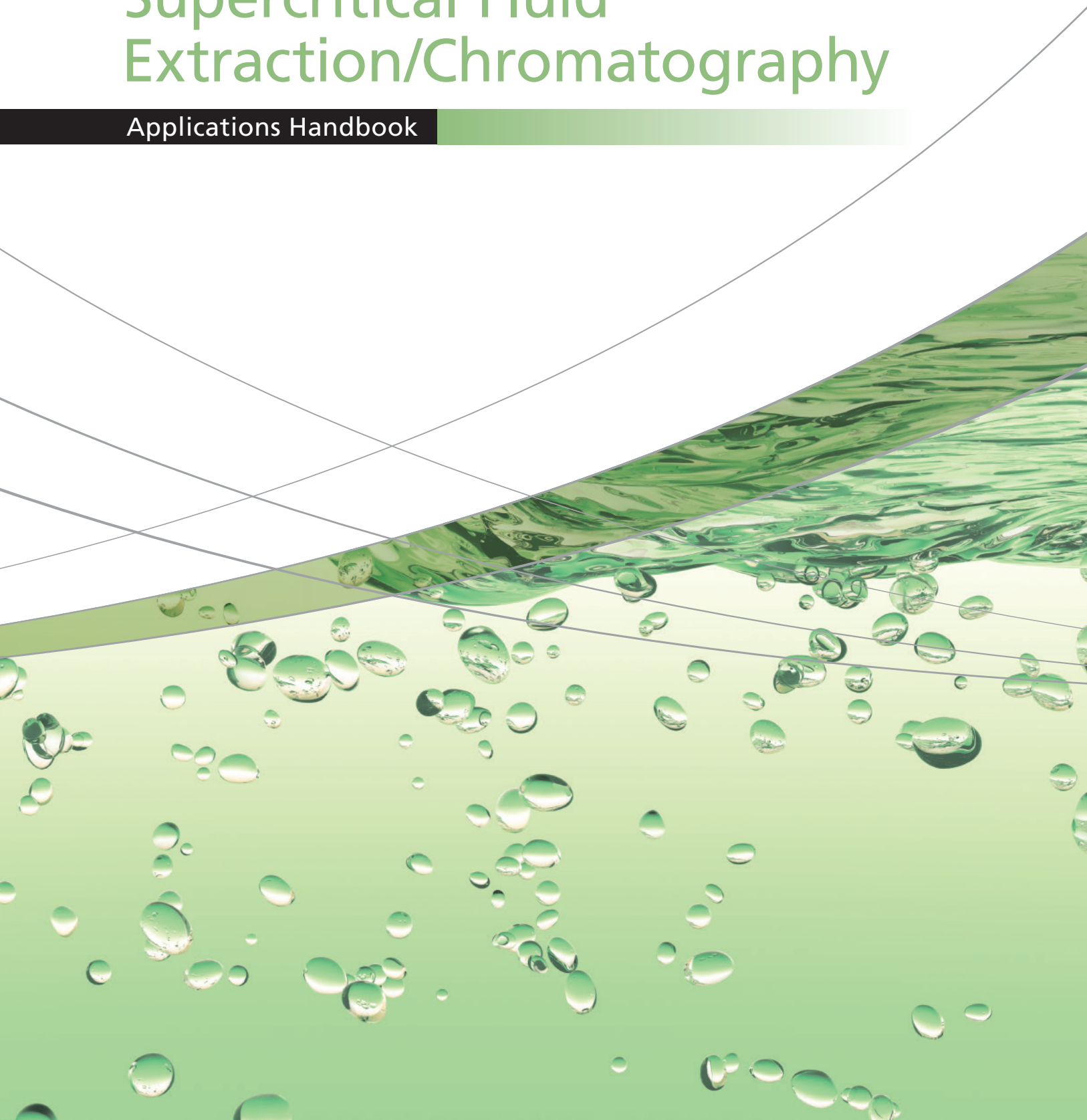
SHIMADZU
Excellence in Science

C10G-E059

Supercritical Fluid Extraction/Chromatography

Applications Handbook



Pharmaceutical and Nutraceutical

SHIMADZU
Excellence in Science

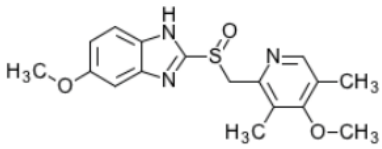
Application News

No.L495

Supercritical Fluid Chromatography

Automated Optimization of Chiral Separation Parameters Using Nexera UC Chiral Screening System

Chiral compounds contain asymmetric carbons in their molecules and are not superimposable on their mirror images. HPLC has been the main method used to separate such chiral compounds, but in recent years, the use of supercritical fluid chromatography (SFC) has been gaining attention. The main mobile phase used for chiral SFC is supercritical carbon dioxide, with low polarity, low viscosity, and high diffusivity, to which polar organic solvents (modifiers) are added to control solubility and polarity. Therefore, chiral compound separation by HPLC, which generally uses normal phase conditions, offers the potential for high speed, low organic solvent consumption, low cost, and low environmental impact. However, chiral SFC requires selecting a variety of separation parameters, such as columns and modifiers, which can involve large amounts of time and effort. This article describes using the Nexera UC chiral screening system to automatically optimize the large number of separation parameters by switching between up to 12 columns and various mixture ratios of four types of modifiers. This can significantly reduce the effort required.



Omeprazole

Fig. 1 Sample Used to Evaluate the Method Scouting Function

Separation Parameters for the Chiral Screening System

Model sample: The structure of omeprazole is shown in Fig. 1. Daicel CHIRALPAK®/CHIRALCEL® series 12 columns for chiral analysis were used for the analysis. These columns offer a line of complementary stationary phase columns that are able to separate a wide variety of chiral compounds. When used in combination with the Nexera UC chiral screening system, which features a method scouting function, optimal chiral separation parameters can be determined easily. In addition, three types of modifiers were used, methanol, ethanol, and a mixture of acetonitrile and ethanol. Details about the separation parameters are indicated in Table 1. The optimal parameters for chiral separation were comprehensively selected from the total of 36 possible combinations of modifiers (3 types) and columns (12 types).

Table 1 Analytical Conditions

Column	: CHIRALPAK®, CHIRALCEL® Series 100 mm L. × 3.0 mm I.D., 3 µm
Mobile Phase	: A; Super critical fluid of CO_2 B; Modifier: Methanol, Ethanol, mixture of Acetonitrile: Ethanol = 3:1 (v:v)
Time Program	: B Conc. 20 % (0 - 8 min) → 40 % (8 - 10 min) → 20 % (10 - 14 min)
Flowrate	: 3 mL/min
Column Temp.	: 40 °C
Injection Volume	: 2 µL
BPR Pressure	: 10 Mpa
Detector	: Photodiode Array Detector (Max Plot 210 - 400 nm)

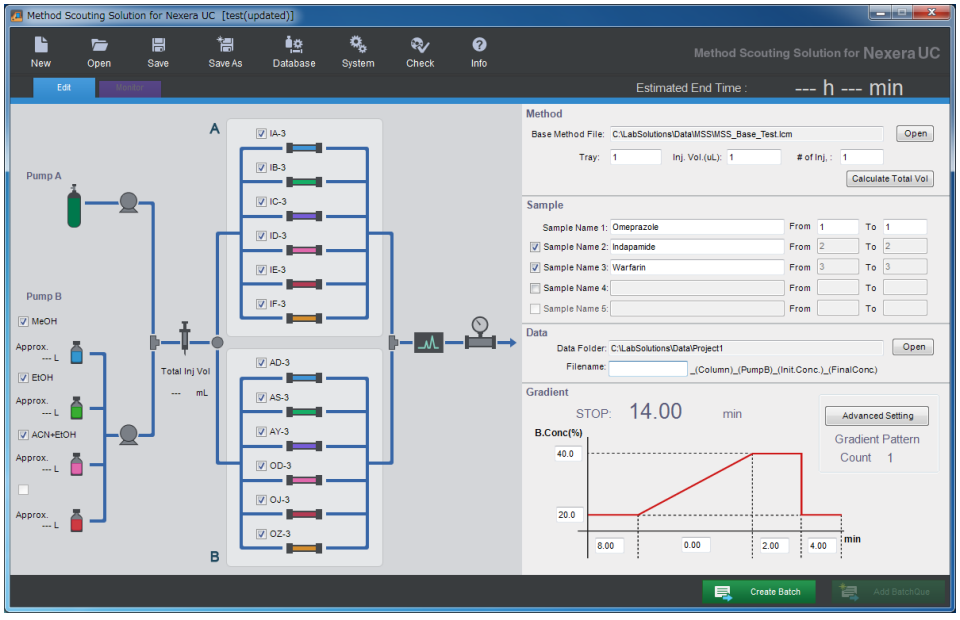
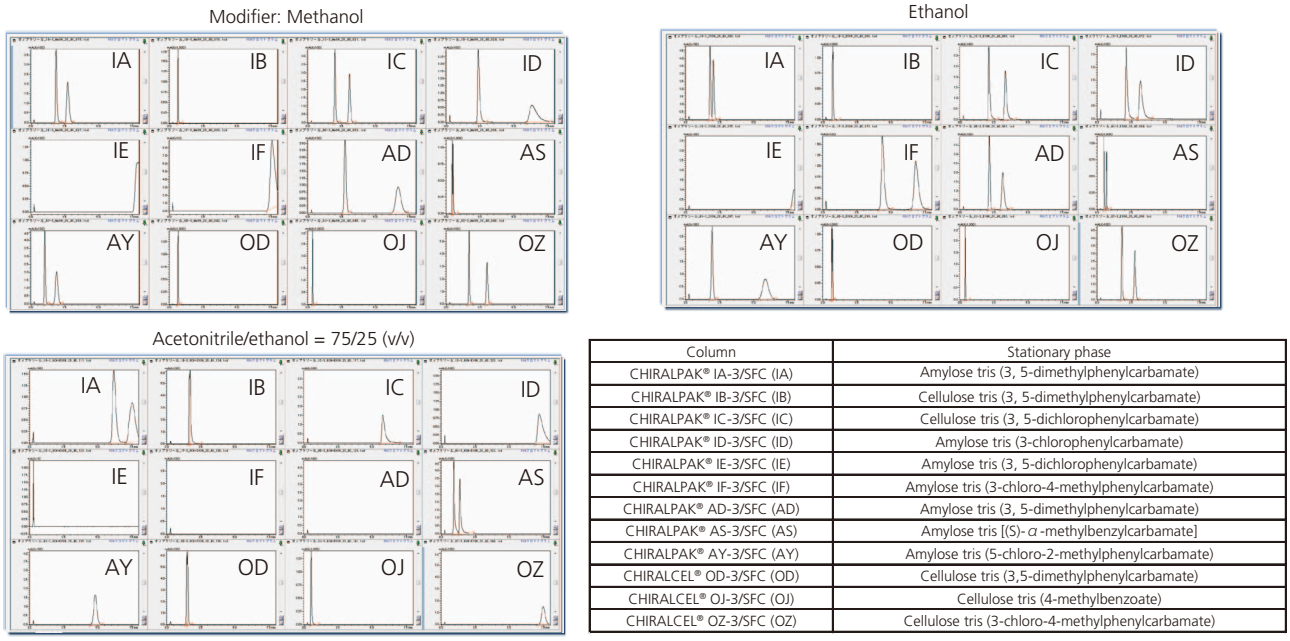


Fig. 2 Method Scouting Solution Operating Screen for Nexera UC

Automated Optimization of Chiral Separation Parameters for Omeprazole

Fig. 3 shows the results from a total of 36 possible combinations of 12 chiral columns and 3 types of modifiers (methanol, ethanol, and acetonitrile/ethanol mixture).

For omeprazole, separation of peaks for two chiral forms were confirmed within 8 minutes of retention. Fig. 4 shows the separation evaluation and optimal parameter ranking results from the optional software. The software automatically ranks all the chromatograms with separation greater than a given criteria (in this case, 1.5). This confirmed the utility of using the Nexera UC chiral screening system to automatically optimize separation parameters for chiral SFC, which otherwise requires a complicated process of selecting analytical conditions.



