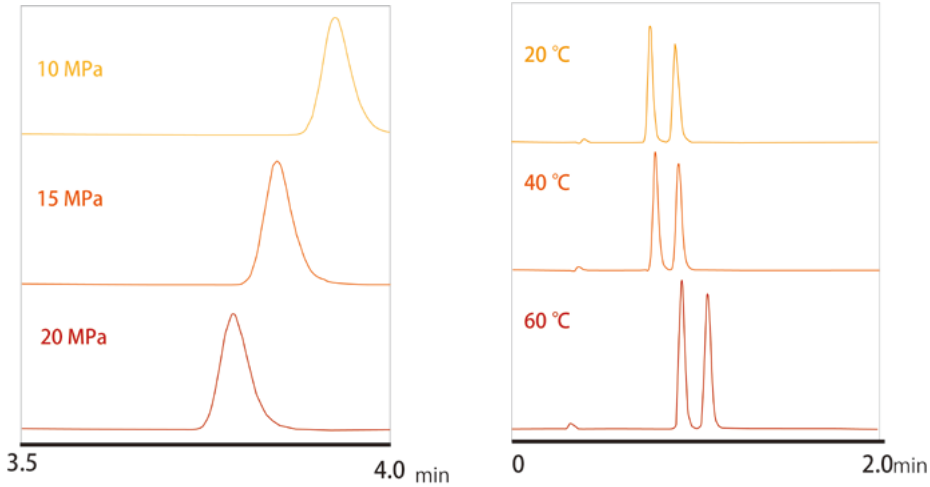


Fig. 29 Effects of Pressure and Temperature on SFC Analysis

3-3 Support for SFC Method Development: Method Scouting Systems

For typical HPLC or SFC analysis, scouting (searching) for optimal separation parameters requires a time-consuming analytical condition optimization process of manually adjusting solutions, replacing columns, and specifying gradient profile settings. In particular, because predicting the retention mode for SFC is more difficult than for regular HPLC, separation parameter scouting must be performed comprehensively.

The separation parameter optimization stage requires a process of checking retention behavior as the type and concentration of solvents and additives are varied. Due to the time and trouble involved in the modifier preparation process, it often results in discarding a greater quantity of unwanted solution.

The Nexera UC/s system described above includes an internal valve for automatically switching between columns and mobile phases/modifiers (Fig. 13) and includes functionality for automatically blending mobile phases to vary the types of solvents, and the types and concentrations of additives used. By using the valve to switch between columns with multiple separation modes, it can comprehensively scout separation parameters. Fig. 30 shows the mobile phase blending process used to automatically prepare modifiers. The settings were configured to pump 5 % of solution B, a 100 mmol/L ammonium formate-methanol solution, with respect to water as solution A, so that 5 mmol/L aqueous ammonium formate solution is pumped from pump B. Fig. 31 shows the peak shape improvements achieved by using the above method to automatically prepare modifiers containing water.

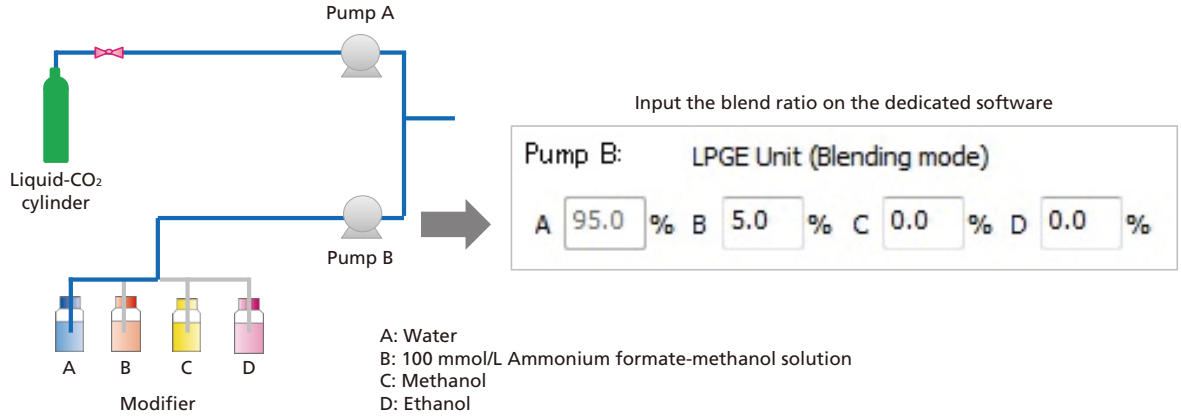
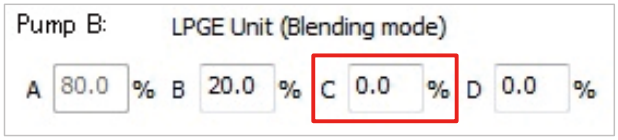
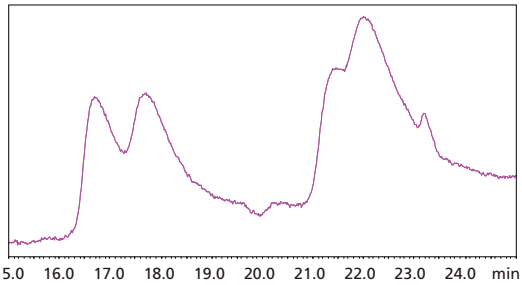


