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Technical Report

Extraction and Fractionation of Functional Ingredients with Online SFE-SFC

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Abstract:

This article describes a preparative online supercritical fluid extraction-supercritical fluid chromatography (SFE-SFC) system that combines an extraction step using supercritical carbon dioxide as the solvent, a separation step using supercritical carbon dioxide as the mobile phase, and a fractionation step using a preparative-scale fraction collector (FRC). The system automates extraction, separation, and fractionation steps and offers numerous advantages including simplified sample pretreatment, unstable ingredient analysis, and high-sensitivity analysis. These features are optimal for the preparative isolation of functional ingredients that would otherwise require complex extraction and purification procedures. This article describes the basic operating principles and characteristics of this preparative online SFE-SFC system along with results from an extraction and fractionation of a functional ingredients.

Keywords: SFC, supercritical fluid chromatography, SFE, supercritical fluid extraction, online SFE-SFC, preparative SFC, fraction collector

1. Challenges for the Preparative Isolation of Functional Ingredients

Diet is now widely recognized as critical for the prevention of lifestyle diseases and maintaining a healthy life, and food ingredients that exhibit regulatory effects such as disease prevention and aging prevention are an area of increasing interest. Lycopene (Fig. 1) is a carotenoid found in large quantities in tomatoes and one of these functional ingredients.

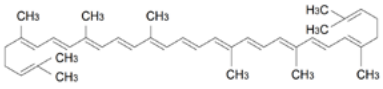


Fig. 1 Structural Formula of Lycopene

Elucidating the physiological effects of lycopene is an active field of research that routinely purifies lycopene from real sources. However, lycopene is vulnerable to decomposition and isomerization by heat and light and should be extracted and isolated quickly at or below 40 °C while shielded from light. Conventional LC-based methods of isolating lycopene often use multiple separate instruments for extraction, separation, and fractionation, which involve many processes and raises concerns about sample decomposition. Another issue with these LC-based methods is the time needed to concentrate fractionated samples that contain large volumes of solvent.

2. Online SFE-SFC Fractionation System Using Supercritical Carbon Dioxide

The issues for the fractionation of functional ingredients that were identified in the previous section can be addressed effectively by using supercritical carbon dioxide as the solvent during ingredient extraction and separation. Supercritical fluid is a material for which the temperature and pressure are at or over the critical point, and has high diffusivity and low viscosity similar to a gas along with the solubility of a liquid. The low critical points of carbon dioxide (critical temperature: 31.1 °C, critical pressure: 7.38 MPa) make it easy to handle, while its non-flammability, inactivity, and low cost have led to widespread industrial use in the decaffeination of coffee beans and other applications. Supercritical carbon dioxide fluid is also used for analytical applications as an extraction solvent in supercritical fluid extraction (SFE) and as the principal mobile phase in supercritical fluid chromatography (SFC).

The preparative online SFE-SFC system, which uses supercritical carbon dioxide, extracts ingredients by SFE, introduces these ingredients directly to a column for separation by SFC, then uses a fraction collector (FRC) for recovery. A flow diagram of the system is shown in Fig. 2.

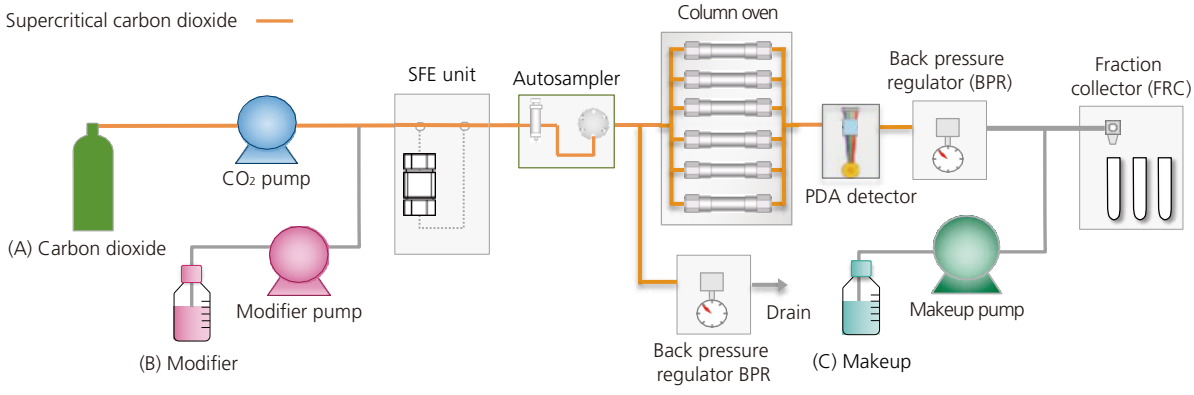


Fig. 2 Flow Diagram

[1] Analytical & Measuring Instruments Division

The preparative online SFE-SFC system automatically extracts, separates, purifies, and recovers target ingredients, and only requires the user to place an extraction vessel containing the sample in the system. Because sample ingredients are extracted and separated without exposure to light and air, the preparative online SFE-SFC system is well-suited to isolating ingredients vulnerable to oxidation and light-sensitive ingredients such as carotenoids. The online fraction collector enables the preparative isolation of specific ingredients. A specially designed gas-liquid separator that discharges gaseous carbon dioxide from the eluate liquid is mounted on the fraction collector and ensures efficient liquid recovery.
The operating principles and characteristics of this system are described below. For more information about this online SFE-SFC system, please see Technical Report C190-E205, "Online Supercritical Fluid Extraction-Supercritical Fluid Chromatography (Online SFE-SFC)."