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. 3 Comparison of Separation Using Different Combinations of 12 Chiral Columns and 3 Modifiers

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

Separation Parameters for Rank 1
Column: CHIRALCEL® OZ-3/SFC
Modifier: Methanol

Separation Parameters for Rank 2
Column: CHIRALPAK® IC/SFC
Modifier: Methanol

Separation Parameters for Rank 3
Column: CHIRALPAK® IC/SFC
Modifier: Ethanol

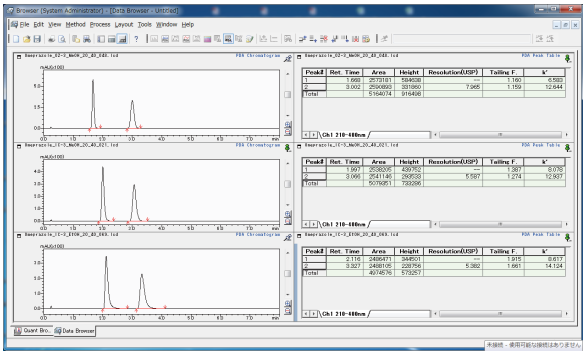


Fig. 4 Evaluation of Separation Parameters and Chiral Separation Chromatogram Using Optimized Parameters

* CHIRALPAK® and CHIRALCEL® are registered trademarks of Daicel Corporation.

First Edition: Oct. 2015

Shimadzu Corporation
www.shimadzu.com/an/

SHIMADZU
Excellence in Science

Application News

No.L499A

Supercritical Fluid Extraction / Chromatography

Application of Online SFE-SFC-PDA for Cleaning Validation

Cleaning validation is a process step that is extremely important for ensuring high quality and safety at pharmaceutical manufacturing sites. Cloth used for surface wiping, called a swab, is used to wipe a given part of a piece of manufacturing equipment, and analysis of the wiped area of the swab is performed by using high-performance liquid chromatography (HPLC) or a total organic carbon analysis (TOC). Evaluations using HPLC have been increasingly used in recent years because HPLC enables determination of individual compounds. Prior to analysis, an extraction procedure must be performed on the swab. Using supercritical fluid extraction (SFE) as the pretreatment method allows for simple and quick target component extraction. Using supercritical fluid chromatography (SFC) after SFE also means that analysis results can be obtained simply by preparing the sample for SFE, which unifies the work flow from pretreatment to analysis. Please see Application News L496 for an overview of online SFE-SFC. This article describes the process of column selection using the Nexera-UC Chiral Screening System as the first step in analysis of the target compound alkylbenzenesulfonate.

Analytical Column Selection

For SFC analysis, selection of the optimal column for the sample has a substantial effect on analysis reliability. We performed SFC separation of alkylbenzenesulfonate in four different columns under the conditions shown in Table 1 and Fig. 1, and chose the Shim-pack UCX-SIL analytical column as it had the best peak shape. Based on an investigation of gradient profiles, we also found a relatively steep gradient profile is suitable for quantitative analysis as the properties of alkylbenzenesulfonate, which have different length of carbon chains, mean the significant peak broadening if the gradient slope is not steep. Based on this information, we optimized analytical conditions using the Shim-pack USX-SIL column and performed online SFE-SFC analysis of a sample from a swab.

Table 1 SFC Analytical Conditions for Column Selection

Column	: Shim-pack UCX series columns (250 mm L. × 4.6 mm I.D., 5 µm) (i) UCX-RP (ODS with polar group), (ii) UCX-GIS (ODS), (iii) UCX-SIL, (iv) UCX-DIOL
Mobile Phase	: A: CO_2; B: Methanol
Time Program	: Shown in the figure
Flowrate	: 3.0 mL/min
Column Temp.	: 40 °C
Back Pressure	: 15 MPa
Wavelength	: 220 nm
Injection Vol.	: Shown in figure

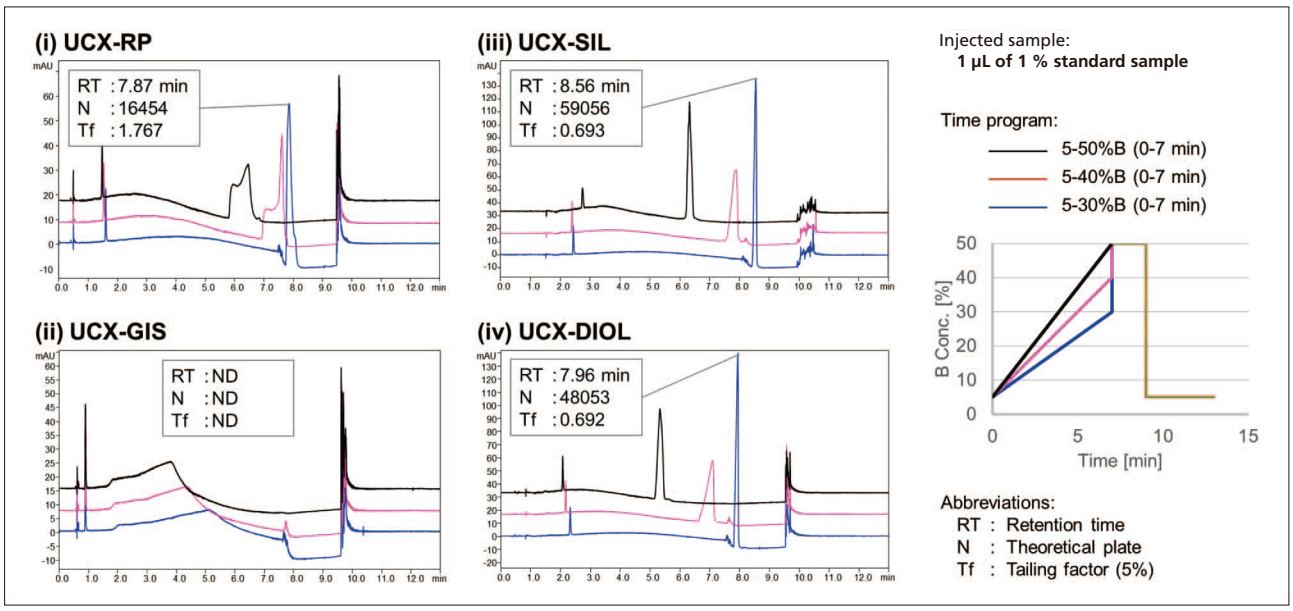


Fig. 1 Comparison of SFC Separation of Standard Alkylbenzenesulfonate in Four Different Columns

Online SFE-SFC Analysis of a Swab Containing Alkylbenzenesulfonate

We investigated column selection by the scouting system, chose the Shim-pack UCX-SIL analytical column, optimized each analytical condition for online SFE-SFC analysis, then performed analysis using the conditions shown in Table 2 below.

Table 2 Analytical Conditions for Online SFE-SFC

[Sample Preparation]	
A total of 10 to 500 µg standard samples in methanol were dropped onto swabs.	
The swabs were enclosed into an extraction vessel and set to the SFE unit.	
[Static Extraction]	
Extraction Time	: 3 min
Mobile Phase	: A: CO_2; B: 0.1 % (w/v) Ammonium Formate in Methanol
B Conc.	: 10 %
Flowrate	: 3.0 mL/min
Back Pressure	: 15 MPa
[Dynamic Extraction]	
Extraction Time	: 3 min
Mobile Phase	: A: CO_2; B: Methanol
B Conc.	: 10 %
Flowrate	: 3.0 mL/min
Back Pressure	: 15 MPa
[SFC]	
Column	: Shim-pack UCX-SIL (250 mm L. × 4.6 mm I.D., 5 µm)
Mobile Phase	: A: CO_2; B: Methanol
Time Program	: 10 %B (0-2 min), 10-60 %B (2-7 min), 60 %B (7-9 min), 10 %B (9-13 min)
Flowrate	: 3.0 mL/min
Column Temp.	: 40 °C
Back Pressure	: 15 MPa
Wavelength	: 220 nm

The peak for the surfactant alkylbenzenesulfonate was well-separated and detected as shown in Fig. 2 below. Fig. 3 shows the results of performing repeated SFE-SFC analyses from the same swab to which had been added an equivalent of 100 ng of alkylbenzenesulfonate. Since there was almost no alkylbenzenesulfonate peak evident from the second and later sample extractions, the extraction procedure was almost entirely complete after the first SFE. Fig. 4 shows the results of adding amounts of alkylbenzenesulfonate to swabs in the range of 10 to 500 µg, and checking linearity. Within this range, the coefficient of determination that represents linearity was 0.996. Fig. 5 shows the result of five consecutive analyses of separate swabs to which were added 100 µg of alkylbenzenesulfonate. Considering the process including extraction, the repeatability of retention times was 0.19 %RSD, and repeatability of peak area was 5.76 %RSD. Based on these results, we confirmed the usefulness of the Nexera-US Online SFE-SFC System in this application.

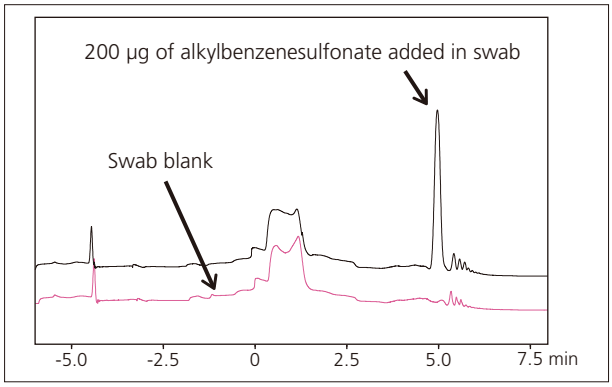


Fig. 2 Online SFE-SFC Analysis of Alkylbenzenesulfonate

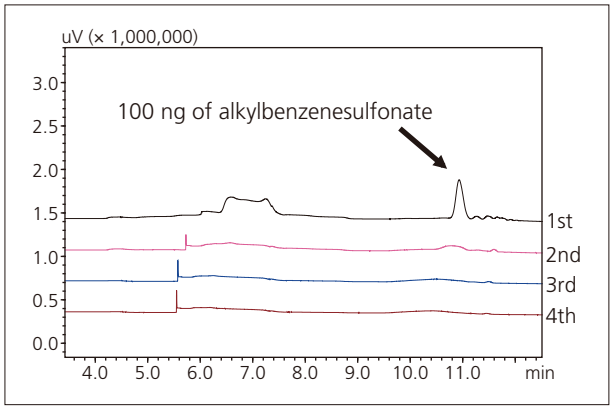


Fig. 3 Confirmation of Online SFE Extraction Efficiency

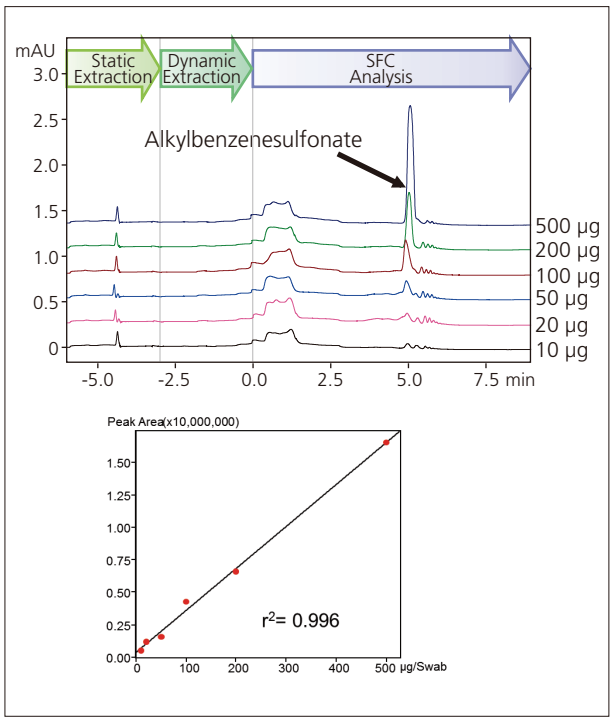


Fig. 4 Linearity of Online SFE-SFC Analysis Using a Swab

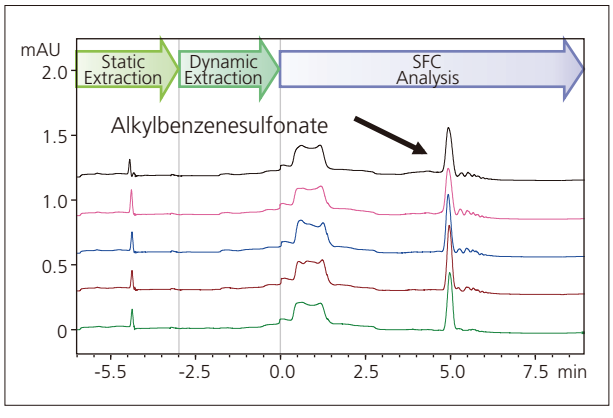


Fig. 5 Repeatability of Online SFE-SFC Analysis Using a Swab

Note: Swab samples were provided by DAIICHI SANKYO COMPANY, LIMITED.