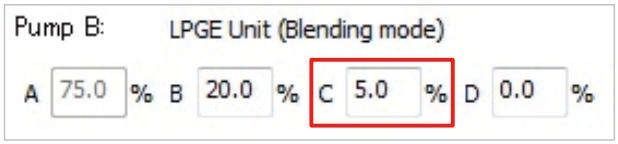
Fig. 30 Effects of Pressure and Temperature on SFC Analysis

In case 0% ratio of water as modifier





In case 5% ratio of water as modifier



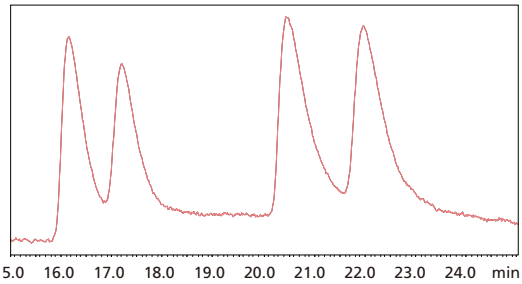


Fig. 31 Peak Shape Improvements Achieved by Adding Water

Using dedicated Method Scouting Solution software, separation parameters can be scouted comprehensively for multiple analytical conditions (Fig. 32). Based on the chromatogram obtained, optional software is used to evaluate separation and rank the optimized parameters (Fig. 33). The software automatically identifies all chromatograms with resolution greater than a specified criterion (1.5 in this case) and then ranks the resolution in those chromatograms.

For more information about using a Nexera UC/s system to optimize separation parameters, refer to Technical Report (C190-E212) "Improving Separation and Method Development Efficiency Using the Nexera UC/s UHPLC/SFC Switching System"

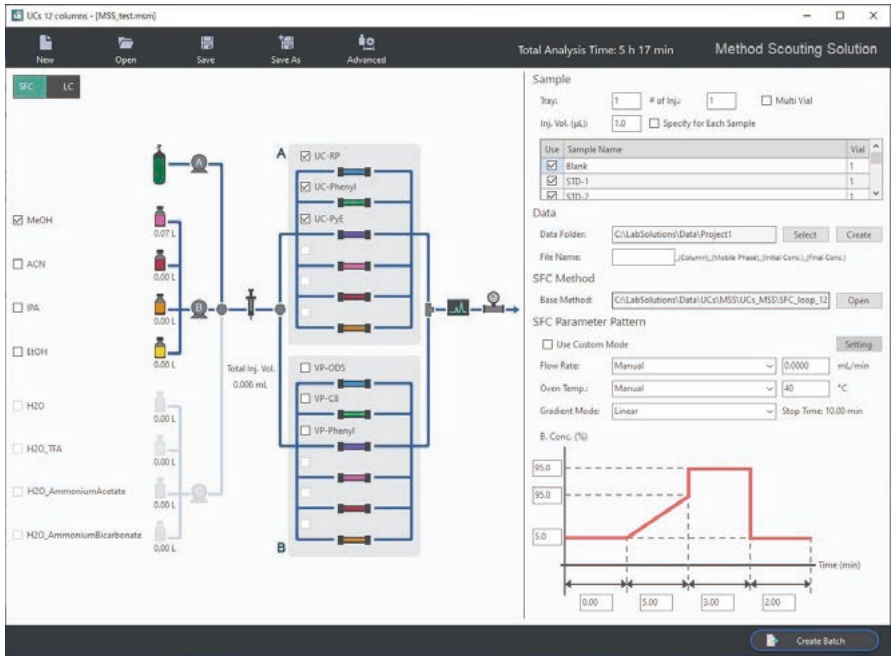


Fig. 32 Method Scouting Solution Operating Window for Nexera UC/s

Ranking	Run No.	Analytical Condition	Resolution	Separatoin factor	Symmetry factor		Retention factor		Area%		Peak number
					Peak1	Peak2	Peak1	Peak2	Peak1	Peak2	
1	32	Omeprazole_OZ-3_MeOH_20_40	7.965	1.921	1.16	1.159	6.583	12.644	49.829	50.171	2
2	17	Omeprazole_IC-3_MeOH_20_40	5.587	1.602	1.387	1.274	8.078	12.937	49.971	50.029	2
3	16	Omeprazole_IC-3_EtOH_20_40	5.382	1.639	1.915	1.661	8.617	14.124	49.984	50.016	2
4	31	Omeprazole_OZ-3_EtOH_20_40	5.377	1.599	1.169	1.162	7.229	11.561	49.778	50.222	2
5	1	Omeprazole_AD-3_EtOH_20_40	3.996	1.509	1.257	1.404	8.779	13.25	50.054	49.946	2
6	8	Omeprazole_AY-3_MeOH_20_40	3.55	2.08	1.178	1.145	3.652	7.597	49.974	50.026	2
7	11	Omeprazole_IA-3_MeOH_20_40	3.428	1.523	1.464	1.312	7.435	11.327	49.973	50.027	2
8	4	Omeprazole_AS-3_EtOH_20_40	2.515	1.673	1.657	1.518	1.244	2.081	49.754	50.246	2
9	10	Omeprazole_IA-3_EtOH_20_40	1.586	1.157	1.322	1.279	7.115	8.234	49.347	50.653	2

Fig. 33 Using Software to Rank Separation Parameters

Chapter 4 Examples of SFC Solutions

Due to the properties of supercritical carbon dioxide, SFC not only offers a solution for separating substances that are difficult to separate by HPLC, but also can be used for pretreatment, as described above. This chapter describes examples of using SFC and the SFE pretreatment method.

4-1 Analyzing Triglycerides by SFC

4-2 Separating Chiral Compounds by SFC

4-3 Analyzing Mycotoxins in Food by Online SFE-SFC

4-4 Extracting Pesticides from Brown Rice by Offline SFE

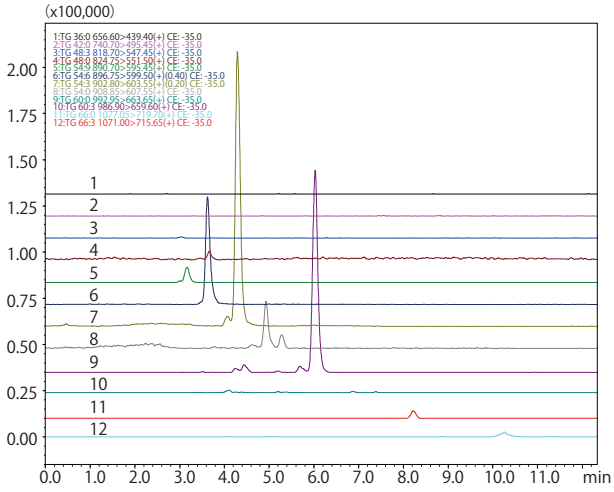
4-1 Analyzing Triglycerides by SFC

Triglycerides are a type of neutral fat decomposed from fat and other tissue to supply energy to cells when needed. Triglycerides are a family of structurally similar compounds with low polarity, which makes them considered difficult to analyze by conventional HPLC. In this example, SFC was used to analyze the triglycerides contained in vegetable oil. For more details about this example, refer to Application News No. C189 "Analysis of Triglycerides Using the Nexera UC Supercritical Fluid Chromatograph"

The composition of triglycerides targeted in this analysis are shown in Table 7. For separation modes that use a retention mechanism based on hydrophobic interactions, such as used in ODS columns, a longer carbon chain length in the acyl group will result in greater retention, whereas more double bonds, given the identical carbon chain length, will result in less retention. Fig. 34 shows a chromatogram from analyzing fish oil diluted with hexane by 10,000 times. The food-grade oil contains many different triglycerides, which comprise different types of acyls depending on their source. In reality, an extremely large number of molecular types exist.

Table 7 Target Components and MRM

Compounds	Acyl composition		MRM
Trilaurin	TG 36:0	C12:0/C12:0/C12:0	ESI(positive)	656.60>439.40
Trimyristin	TG 42:0	C14:0/C14:0/C14:0	ESI(positive)	740.70>495.45
Tripalmitolein	TG 48:3	C16:1/C16:1/C16:1	ESI(positive)	824.75>551.50
Tripalmitin	TG 48:0	C16:0/C16:0/C16:0	ESI(positive)	818.70>547.45
Trilinolenin	TG 54:9	C18:3/C18:3/C18:3	ESI(positive)	908.85>607.55
Trilinolein	TG 54:6	C18:2/C18:2/C18:2	ESI(positive)	902.80>603.55
Triolein	TG 54:3	C18:1/C18:1/C18:1	ESI(positive)	896.75>599.50
Tristearin	TG 54:0	C18:0/C18:0/C18:0	ESI(positive)	890.70>595.45
Trieicosenoin	TG 60:3	C20:1/C20:1/C20:1	ESI(positive)	986.90>659.60
Triarachidin	TG 60:0	C20:0/C20:0/C20:0	ESI(positive)	992.95>663.65
Trierucin	TG 66:3	C22:1/C22:1/C22:1	ESI(positive)	1071.00>715.65
Tribehenin	TG 66:0	C22:0/C22:0/C22:0	ESI(positive)	1077.05>719.70



1. TG 36:0 (Trilaurin)
2. TG 42:0 (Trimyristin)
3. TG 48:3 (Tripalmitolein)
4. TG 48:0 (Tripalmitin)
5. TG 54:9 (Trilinolenin)
6. TG 54:6 (Trilinolein)
7. TG 54:3 (Triolein)
8. TG 54:0 (Tristearin)
9. TG 60:3 (Trieicosenoin)
10. TG 60:0 (Triarachidin)
11. TG 66:3 (Trierucin)
12. TG 66:0 (Tribehenin)

Fig. 34 Chromatogram of Fish Oil

4-2 Separating Chiral API Molecule using SFC

Optically active (chiral) compounds contain asymmetric carbon structures in their molecules that cannot be superimposed on their mirror images. Such isomers have identical chemical and physical properties, but often have been confirmed to have activity differences, especially in biological organisms. Consequently, there is a growing need to analyze them during drug synthesis for drug discovery in the pharmaceutical industry.