Second Edition: Feb. 2016
First Edition: Jan. 2016

SHIMADZU

Shimadzu Corporation
www.shimadzu.com/an/

SHIMADZU
Excellence in Science

Application News

No.L501

Supercritical Fluid Extraction / Chromatography

Analysis of Vitamin E in a Commercial Supplement by Offline SFE-SFC-PDA

Vitamin E, also called tocopherol, is a fat-soluble vitamin and an important chemical substance that exhibits an antioxidant effect, particularly in the human body. There are four tocopherols (α, β, γ and δ) that differ based on the number and position of methyl groups. The α-tocopherol exhibits the strongest antioxidant activity, and this is the tocopherol form found in most commercial supplements as vitamin E. Since it is highly fat-soluble, a quick and simple extraction method using supercritical fluid is expected to be applicable. In this article, we introduce a procedure for α-tocopherol pretreatment that uses supercritical fluid extraction (SFE).

Offline SFE System

While the online SFE-SFC system has already been described in several Application News articles, many have expressed the desire to combine SFE with existing analytical methods other than SFC, and SFE has gained attention for its flexibility in terms of sample handling. The advantages of SFE are as follows.

1. Quick and highly efficient extraction using supercritical fluid that is highly permeable and has a high diffusion rate.
2. Extraction of unstable compounds under mild temperature conditions with light-shielding.
3. Low cost compared to solvent extraction.
4. Complete automation of the extraction procedure.
5. Easy handling of the extraction sample.
6. Compatible with various analysis methods.

Fig. 1 shows a flow diagram for an offline SFE system. A supercritical state is present upstream of the BPR back-pressure control unit. Valves inside the SFE unit are controlled to switch between static extraction via enclosure of supercritical fluid in the vessel and dynamic extraction via passage of supercritical fluid through the vessel, which enables quick and highly efficient extraction of the target compounds.

A HPLC pump with a low-pressure GE valve installed is used in the solvent delivery system, and the extraction conditions can be optimized by changing the type of modifier (maximum of four types, including eluent from the trap column) and the concentration relative to carbon dioxide. Extract is retained in the trap column, and the low-pressure GE valve on the solvent delivery pump is switched to the solvent suitable for elution from the trap column. Then the eluent is collected in test tubes with a fraction collector.

SFE Treatment for α-Tocopherol

The commercial supplement used as an actual sample may be present as a paste inside the capsule and may be moisture absorbent. As shown in Fig. 2, we mixed 275 mg of paste supplement with 1 g of Miyazaki Hydro-Protect, which is a dehydrating agent for SFE sold by Shimadzu, and transferred this mixture to the SFE extraction vessel.

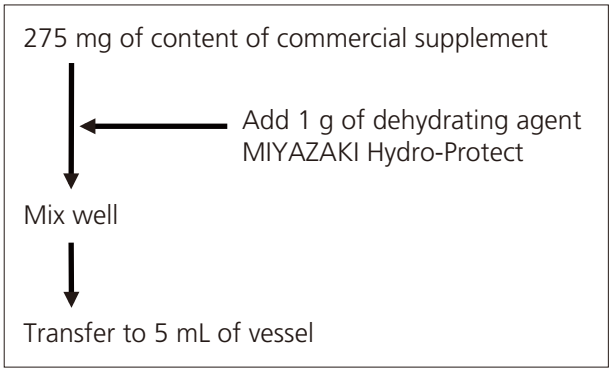


Fig. 2 Preliminary Pretreatment for Supplement Sample Before SFE

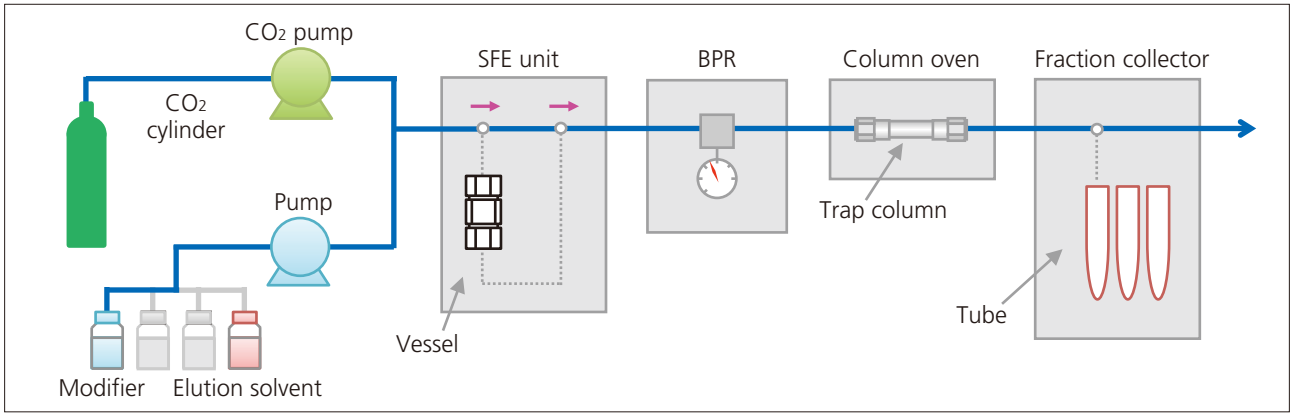


Fig. 1 Flow Diagram of Supercritical Fluid Extraction (SFE) System

The conditions used for SFE are shown in Table 1. We investigated column selection, chose the Shim-pack UCX-SIL analytical column, optimized each analytical condition for online SFE-SFC analysis, then performed analysis using the conditions shown in Table 2.

Table 1 SFE Conditions for α-Tocopherol

Offline SFE:	
Extraction Vessel	: 5 mL
Extraction Solvent	: CO_2
Flowrate	: 5 mL/min
Temperature	: 40 ˚C
Back Pressure	: 15 MPa
Extraction Time	: 15 min (Static 2 min → Dynamic 3 min) × 3 times
Trap & Pressure Down Conditions	
Trap Column	: Shim-pack VP-ODS (50 mm L. × 4.6 mm I.D.)
Temperature	: 60 ˚C
Pressure Down Time	: 10 min (15 - 25 min)
Recovery Conditions	
Elution Solvent	: Hexane
Flowrate	: 2 mL/min
Temperature	: 60 ˚C
Fraction Time	: 3.5 min (25 - 28.5 min)

SFE Evaluation of α-Tocopherol in a Commercial Supplement

For the α -tocopherol extract obtained through offline SFE, we performed SFC under the conditions shown in Table 2 then evaluated the extraction procedure. Extract was mixed with hexane to make up 10 mL before being used for SFC analysis. A representative SFC chromatogram is shown in Fig. 3.

Table 2 SFC Conditions for α-Tocopherol

SFC Conditions:	
Column	: Nacalai COSMOSIL Cholester (250 mm L. × 4.6 mm I.D., 5 µm)
Flowrate	: 3 mL/min
Modifier	: IPA
Gradient	: 2 % (0 min) - 20 % (10 min) - 50 % (10 - 12 min)
Temperature	: 40 ˚C
Back Pressure	: 15 MPa
Injection Volume	: 2 µL

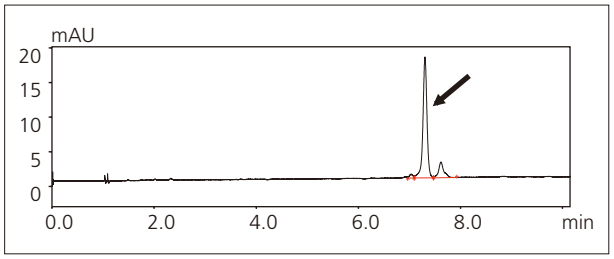


Fig. 3 SFC Analysis of α-Tocopherol Obtained by SFE from a Commercial Supplement

First, we used a standard product to evaluate the suitability of the α-tocopherol SFC conditions used for evaluation of offline SFE. Fig. 4 shows the linearity in the sample concentration range of 0.5 µg/L to 2.0 µg/L, and Table 3 shows the repeatability at a concentration of 1.0 µg/L. Good linearity and sufficient repeatability in terms of retention time, peak area and peak height were obtained.

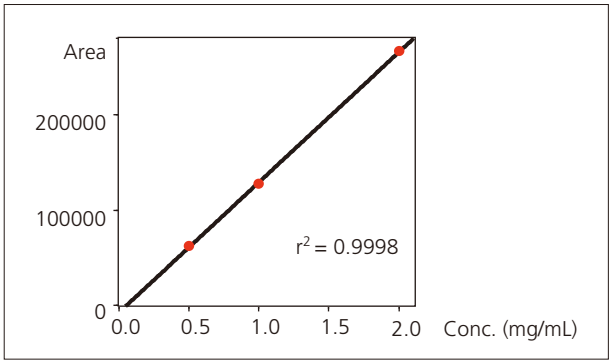


Fig. 4 Linearity for Standard α-Tocopherol Obtained by SFC

Table 3 Repeatability for Standard α-Tocopherol Obtained by SFC (n=6)

No	Retention Time (min)	Area	Height
Average	7.242	127,338	19,682
RSD (%)	0.057	0.573	0.274

Table 4 shows the repeatability of the quantitative α-tocopherol result obtained by repeated SFE treatment, and α -tocopherol recovery relative to the theoretical value (7.4 mg). Fig. 5 shows the overlaid chromatograms for α-tocopherol. Good recovery and repeatability was confirmed after just one extraction, showing that offline SFE is effective for vitamin E compound extraction.

Table 4 Repeatability and Recovery of α-Tocopherol in a Commercial Supplement Using SFE

No	Conc. (mg/mL)	Recovery (%)
1	0.776	104.46
2	0.780	105.00
3	0.772	103.92
4	0.790	106.35
5	0.761	102.44
6	0.758	102.04
Average	0.773	
RSD (%)	1.549	

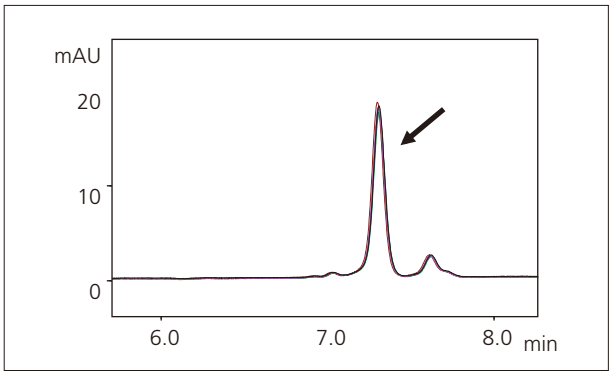


Fig. 5 Overlaid Chromatograms for α-Tocopherol After SFE

First Edition: Jan. 2016

Shimadzu Corporation
www.shimadzu.com/an/

SHIMADZU
Excellence in Science

Application News

No. L517

Supercritical Fluid Chromatography

Analysis and Evaluation of Chiral Drugs in Biological Samples Using the Nexera UC-MS/MS System

As introduced in Application News No. L495, the optimization for chiral separation using supercritical fluid chromatography (SFC) starts from employing column scouting to find the column and mobile phase appropriate to separation. This article introduces an example of the selectivity and sensitivity of drug level monitoring in a biological sample and the evaluation results of the analysis method, as an application to the pharmacokinetics research of chiral separation using SFC/MS/MS, after having selected an appropriate column.