HPLC is the main method used to separate chiral compounds by chromatography, but there are concerns about the cost and environmental impact of the organic solvents used for separation by normal-phase chromatography. The supercritical carbon dioxide used in SFC is nonpolar and has similar properties to n-hexane normally used as a mobile phase in normal-phase chromatography. That makes it possible to migrate chiral separation methods from conventional normal-phase chromatography to SFC. However, that requires selecting from a wide variety of columns and modifiers during optimization of chiral compound separation parameters for SFC. Therefore, the following is an example of using a Nexera UC chiral screening system to determine the optimal chiral separation parameters for omeprazole. For more information about the analysis, refer to Application News No. L495 "Automated Optimization of Chiral Separation Parameters Using Nexera UC Chiral Screening System"

The Nexera UC chiral screening system can automatically switch between up to twelve columns, four types of modifiers, and various mixture ratios thereof to automatically optimize from a large number of possible separation parameters. Each parameter setting is specified using the Method Scouting Solution software shown in Fig. 32. The columns used are listed in Table 8.

Table 8 Chiral Columns Used for Separation Parameter Optimization

Column	Stationary Phase
CHIRALPAK® IA-3/SFC (IA)	Amylose tris (3, 5-dimethylphenylcarbamate)
CHIRALPAK IB-3/SFC (IB)	Cellulose tris (3, 5-dimethylphenylcarbamate)
CHIRALPAK IC-3/SFC (IC)	Cellulose tris (3, 5-dichlorophenylcarbamate)
CHIRALPAK ID-3/SFC (ID)	Amylose tris (3-chlorophenylcarbamate)
CHIRALPAK IE-3/SFC (IE)	Amylose tris (3, 5-dichlorophenylcarbamate)
CHIRALPAK IF-3/SFC (IF)	Amylose tris (3-chloro-4-methylphenylcarbamate)
CHIRALPAK AD-3/SFC (AD)	Amylose tris (3, 5-dimethylphenylcarbamate)
CHIRALPAK AS-3/SFC (AS)	Amylose tris [(S)-*α*-methylbenzylcarbamate]
CHIRALPAK AY-3/SFC (AY)	Amylose tris (5-chloro-2-methylphenylcarbamate)
CHIRALCEL® OD-3/SFC (OD)	Cellulose tris (3,5-dimethylphenylcarbamate)
CHIRALCEL OJ-3/SFC (OJ)	Cellulose tris (4-methylbenzoate)
CHIRALCEL OZ-3/SFC (OZ)	Cellulose tris (3-chloro-4-methylphenylcarbamate)

Fig. 35 shows the results of separation patterns from a total of 36 possible combinations of 12 chiral columns and 3 types of modifiers (methanol, ethanol, and acetonitrile/ethanol mixture). Then optional software was used to rank the results, with chromatograms for the top three shown in Fig. 36. As a result, the parameters were successfully optimized while reducing the amount of work involved in the tedious process of optimizing separation parameters for chiral analysis by SFC.

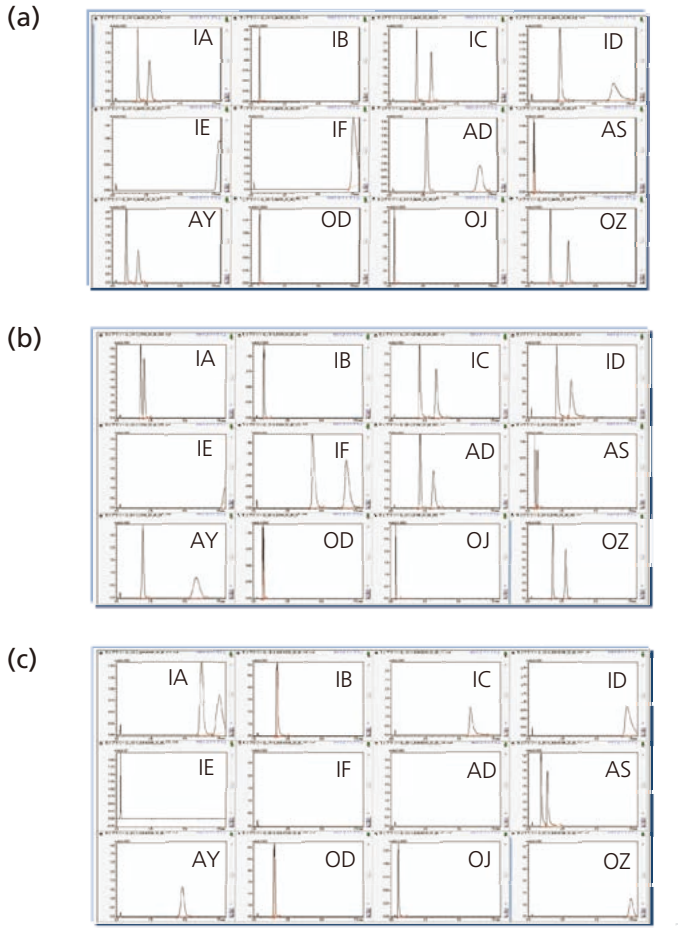
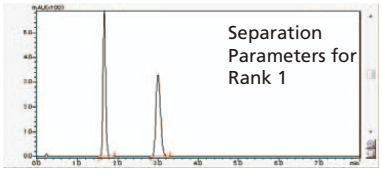
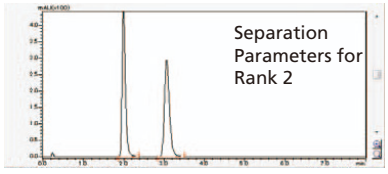