Y. Watabe, T. Hattori, T. Iida

Analysis of Omeprazole in a Plasma Sample

The applicability of human plasma matrix to SFC was evaluated taking an example of enantiomeric drug omeprazole, well-known as a proton pump inhibitor. Fig. 1 shows the chemical structure of omeprazole. Fig. 2 shows the pretreatment procedure employed for the blood plasma sample. Table 1 lists the analytical conditions. CHIRALPAK® IC-3 from Daicel Company, which exhibited good separation when utilized in Application News No. L495 was used as the column. Detection was performed using the LCMS-8050 triple quadrupole mass spectrometer.

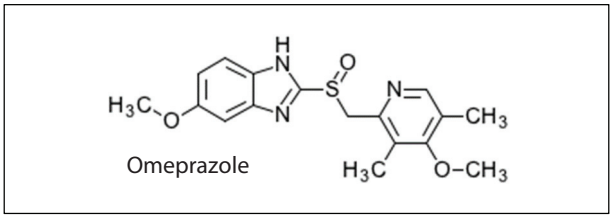


Fig. 1 Omeprazole Structure

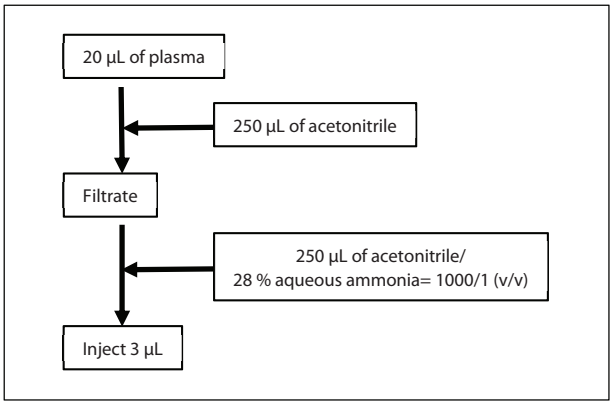


Fig. 2 Plasma Sample Pretreatment Procedure

Table 1 Analytical Conditions

Column	:	CHIRALPAK®, IC-3 (100 mm L. × 3.0 mm I.D., 3 µm)
Mobile phase	:	A) Super critical fluid of CO_2 B) Modifier: Methanol A/B = 5/1 (v/v for omeprazole, isocratic) = 4/1 (v/v for rabeprazole, isocratic)
Flow rate	:	3 mL/min
Column temp.	:	40 °C
Injection volume	:	3 µL
BPR pressure	:	10 MPa
BPR temp.	:	50 °C
Detector	:	LCMS- 8050 (ESI, MRM mode)
Make-up	:	Methanol
Make-up flow rate	:	0.1 mL/min
MRM	:	(+) *m/z* 346.1 > 198.1 (for omeprazole) (+) *m/z* 359.9 > 150.1 (for rabeprazole)

Calibration curve was created based on human plasma samples that contained 1, 2, 10, 2 and 100 µg/L of standard omeplazole to confirm the linearity of loaded amounts.
Fig. 3 and Fig. 4 show the MRM chromatograms for 2 µg/L and 20 µg/L respectively. Among the optically separated peaks, (A) is the fast-eluting isomer and (B) is the slow-eluting isomer. The linearity (r^2) obtained after correcting by 1/(concentration squared) was favorable at 0.99996 for omeprazole (A) and 0.99998 for omeprazole (B).

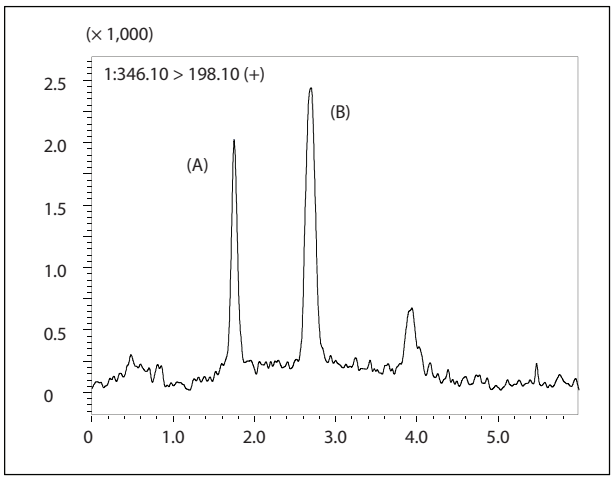


Fig. 3 Omeprazole Added to Human Plasma (2 µg/L)

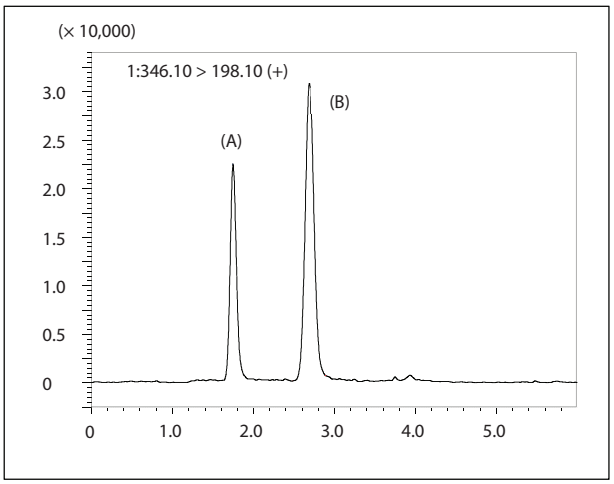


Fig. 4 Omeprazole Added to Human Plasma (20 µg/L)

The repeatability of the area values at 2 µg/L obtained from five repetitions was favorable with RSD values of 4.4 % for both omeprazole (A) and (B). At 10 µg/L, the recovery rates calculated from the results of stock solution analyses were 101.1 % and 100.5 % respectively.

Analysis of Rabeprazole in a Plasma Sample

Rabeprazole, known as a gastric acid secretion inhibitor, has a similar chemical structure to omeprazole, suggesting the possibility of successful chiral separation under similar analytical conditions including the same analytical column. Here we attempted to analyze rabeprazole in a plasma sample based on the analytical conditions used for omeprazole in the previous section. The chemical structure of rabeprazole is shown below. The structural similarity to omeprazole is easily recognized. As shown in Table 1, analysis was successful by merely changing the modifier concentration and the MRM settings.

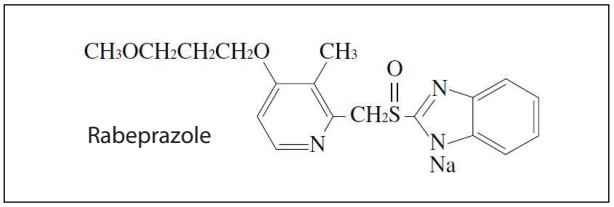


Fig. 5 Rabeprazole Structure

Calibration curve was crated based on human plasma samples that contained 0.3, 1, 3, 10 and 30 µg/L of standard raberlazole to confirm the linearity of loaded amounts. Fig. 6 and Fig. 7 show the MRM chromatograms for 3 µg/L and 30 µg/L respectively. As in Fig. 3 and Fig. 4, (A) is the fast-eluting isomer among the optically separated peaks and (B) is the slow-eluting isomer.
The linearity (r^2) obtained after correcting by 1/(concentration squared) was favorable at 0.99996 for rabeprazole (A) and 0.99999 for rabeprazole (B).

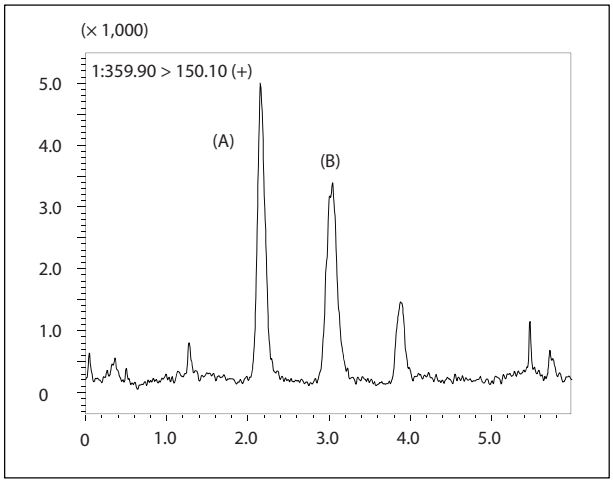


Fig. 6 Rabeprazole Added to Human Plasma (3 µg/L)

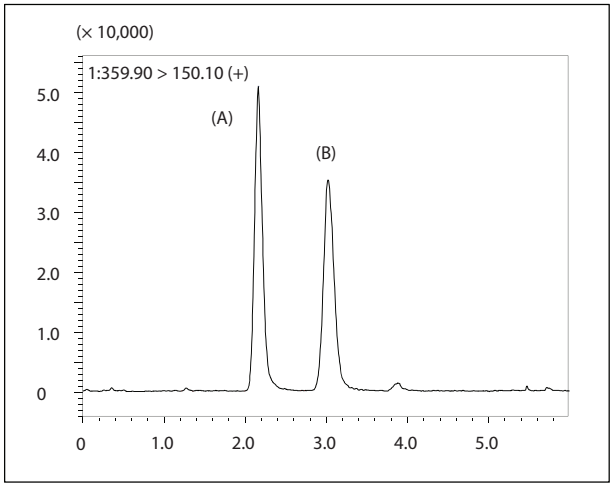


Fig. 7 Rabeprazole Added to Human Plasma (30 µg/L)

The repeatability of the area values at 10 µg/L obtained from five repetitions was favorable with RSD values of 1.8 % and 2.4 % for rabeprazole (A) and (B) respectively. The recovery rates calculated from the results of stock solution analyses were 102.5 % and 100.1 % respectively. Table 2 summarizes the linearity, peak area repeatability, and recovery rate for each compound. These results verify the applicability of this method to the practical analysis of plasma samples.

Table 2 Evaluation Results

	Linearity (r^2)	Area Repeatability (%RSD)	Recovery Rate (%) (4)
Omeprazole (A)	0.99996 (1)	4.4 (3)	101.1
Omeprazole (B)	0.99998 (1)	4.4 (3)	100.5
Rabeprazole (A)	0.99996 (2)	1.8 (4)	102.5
Rabeprazole (B)	0.99999 (2)	2.4 (4)	100.1

(1) 1 to 100 µg/L, (2) 0.3 to 300 µg/L, (3) 2 µg/L, (4) 10 µg/L

Notes: This product has not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan. It cannot be used for the purpose of medical examination and treatment or related procedures.

First Edition: Mar. 2017

Shimadzu Corporation
www.shimadzu.com/an/

SHIMADZU
Excellence in Science

Application News

No. L519

Supercritical Fluid Chromatography

Analysis of Choline and Acetylcholine in Rat Cerebrospinal Fluid Samples Using the Nexera UC-MS/MS System

Choline, which is a structural element of cell membranes, and acetylcholine, which is known as a neurotransmitter, are both familiar compounds in the field of bioanalysis. Since acetylcholine is biosynthesized in the body from choline, it is possible to estimate the quality of internal activity by monitoring both of these compounds. This article focuses on the SFC analysis of these compounds in a rat cerebrospinal fluid sample by direct injection of the cerebrospinal fluid to the Nexera UC SFC system. Also introduced is automatic extraction and analysis of a cerebrospinal fluid sample impregnated into filter paper, in consideration of convenience and durability for storage and transport, using the Nexera UC online SFE-SFC-MS/MS system.