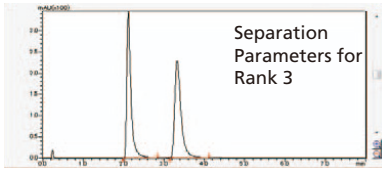
Fig. 35 Chiral Screening Separation Results
Modifiers: (a) Methanol, (b) Ethanol, and (c) Acetonitrile/ethanol = 75/25 (v/v)



Column: CHIRALCEL OZ-3/SFC
Modifier: Methanol



Column: CHIRALPAK IC/SFC
Modifier: Methanol



Column: CHIRALPAK IC/SFC
Modifier: Ethanol

Fig. 36 Chromatograms for Top Three Separation Parameters

4-3 Analyzing Mycotoxins in Food using Online SFE-SFC

Mycotoxins are contaminants present in a variety of foods and animal feed that have been reported to affect the health of humans and animals. Therefore, to minimize health problems in people and animals, Europe has specified maximum allowable limit values for mycotoxins in food and feed products. However, conventional mycotoxin pretreatment methods require extraction and other tedious steps. This section describes an example of using SFE to extract mycotoxins from food and then analyze it online.

An overview diagram of the system used for analysis is shown in Fig. 37. Peanut butter containing a certified standard sample of aflatoxin substances and corn containing a certified standard sample of mycotoxin substances were used as samples. A dehydrating agent* was mixed with the peanut butter. One gram of the corn was weighed out. Then the samples were placed in the extraction vessel to extract components by SFE (Fig. 38).