Y. Watabe, T. Iida

SFC-MS/MS Analysis

A CN column provided favorable separation of choline and acetylcholine in SFC-MS/MS analysis. Calibration curves were created from the peak area values from six times repeated analyses for each of the three concentrations of 10, 100, and 1000 µg/L. Good linearity was obtained and the quantitation limit (LOQ, ASTM method) was 30 µg/L for choline and 10 µg/L for acetylcholine. Table 1 lists the conditions of SFC-MS/MS analysis. Fig. 1 shows the structural formula of choline and acetylcholine and Fig. 2 shows the obtained calibration curves.

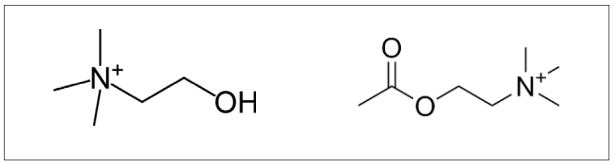


Fig. 1 Structure of Choline (Left) and Acetylcholine (Right)

Table 1 SFC-MS/MS Analytical Conditions

Column	:	Inertsil CN-3 250 mm L. × 4.6 mm I.D., 5 µm
Mobile phase	:	A) Supercritical fluid of CO_2 B) Modifier: Methanol containing 20 mmol/L ammonium formate / water =95/5 (v/v)
Time program	:	B Conc. 10 % (0 min) → 25 % (10 min) → 50 % (10.1-12 min) → 10 % (12.1-15 min)
Flow rate	:	2.5 mL/min
Column temp.	:	40 °C
Injection volume	:	1 µL
BPR pressure	:	10 Mpa
BPR temp.	:	50 °C
Detector	:	LCMS-8050 (ESI, MRM mode)
Make-up	:	Methanol
Make-up flow rate	:	0.2 mL/min
MRM transitions	:	(+) *m/z* 104.1>60.1 (for choline) (+) *m/z* 146. 1>87.1 (for acetylcholine)

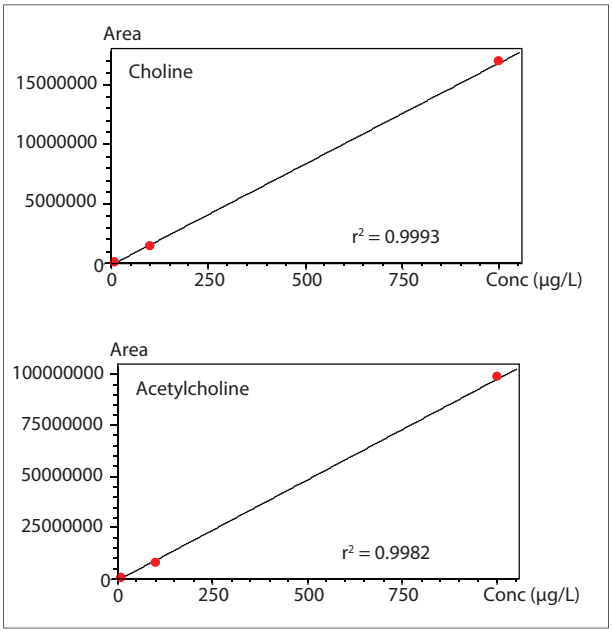


Fig. 2 Calibration Curves of Choline and Acetylcholine

The retention time and peak area repeatabilities after six repetitions at each concentration of 10, 100, and 1000 µg/L was confirmed at calibration curve creation and the results are summarized in Table 2. The linearity (r^2) was 0.9993 for choline and 0.9982 for acetylcholine. Fig. 3 shows the MRM chromatograms for 100 µg/L.

Table 2 Repeatabilities of Choline and Acetylcholine Standards (n = 6)

		Retention time (%RSD)	Peak area (%RSD)
Choline	10 µg/L	0.22	7.5
Choline	100 µg/L	0.05	1.7
Choline	1000 µg/L	0.07	2.2
Acetylcholine	10 µg/L	0.07	5.7
Acetylcholine	100 µg/L	0.06	4.2
Acetylcholine	1000 µg/L	0.07	6.0

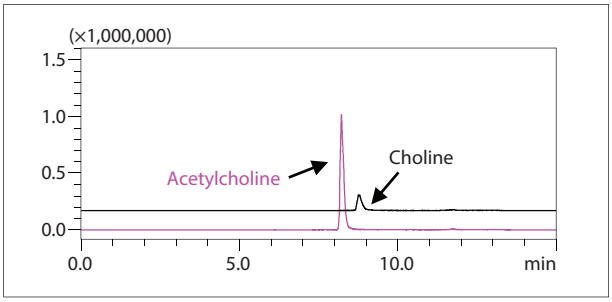


Fig. 3 Choline and Acetylcholine Standards (100 µg/L)

Next, by employing the microdialysis method in which biological compounds are continuously sampled from an awake animal via the semipermeable membrane of a minute dialytic probe connected to a pump, cerebrospinal fluid was sampled from a rat and directly delivered to SFC analysis. The injection volume of cerebrospinal fluid was set to 1 µL due to concerns regarding the miscibility between the aqueous sample and low polar supercritical carbon dioxide, which is the main component of the mobile phase used in SFC. With respect to acetylcholine, the LOQ determined according to the ASTM method was about 10 µg/L. Since the calculated concentration was less than the LOQ, only peak identification was performed. As shown in Table 3, the retention time and peak area repeatabilities were favorable for the six repeated analyses of choline. Fig. 4 shows the chromatograms resulting from SFC analysis of the cerebrospinal fluid sample.

Table 3 Choline Quantitative Value in Rat Cerebrospinal Fluid Sample and Repeatabilities (n = 6)

	Retention time (%RSD)	Peak area (%RSD)
Choline (Concentration 229.6 µg/L)	0.10	3.1

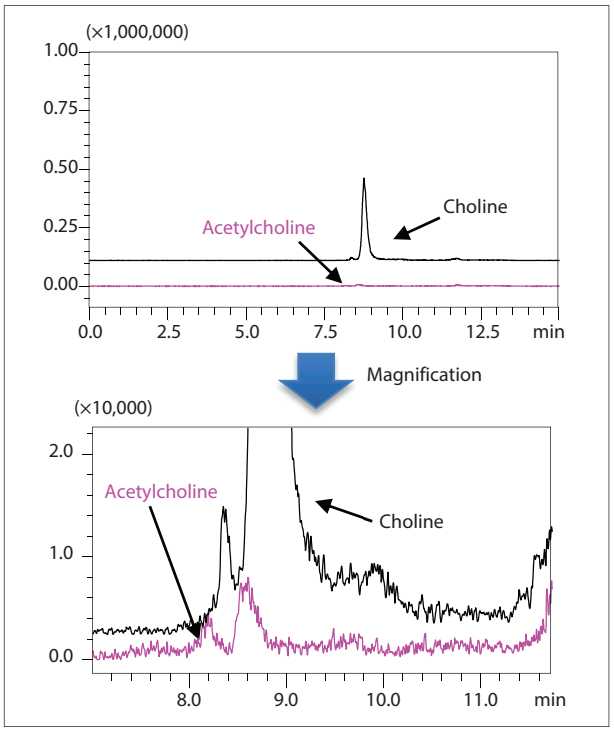


Fig. 4 SFC Analysis of Choline and Acetylcholine in a Cerebrospinal Fluid Sample

Online SFE-SFC-MS/MS Analysis

Next, a sample was prepared by impregnating cerebrospinal fluid sample into filter paper and drying the paper. SFE-SFC-MS/MS analysis was then performed on the sample. The convenience of this method is gaining attention not only because of easy of sample handling but also because of improved miscibility concerns between a mobile phase of low polar supercritical carbon dioxide and an aqueous sample solvent containing a biological sample. Table 4 lists the conditions used in online SFE-SFC-MS/MS analysis.

Table 4 Online SFE-SFC-MS/MS Conditions

Vessel	:	0.2 mL (1 µL of sample was added to filter paper)
Extractant	:	A) Supercritical fluid of CO_2 B) Methanol containing 20 mmol/L ammonium formate / water = 95/5 (v/v) A/B = 9/1 (v/v)
Flow rate	:	2.5 mL/min
Extraction time	:	Static (0-3 min) – Dynamic (3-6 min) – Static (6-8 min) - Dynamic (8-11 min) – Static (11-13 min) – Dynamic (13-16 min)
BPR pressure	:	10 Mpa
Extraction temp.	:	60 °C
Time program	:	B Conc. 10 % (16 min) → 25 % (26 min) → 50 % (26.1-28 min) → 10 % (28.1-31 min)

* SFC-MS/MS conditions are identical to Table 1 except for the time program.

Fig 5. shows the result obtained from online SFE-SFC-MS/MS analysis of a sample created by dropping 1 µL of 100 µg/L standard solution onto filter paper (GA-200 by ADVANTEC). Fig. 6 shows the result obtained by processing the rat cerebrospinal fluid sample in the same manner. The peak obtained for acetylcholine was small like the SFC analysis result, however, since the baseline noise level was improved in comparison, improved LOQ was obtained. Because the S/N value of corresponding peak to acetylcholine was more than 15 based on the baseline noise determined by ASTM method, a simple quantitative calculation was made based on the 100 µg/L standard data in the same way as the more concentrated choline. The obtained choline concentration of 297 µg/L was close to the SFC result and suggested that extraction in online SFE was performed efficiently. For acetylcholine, a calulation result of 1.7 µg/L was obtained from the peak area.

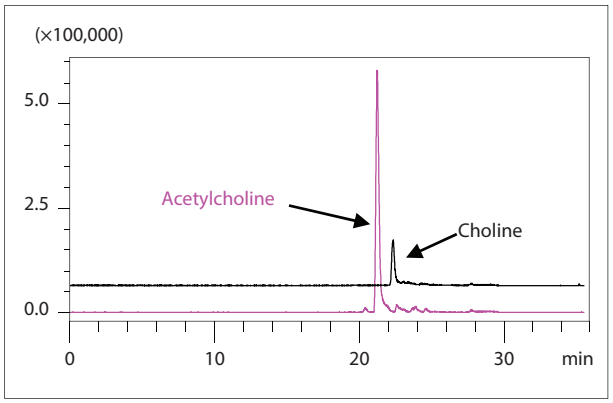


Fig. 5 Online SFE-SFC Analysis of Choline and Acetylcholine Standards

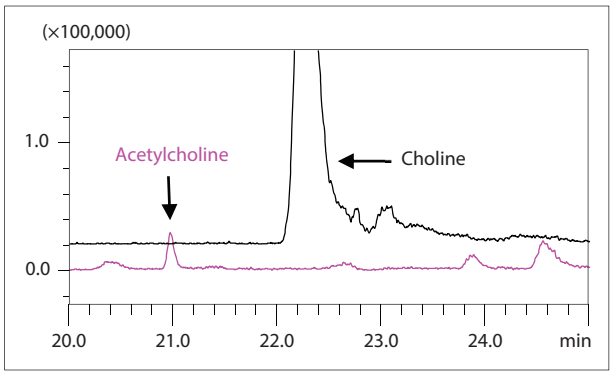


Fig. 6 Online SFE-SFC Analysis of Choline and Acetylcholine in a Cerebrospinal Fluid Sample

First Edition: Mar. 2017

SHIMADZU

Shimadzu Corporation
www.shimadzu.com/an/

SHIMADZU
Excellence in Science

Application News

No.L496

Supercritical Fluid Extraction / Chromatography

Analysis of Unstable Compounds Using Online SFE-SFC

Supercritical fluids have characteristics of both gas and liquid; low viscosity, high diffusivity and solubility. In particular, carbon dioxide becomes a supercritical fluid at a relatively modest critical point (31.1 °C and 7.38 MPa). Due to its low toxicity, inertness, easy availability, and low cost, supercritical carbon dioxide fluid is used in a wide variety of fields. Analytical applications using it include supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC).

Previously SFE and SFC were offline operations for pretreatment or analysis, respectively, and treated as completely separate workflows. However, now SFE and SFC can be connected online using the Nexera UC system, which allows integration of all the processes from pretreatment to data acquisition into a single workflow. This article describes using the Nexera UC system for online SFE-SFC analysis.

Online SFE-SFC

A flow diagram of online SFE-SFC analysis is shown in Fig. 1. Online SFE-SFC involves online introduction of components extracted from an extraction vessel using supercritical fluid to an SFC analytical column, where they are separated and then detected accordingly. The entire process, from extraction to data acquisition, is performed by switching flow lines using a valve inside the SFE unit. Two types of extraction operations are involved. After supercritical fluid is introduced to the extraction vessel, static extraction is performed where components are extracted while fluid flow is stopped. Then dynamic extraction is done to extract components while pumping fluid through the extraction vessel. In the case of online SFE-SFC, the sample is transported through the analytical column during dynamic extraction.
Consequently, 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. That can significantly reduce the time and effort required for the various operations involved in the 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 eliminates need for preparing sample solutions, which means it eliminates possibility of dilution of target components by the sample solvent, thus providing an easy way of increasing sensitivity.

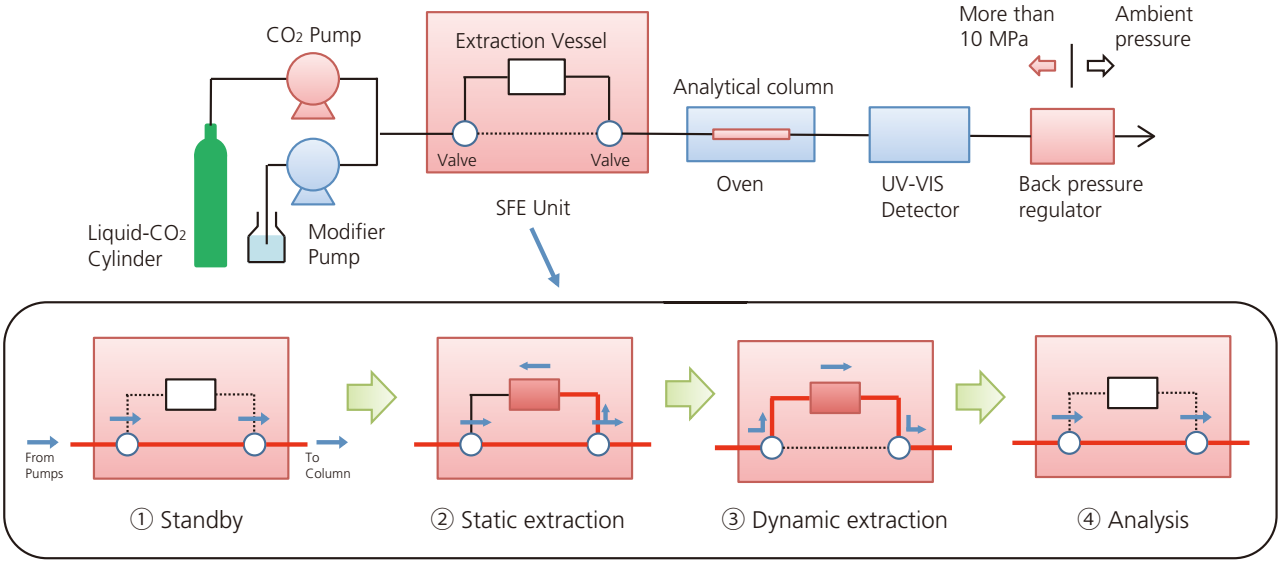


Fig. 1 Process Flow Diagram of Online SFE-SFC System

Online SFE-SFC Analysis of Reduced Coenzyme Q10

Fig. 2 shows the structure of the reduced coenzyme Q10 (ubiquinol). It is easily oxidized to form oxidized coenzyme Q10 (ubiquinone). In this case, both solvent extraction-SFC and online SFE-SFC were used to analyze the reduced coenzyme Q10 contained in a supplement capsule.

Pretreatment operations and analytical conditions for the solvent extraction-SFC analysis are indicated in Fig. 3 and Table 1.

Chromatograms from analyzing the supplement and the oxidized coenzyme Q10 standard sample are shown in Fig. 4.

Table 1 Analytical Conditions for Solvent Extraction-SFC

System	: Nexera UC SFC-UV System
Column	: Shim-pack UC-RP (150 mm L. × 4.6 mm I.D., 3 µm)
Column Temp.	: 40 ˚C
Modifier	: MeOH
Flowrate	: 3 mL/min
Time Program	: 5 % (0 min) → 50 % (5 - 8 min)
BPR	: 10 MPa
Detector	: UV-VIS (220 nm)
Inj. Vol.	: 1 µL

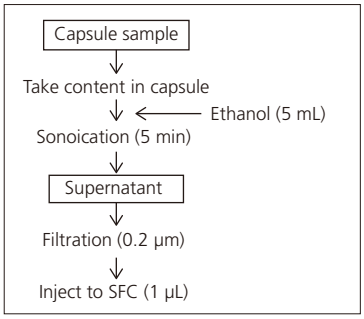


Fig. 3 Pretreatment

Analytical conditions for online SFE-SFC are indicated in Table 2.

About 5 µL each of the liquid sealed inside the supplement capsule and the standard sample of oxidized coenzyme Q10 were dripped onto filter paper. Then a portion of the filter paper was cut with a punch-out device and placed in the extraction vessel for analysis by online SFE-SFC. Chromatograms from analyzing the supplement and the oxidized coenzyme Q10 standard sample are shown in Fig. 5.

Table 2 Analytical Conditions for Online SFE-SFC

System	: Nexera UC Online SFE-SFC-UV System	
SFE		
Extraction Vessel	: 0.2 mL	
Static Extraction	: Time	; 0 - 2 min,
	: B.Conc.	; 5 %
	: BPR	; 10 MPa
	: Flowrate ; 3 mL/min	
Dynamic Extraction	: Time	; 2 - 4 min,
	: B.Conc.	; 5 %
	: BPR	; 10 MPa
	: Flowrate ; 3 mL/min	
SFC		
Column	: Shim-pack UC-RP (150 mm L. × 4.6 mm I.D., 3 µm)	
Column Temp.	: 40 ˚C	
Mobile Phase	: A; CO_2	
	: B; MeOH	
Flowrate	: 3 mL/min	
Time Program	: 5 % (4 min) → 50 % (9 - 13 min)	
BPR	: 10 MPa	
Detector	: UV-VIS (220 nm)	

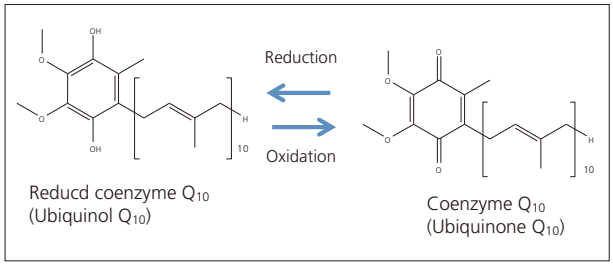


Fig. 2 Structural Formulas

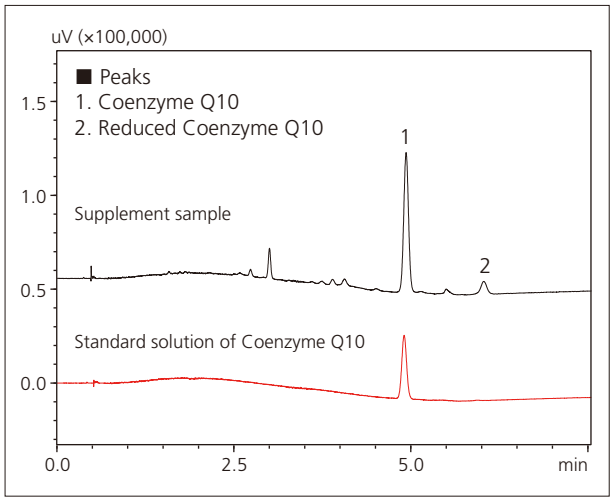


Fig. 4 Chromatograms Obtained by Solvent Extraction-SFC

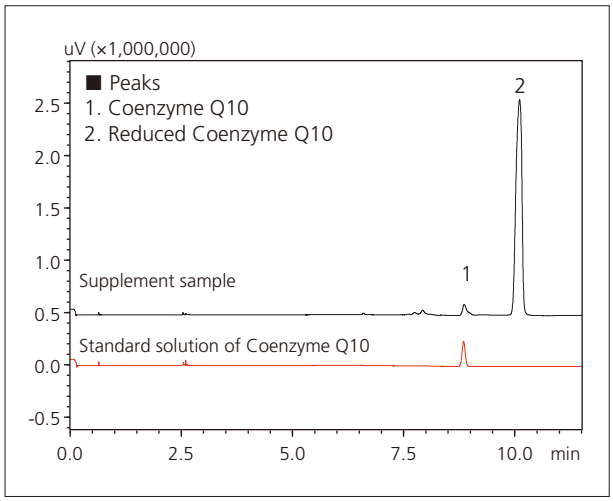


Fig. 5 Chromatograms Obtained by Online SFE-SFC

The results show that the coenzyme Q10 was oxidized during extraction with solvent extraction-SFC, but not oxidized and remained as the reduced coenzyme Q10 form throughout extraction, separation, and detection steps with online SFE-SFC. This shows how online SFE-SFC is an extremely unique analytical technique that can be used to analyze unstable compounds without altering their original form.

First Edition: Oct. 2015

SHIMADZU

Shimadzu Corporation

www.shimadzu.com/an/

SHIMADZU
Excellence in Science

PO-CON1618E

A novel approach to the analysis of multivitamin by online supercritical fluid extraction/supercritical fluid chromatography

Pittcon 2016 830-12

Qiang Li, Hongyuan Hao, Taohong Huang,
Shin-ichi Kawano, Yuki Hashi
Analytical application centre, Shimadzu Globule COE,
Shimadzu (China) Co., Ltd, Shanghai, China

A novel approach to the analysis of multivitamin by online supercritical fluid extraction/supercritical fluid chromatography

Introduction

Vitamin is a series of basic trace substances which could maintain normal life forms of the animal body. Due to the chemical structure, fat-soluble vitamins such as vitamin A, vitamin E, etc. have strong hydrophobicity, low solubility in polar organic solvents. The analytical methods of those compounds are various, such as vitamin A by reversed phase liquid chromatography (RP-HPLC), vitamin D by normal phase liquid chromatography (NP-HPLC), and vitamin E normally used gas chromatography. Because of original method diversity, it is difficult to develop a new method of simultaneous analysis for fat-soluble vitamins. Supercritical Fluid Chromatography (SFC) is an unconventional chromatographic separation technology by using supercritical fluid and a small amount of modifier as mobile phase. Supercritical CO_2 ($scCO_2$) with its character of safe, inexpensive, non-toxic, facile, chemical inertness and other factors become the main mobile phase of SFC. Supercritical fluid ($scCO_2$) with low viscosity, high diffusivity and solubility characteristics is used in a wide variety of fields. Nexera UC Online SFE - SFC system is the latest products of supercritical fluid chromatograph in Shimadzu, which realized SFE (supercritical flow extraction) and SFC online combination, and simplify and unify the pretreatment method with high automation, extraction efficiency, and repeatability. In this study, a simultaneous analytical method for fat-solvable vitamins in drug and health care food was developed by using Nexera UC. It provides effective analysis and detection means for a variety of fat-soluble vitamins, and can be the reference for the quantitative study of this kind of material.