* "Miyazaki Hydro-Protect" Pat. No. 3645552

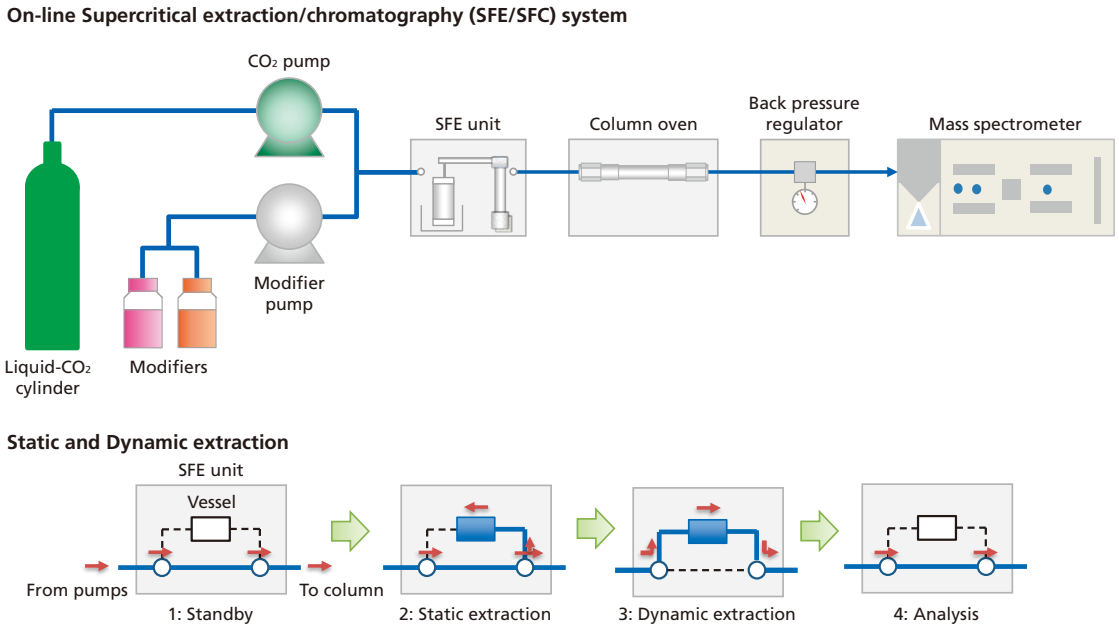


Fig. 37 Overview Diagram of System Used to Analyze Mycotoxins

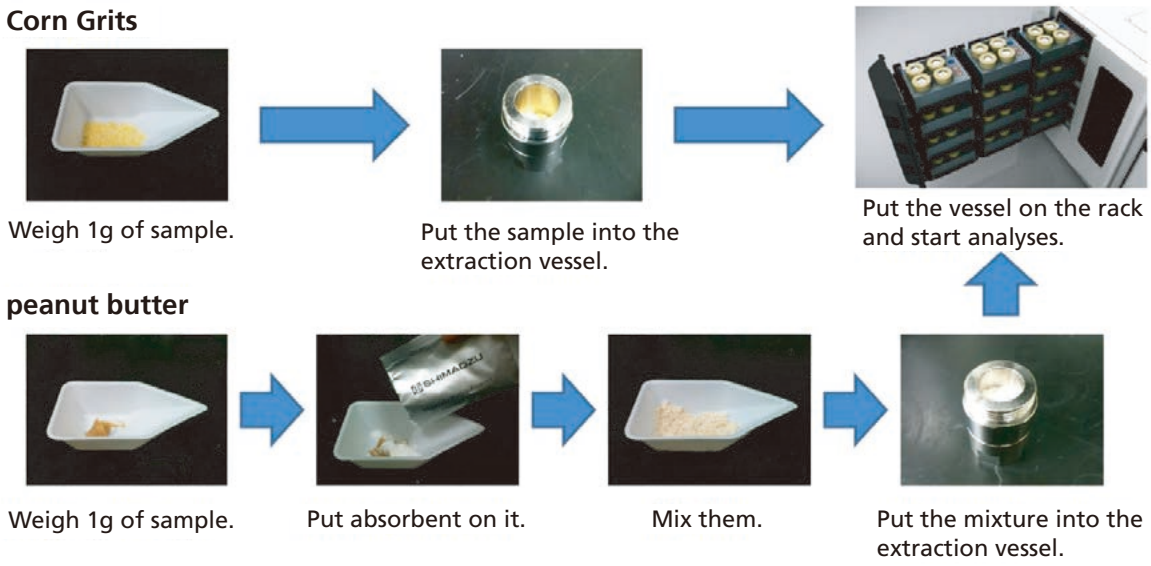


Fig. 38 SFE Extraction Preparation for Mycotoxin Analysis

Results from measurements using extraction conditions and analytical conditions listed in Table 9 are shown in Fig. 39. The results confirm that SFE extraction can be used for mycotoxin components in actual food.

Table 9 Analytical Conditions

[SFE] Nexera UC		
Extraction vessels	5 mL	
Static extraction	Extraction time	2min
	B. Conc.	Corn: 20% (0-1 min), 0% (1.01-2min) Peanut Butter: 30% (0-1 min), 0% (1.01-2min)
	BPR	A: 150 bar (to column) B: 152 bar (to waste)
	Flow rate	5.0 mL/min
Dynamic extraction	Extraction time	2min
	B. Conc.	0% (2-4min)
	BPR	A: 150 bar (to column) B: 152 bar (to waste)
	Flow rate	5.0 mL/min

[SFC] Nexera UC	
Column	Cosmosil π-NAP (150 mmL. x 4.6 mm, 5 μm)
Mobile phase	A: CO_2 B: 10 mmol/L ammonium acetate methanol solution
Flow rate	3.0 mL/min
Gradient program	B. Conc.: 0% (4-6 min) – 50% (20min)
Make-up solution	10 mmol/L ammonium acetate methanol solution
Make-up flow rate	0.1 mL/min
Column temp.	40 ˚C
BPR pressure	A: 150 bar (to column) B: 400 bar (to waste)

[MS Detection] LCMS-8060						
Ionization	ESI					
DL temperature	100 ˚C					
Block heater temp.	300 ˚C					
Interface temp.	300 ˚C					
Nebulizing gas flow	3 L/min					
Drying gas flow	5 L/min					
Heating gas flow	5 L/min					
MRMtransition	Aflatoxin B1	Positive	313.10 > 285.20 313.10 > 269.10 313.10 > 241.10	Zearalenone	Negative	317.20 > 175.20 317.20 > 131.10 317.20 > 273.25
	Ochratoxin A	Positive	404.00 > 239.05 404.00 > 220.95 404.00 > 193.25	Deoxynivalenol	Negative	355.10 > 59.10 355.10 >265.30

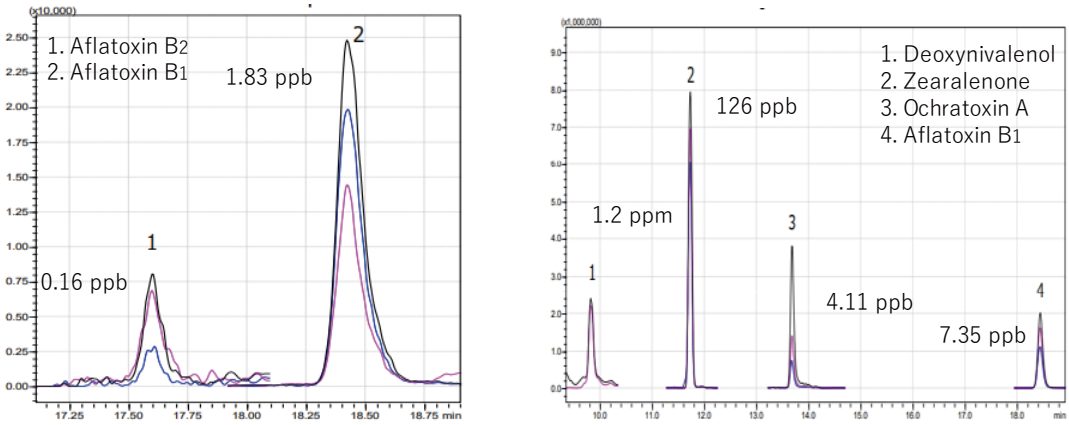


Fig. 39 Results from Analyzing Mycotoxins in Food

4-4 Extracting Pesticides from Brown Rice using Offline SFE

QuEChERS is a well-known method for pretreating agricultural products for residual pesticide analysis that prioritizes simplicity and speed. While special kits are available for the QuEChERS method, the kits require a large number of process steps, including reagent addition, solvent extraction, purification by dispersive solid phase extraction, and centrifugal separation. In this example, a Nexera UC offline SFE system was used to extract target pesticides from agricultural products and then analyze them by GC-MS. For more details about the analysis, refer to Application News No. L502 "Analysis of Residual Pesticides in Agricultural Products Using Nexera UC Off-Line SFE-GC/MS System"