Methods and Materials

Sample Preparation

The analytical method for 5 kinds of fat-soluble vitamins was established in this study. Take the five standard include vitamin A acetate (VAA), vitamin A palmitate (VAP), vitamin E acetate (VEA), vitamin D2 (VD2), and vitamin D3 (VD3) and dissolves with n-hexane, diluted to a series of mixture concentration samples with ethanol. Then, dropped them to extraction tank and analyzed for standard curve. For commercially available vitamin A, vitamin E gelatin pearl, capsule and tablets, take out the contents into extraction tank to analyzed.

Experimental condition

Instrument

Nexera UC Online SFE-SFC system

configuration:

SFE-30A (SFE module), LC-30ADSF (CO_2 deliver pump), LC-20ADXR (modifier deliver pump), DGU-20A5 (degasser), CTO-20AC (column oven), SFC-30A×2 (back pressure adjustment module), SPD-20A (UV detector), CBM-20A (system controller), LabSolutions Ver5.8 (workstation).

A novel approach to the analysis of multivitamin by online supercritical fluid extraction/supercritical fluid chromatography

SFE condition	
Extraction agent	: $scCO_2$
modifier	: MeOH(5%)
flow rate	: 5 mL/min
static extraction	: 3 min
dynamic extraction	: 3 min
SFE temperate	: 50 °C
back pressure	: A-14.8 MPa, B-15 MPa
SFC condition	
Column	: GL Science ODS-P 4.6 mm I.D.×250 mm L., 5 µm
Mobile phase A	: $scCO_2$
Mobile phase B	: MeOH
Gradient program	: 0%B (6 min)-2%B (9 min)-10%B (16 min)-50%B(16.1-17 min)
Flow rate	: 3 mL/min
Oven temperature	: 40 °C
back pressure	: 10 MPa
detector wavelength	: 325 nm; 284 nm

Results and Discussion

Supercritical fluid extraction

Samples were loaded to the extraction vessel, and then set in a supercritical fluid extraction module for extraction. Liquid CO_2 and modifier of methanol (98/2, v/v) were delivered through the pumps into the extraction vessel (Figure 1), and changed to supercritical fluid under the setting of temperature and pressure. Methanol, as modifier, is to adjust the polarity, solubility and other properties of supercritical fluid to improve the extraction efficiency. Kept the vessel filled with supercritical fluid in 3 min at a stable temperature and pressure for static extraction.

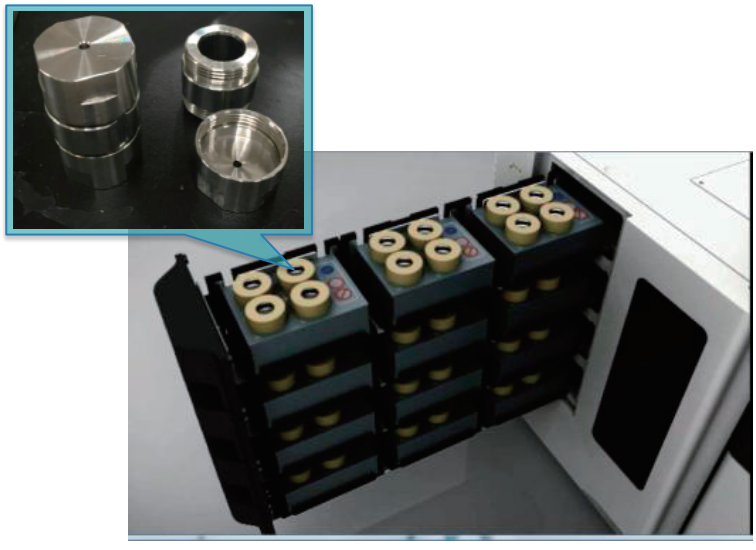


Figure 1 Extraction vessel and SFE

A novel approach to the analysis of multivitamin by online supercritical fluid extraction/supercritical fluid chromatography

Then, through SFE unit flow switch valve, supercritical fluid flow through the extraction vessel and extracted components from sample in 3 min by dynamic extraction. In the process of dynamic extraction, extract was directly introduced into subsequent SFC system. SFC separation and analysis was start after the completion of the extraction. The whole process of online SFE - SFC is shown in figure 2.

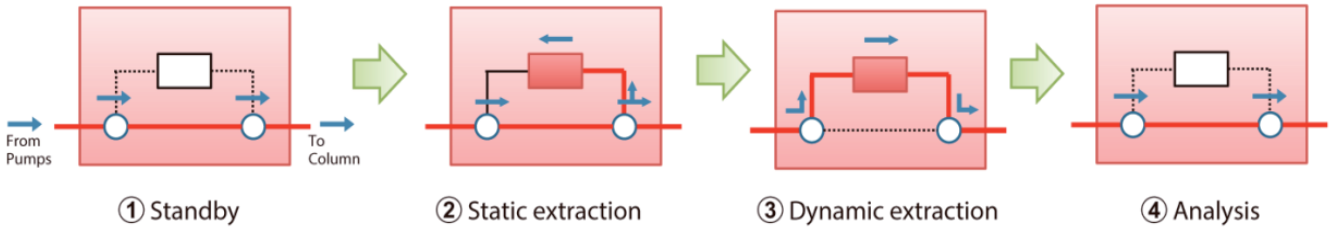


Figure 2 Pretreatment processing of SFE

For estimating the extraction efficiency of 5 compounds under the condition of setting, repeated extraction and analysis for the same vessel was performed. Peak area of every compound was calculated and peak area ratio of first extraction to total three times was recorded in table 2 to show the extraction yield of every compounds. The results showed that the SFE extraction yield of 5 vitamins were above 85% under the condition of settings.

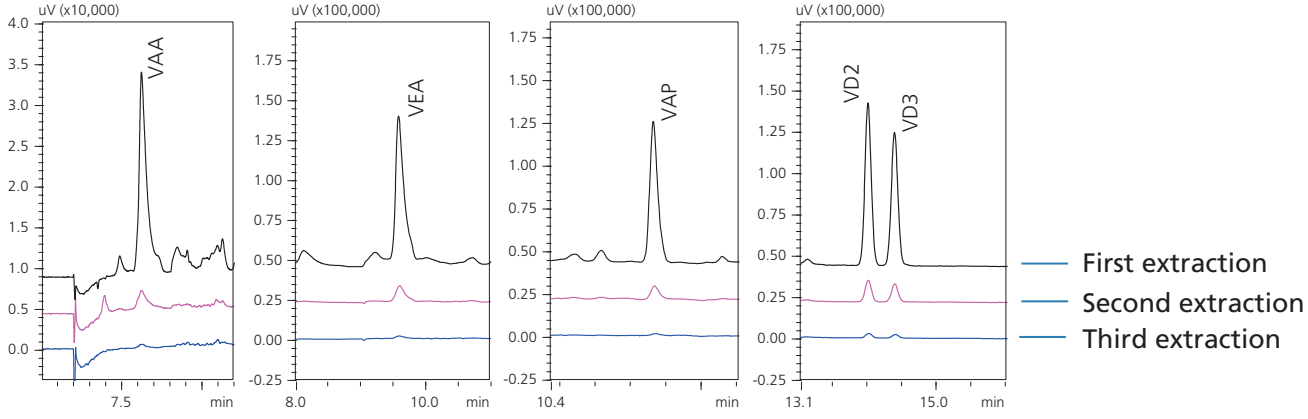


Figure 3 Chromatographs of three extractions for 5 vitamins

Table 1 Yield of three extractions for 5 vitamins

	VAA	VEA	VAP	VD2	VD3
1st extraction	94.2	88.6	89.3	86.2	86.0
2nd extraction	5.8	10.1	9.7	11.6	12.0
3rd extraction	0	1.3	1.0	2.2	2.0

A novel approach to the analysis of multivitamin by online supercritical fluid extraction/supercritical fluid chromatography

Supercritical Fluid Chromatography

The online SFE-SFC analytical results of 5 fat-soluble vitamins were showed in Fig 4. Vitamin A acetate and other four compounds were isolated obviously. The standard curves of absolute amount of compound added in extraction vessel to the detector response shown in Figure 5, it indicates 5 compounds with good linearity in their respective concentration, and regression coefficient of R2 in 0.997-0.999.

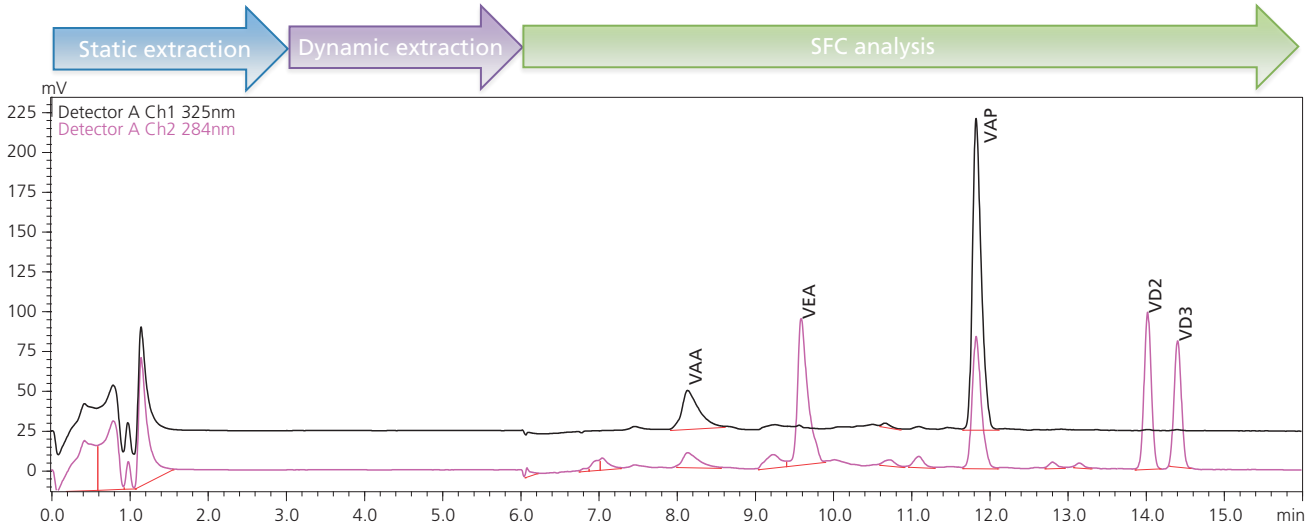


Figure 4 Chromatogram of simultaneous analysis for 5 vitamins

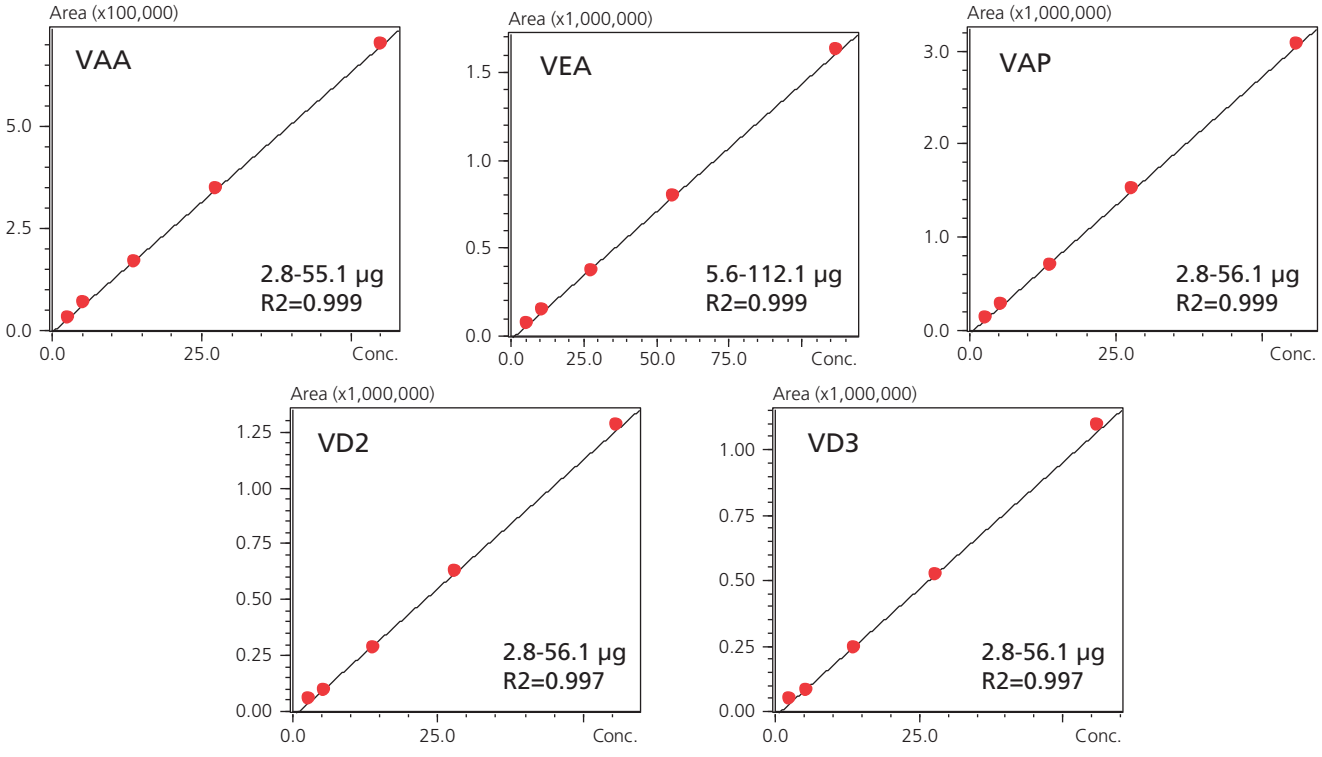


Figure 5 Calibration curves of 5 vitamins

A novel approach to the analysis of multivitamin by online supercritical fluid extraction/supercritical fluid chromatography