In the offline SFE system, the extraction vessel was filled with 1 g of the agricultural product crushed with a mixer and mixed with 1 g of a dehydrating agent*. Using a specially designed rack changer enables successive extraction from up to 48 samples. A standard mixture solution of GC/MS target pesticides (Hayashi Pure Chemical PL2005 Pesticide GC/MS Mix I-VI, 7) was added to ground brown rice to make a sample concentration of 100 ng/g before extraction by SFE. The resulting extracts were then analyzed by GC-MS. The MRM chromatogram obtained from GC/MS analysis is shown in Fig. 40, with the partition coefficients and recovery rates for each pesticide indicated in Fig. 41. Excellent recovery rates (70 to 120 %) were achieved for all pesticides.

* "Miyazaki Hydro-Protect" Pat. No. 3645552

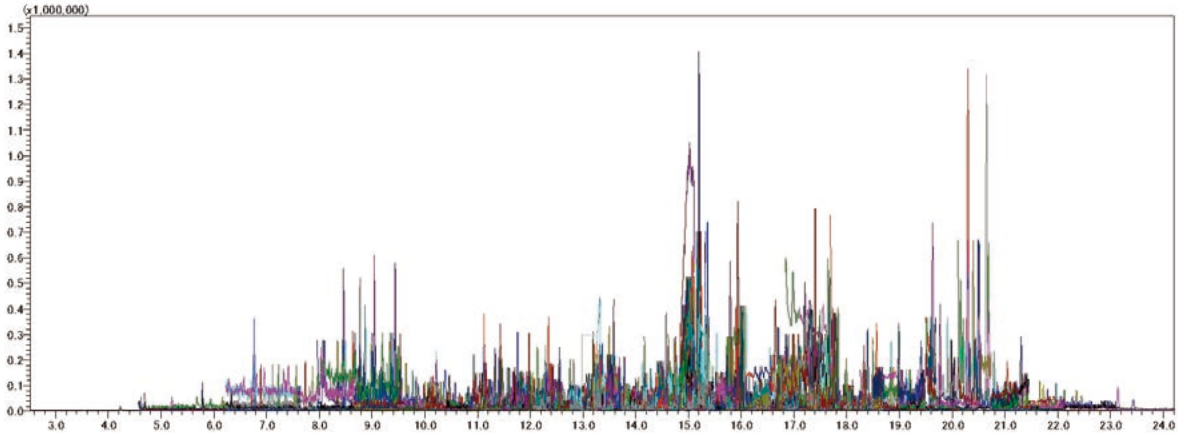


Fig. 40 MRM Chromatogram of Brown Rice Extract

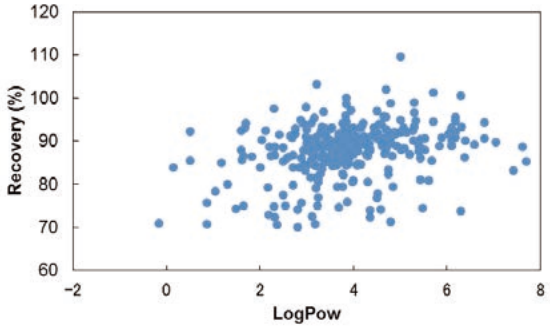


Fig. 41 Pesticide Partition Coefficients and Recovery Rates

Chapter 5 Frequently Asked Questions about SFC

5-1 SFC Installation

Q1: Are there any special precautions for indoor use?

Be careful to discharge exhaust gases into a fume hood to prevent CO_2 concentrations from becoming too high within the laboratory.

Installing a CO_2 meter is also recommended for the purpose of detecting CO_2 gas leaks within the room.

Q2: Is a temperature-controlled (and humidity-controlled) room environment preferable for maintaining the carbon dioxide gas supply temperature?

As long as the instruments are used within the manufacturer's recommended conditions, controlling the room temperature is probably not a significant concern because temperature is controlled in the column oven, but gas cylinders are affected by the ambient temperature where they are installed so care should be given to that temperature.

Q3: Can an LC unit currently in use (Prominence LC-20A series) be used in combination with SFC?

Yes, if the current Prominence unit is operated in combination with a modifier pump. For more details, contact Shimadzu.

Q4: Can CO_2 cylinders typically distributed be used?

Cylinders of 99.9 % CO_2 gas that are typically sold are more than acceptable for use. However, quality can vary so be sure to contact the gas vendor to confirm detailed specifications.

Q5: About how much CO_2 gas is used per analysis?

Assuming ten minutes per analysis and pumping 100 % CO_2 at a flowrate of 3 mL/min, about 30 g is used per analysis. Use that value to recalculate usage based on appropriate changes to the analysis time, modifier ratio (which reduces the proportion of CO_2), and flowrate.

5-2 Samples and Target Components

Q1: Do samples analyzed by SFC need to be dissolved in a liquid like HPLC?