Repeatability and recovery

Add 2 times of LLOQ for each compound into extraction vessel to test repeatability and recovery. Results were shown in table 2.

Table 2 Repeatability and recovery of 5 vitamins (n=6)

	VAA	VEA	VAP	VD2	VD3
Rt (RSD%)	0.24	0.15	0.10	0.04	0.03
Area (RSD%)	13.0	5.2	4.1	4.5	5.9
Recovery (%)	94.4	101.1	100.0	90.5	90.5

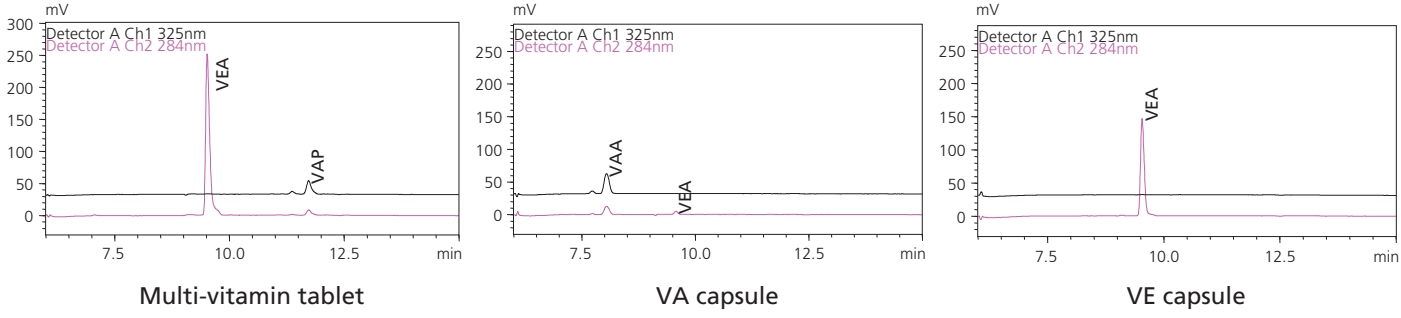


Figure 6 Chromatogram of three real samples which were analysed by using Nexera UC

Conclusions

An Online SFE-SFC method has been developed for quantitative analysis of 5 fat-soluble vitamins in drugs and health care food sample. It provided a new way for simultaneous analysis for 5 vitamins which combined the processing of pretreatment and analysis together. The results showed that this method is rapid and reliable.

First Edition: March, 2016

SHIMADZU

Shimadzu Corporation
www.shimadzu.com/an/

SHIMADZU
Excellence in Science

Upgrade Your Existing UHPLC to an UHPLC/SFC Switching System

Reduce Instrument Purchase Costs and Enable SFC Analysis Immediately

You can now upgrade to an UHPLC/SFC switching system (Nexera UC/s) by adding the applicable SFC units to your existing UHPLC system.
This enables you to perform both UHPLC and SFC analysis with a single system.

Upgrading Lowers Purchasing Costs and Reduces Installation Space

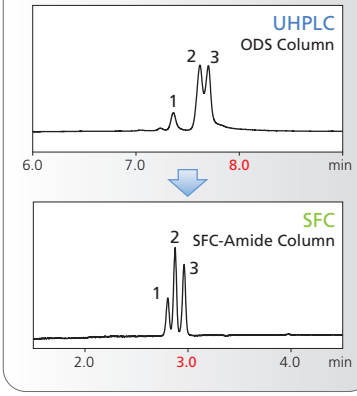
UHPLC System

UHPLC/SFC Switching System (Nexera UC/s)

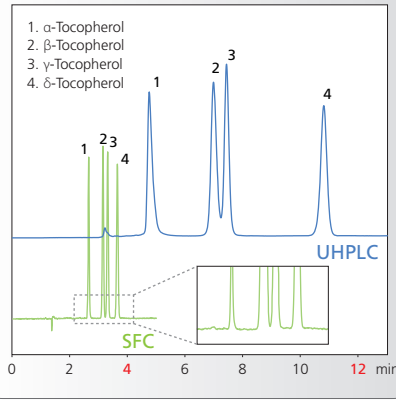
SFC Analysis Improves Separation and Analysis Times, while Reducing Solvent Consumption

In comparison to UHPLC, column efficiency is not impaired in SFC analysis even at a high flow rate. As a result, analysis times are shortened by the increase in speed. At the same time, since the separation characteristics are different, improved separation can be expected for foreign substances and isomers that are not sufficiently separated by UHPLC. Further, the consumed amount of organic solvents can be reduced.

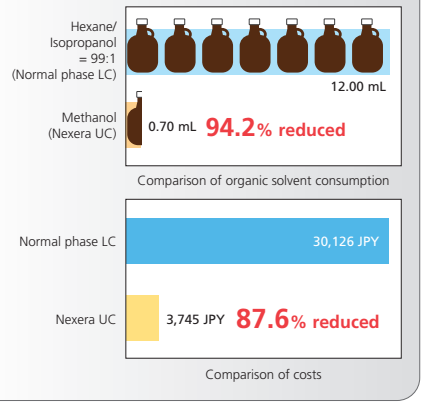
Improved Separation with SFC Analysis



Shortened Analysis Times

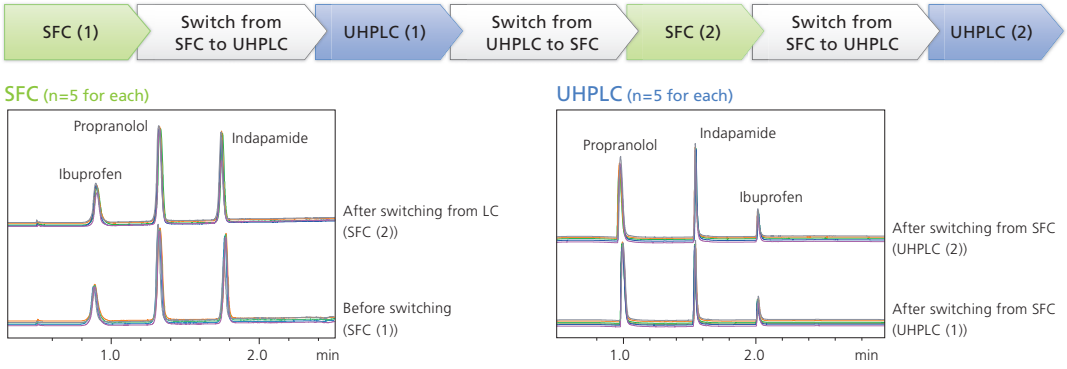


Reduced Solvent Consumption



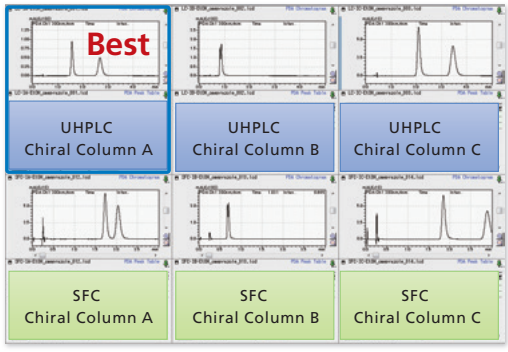
Reliable Analysis Even When Switching Between SFC and UHPLC Modes

SFC and UHPLC utilize significantly different mobile phases and separation characteristics, but analysis can be performed reliably without effects from switching modes by simply purging the flow lines.

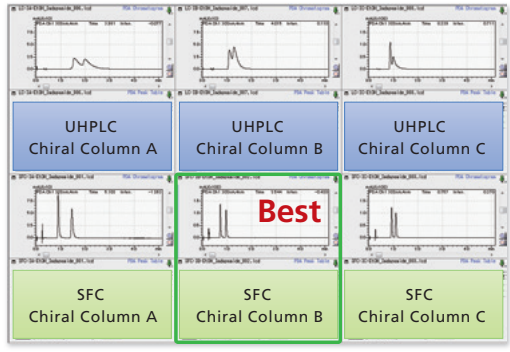


Two Separation Methods Heighten the Efficiency of Examining the Optimal Separation Conditions

Various separation methods, including the separation of chiral compounds and structural isomer, are required in fields such as pharmaceuticals, foods, and the environment. For example, in the case of method scouting with two chiral standard samples, favorable separation is obtained for omeprazole with UHPLC conditions, and imdapamide with SFC conditions. Screening utilizing these two methods makes it possible to construct better analysis conditions in a short time. Switching between SFC and UHPLC analysis methods is easy with the dedicated software.



Omeprazole Method Scouting

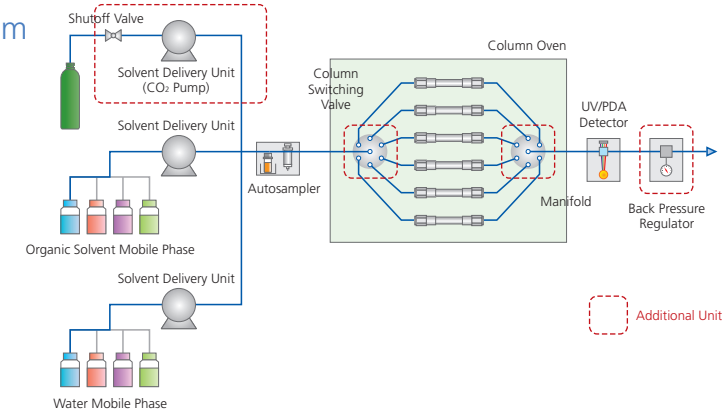


Imdapamide Method Scouting

Kit for Upgrading to an SFC Analysis System

You can upgrade to an UHPLC/SFC switching system (Nexera UC/s) capable of UHPLC and SFC analysis using the existing* solvent delivery unit, autosampler, oven, and detectors.

* The following units can be used in combination when upgrading.
Solvent delivery Unit: LC-30AD
Autosampler: SIL-30AC
Column oven: CTO-20A/20AC
Detector: SPD-20A(V), SPD-M20A
Mass spectrometer: LCMS-2020, LCMS-80X0



SHIMADZU

Shimadzu Corporation

www.shimadzu.com/an/

First Edition: November 2016, Printed in Japan

Find us on

LinkedIn

First Edition: March, 2018

SHIMADZU

Shimadzu Corporation
www.shimadzu.com/an/