In general, samples must be dissolved in a solution before injection, just like for HPLC. However, by connecting it to a supercritical fluid extraction (SFE) system, even components in solid samples can be analyzed.

Q2: For SFC, how is sample solubility in the mobile phase checked?

Due to the polarity of supercritical carbon dioxide, the ideal mobile phase should be n-hexane or a mixture of n-heptane and 2-propanol, but typically samples are dissolved for use in 100 % methanol or other modifier.

Q3: Can samples containing water be injected?

Yes, they can. However, for larger injection volumes, the sample solvent could affect peak shape or retention times.

Q4: Does supercritical carbon dioxide cause any sample decomposition?

Supercritical carbon dioxide is known to cause slight acidity in mobile phases, but there is minimal risk of decomposing or otherwise altering compounds.

Q5: Are there any examples of analyzing blood, urine, or other biological samples by injecting them directly into an SFC system?

Currently, there are probably no previous examples of analyzing blood by direct injection. However, there are examples of analyzing blood soaked in filter paper by SFE-SFC and examples of directly injecting urine samples for SFC-MS analysis.[*8-11]

Q6: Are there any samples that can be analyzed by HPLC, but not SFC?

SFC analysis of amino acids, soluble vitamins, polyphenols, and other highly polar compounds is possible by increasing modifier concentration and adding an acid. However, the resulting peak shapes are not as good as when using HPLC. For preparative applications, SFC offers the advantage of not requiring drying or purification steps after fractionation.

Q7: What type of modifier solvent is typically selected for analyzing compounds that decompose in alcohol or water?

A non-protic solvent is often used in cases where SFC is useful for analyzing samples that decompose or change properties when exposed to moisture. Candidate non-protic solvents include acetonitrile and THF.

5-3 Mobile Phase

Q1: What waste liquids remain after analysis? Are they the solvent with dissolved measurement target substances, modifiers, and make-up solution?

Yes, waste liquids include the solvent containing dissolved measurement target substances, modifiers, and make-up solutions. However, liquid waste bottles will also contain carbon dioxide gas and some solvent consumption occurs due to evaporation.

Q2: What additives can be added to modifiers to improve peak broadening problems when measuring hydrophilic peptides?

There is a report of analysis by using TFA as a method for improving peak shape in peptide analysis by SFC.[*12]

5-4 Columns

Q1: Can HPLC columns also be used for SFC?

SFC columns are generally compatible with fittings used in Shimadzu HPLC systems so it is possible to connect them. However, confirm the pressure capacity and other requirements for actual use with respective column manufacturers.

Q2: Can columns be used for both HPLC and SFC?

Yes, they can. However, it requires particular care regarding purging column liquids after analyses are finished. After using the column for SFC analysis, reduce the pressure inside the column to vaporize any CO_2 in the column or purge it with methanol or ethanol before disconnecting the column. Disconnecting the column with only supercritical carbon dioxide dries out the packing material due to evaporation of liquid in the column. Then if that column is used for HPLC, the high viscosity of the solvent used for the LC mobile phase, such as methanol or water, will cause surface tension around pores in the stationary phase that prevents penetration into the pores and interaction with functional groups. Because the supercritical fluid used for SFC analysis has lower viscosity than the mobile phase solvents used for HPLC, they can penetrate inside pores and interact with functional groups without being affected by surface tension, even if not filled with solvent. If a column used for HPLC analysis is used for SFC, any water in the column must be thoroughly purged with methanol or ethanol, because the water will not mix with the supercritical carbon dioxide.

Q3: Is there a difference in the service life of columns used for SFC and HPLC?

Due to the lower viscosity of supercritical fluid, it applies less force on the packing material. In other words, it applies less back pressure on the column, which tends to result in longer column life than for HPLC.

Q4: Since we cannot predict the elution order for separation by SFC, we do not know which columns are to be used or how they should be used. When screening columns for SFC, what separation parameters should be considered?

For SFC, the same mobile phase parameters can be used for everything from normal-phase columns to reversed-phase columns. Therefore, we recommend first fixing the mobile phase parameters and then checking separation using multiple columns. For advice about selecting columns, refer to 3-1-2 Column Selection. Given the multiple columns, solvent delivery parameters, and separation parameters such as temperature and pressure, there is a huge number of possible parameter combinations. To reduce the amount of work involved in considering separation parameters, you are recommended to use a method scouting system because it automatically sets and executes respective parameter combinations.

5-5 Miscellaneous

Q1: Are retention time and peak intensity repeatability levels comparable to HPLC levels in terms of precision and accuracy?

Repeatability comparisons for HPLC and SFC using identical conditions have resulted in comparable repeatability and quantitative accuracy levels.

Q2: We have been struggling with matrix and ghost peaking problems in our LC/MS analysis. Will the same phenomena occur for SFC as well?

Ionization can be inhibited by the matrix even for SFC/MS. However, since LC and SFC can result in significantly different separation behavior, sometimes SFC/MS can resolve such LC/MS matrix effect problems.

Q3: Can SFE be used for samples that contain non-volatile salts?

Yes, as long as the non-volatile salts are not dissolved by the extraction solvent. However, beware of contamination and precipitation if using a mass spectrometer for detection.

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