(1) Extraction

(1)-1 Extraction vessel delivery and temperature adjustment

Designated extraction vessel is transported to the SFE unit and is temperature controlled until it reaches the set temperature.

(1)-2 Static extraction

When the temperature of the extraction vessel reaches the designated temperature, the supercritical fluid is introduced into the extraction vessel. After the introduction, the fluid is not passed into the extraction vessel, and extraction is performed in a static state.

(1)-3 Dynamic extraction

After the static extraction, extraction is performed while passing the supercritical fluid through the extraction vessel. The extract is removed from the vessel and introduced to the analytical column.

(2) Separation and Detection

After completing the extraction, the extraction vessel is removed from the flow path and extracts are separated by increasing the concentration of modifier in the mobile phase flowing through the column. Ingredients eluted from the column are then detected by a photodiode array detector or mass spectrometer.

(3) Fractionation

Signals from the detector are used to trigger recovery of the liquid portion (fraction) containing the target ingredients into a test tube or vial by the fraction collector. The fractioned range can be set based on signal intensity as well as by retention time.

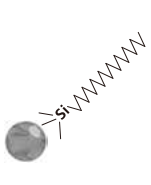
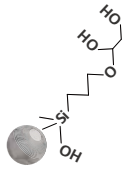
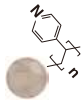
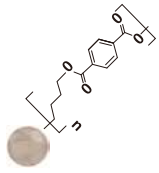
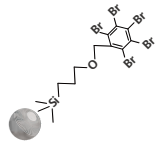
3. Method Scouting

SFC can use various separation modes based on hydrophilic interactions (using a normal-phase column), hydrophobic interactions (using a reversed-phase), and π-π interactions (using a phenyl column). Method scouting is an effective approach to finding the optimum column and mobile phase conditions for separation of a given target ingredient.
The "6 columns set" is a package of columns with a range of separation behaviors that provides the best starting point for scouting these separation conditions. The characteristics of each column in this "6 columns set" are shown in Table 1.
Column scouting with lycopene and β-carotene standards was used to select a column for the extraction and fractionation of lycopene from a processed tomato product. The analytical conditions used in column scouting are shown in Table 2 and the resulting data are shown in Fig. 3.
Each of the six columns showed a different separation pattern. Because carotenoids such as lycopene and β-carotene are aliphatic hydrocarbons of relatively low polarity, they eluted with almost no retention on normal-phase columns UC-Sil II and UC-Diol II, which use highly polar stationary phases. Conversely, lycopene and β-carotene were strongly retained by the UC-PBr column, from which almost none of either compound was eluted.
The UC-PolyBT column produced a low lycopene peak with tailing. This peak shape was probably caused by strong interaction between the stationary phase and the double bonds at either end of the lycopene molecule. Good peak shapes for both lycopene and β-carotene were obtained from the UC-ODS and UC-PolyVP columns that exhibited hydrophobic interaction separation behavior. Of these, the UC-ODS column was selected as it resulted in the best separation of the two compounds.
A comparison of methanol and acetonitrile modifiers produced sharper peaks with acetonitrile, thus acetonitrile was selected as the modifier.

Table 2 Column Scouting Conditions

Column	: Refer to Table 1 (250 mm × 4.6 mmI.D., 5 µm)
Mobile phase	: A; CO_2
	B; Modifier: Methanol, or Acetonitrile
Gradient	: 5 - 40 % (0-10 min) → 40 % (10-15 min) → 5 % (15 -20 min)
Flow rate	: 3.0 mL/min
BPR pressure	: 15 MPa
BPR temp.	: 50 ˚C
Column temp.	: 40 ˚C
Detection	: Photo diode array detector (wavelength = 190-800 nm)
	PDA Chromatogram at 460 nm
Cell	: High pressure cell for SFC (analytical)
Sample	: Lycopene, β-carotene 500 mg/L each (in $CHCl_3$)
Injection	: 3 µL

Table 1 Characteristics of "6 columns set"

	6 columns set					
	UC-ODS	UC-Sil II	UC-Diol II	UC-PolyVP	UC-PolyBT	UC-PBr
Chemistry						
Features	The separation mode is reverse phase. Retention is provided through hydrophobic interaction.	This is excellent for retention of basic compounds and recognition of their tertiary structures.	The separation mode is normal phase. This inhibits non-specific interactions.	A favorable peak shape is obtained even without acid-base additives.	This is excellent for resolving aromatic compounds through π-π interactions.	With ODS, separation of poorly retained compounds is improved.

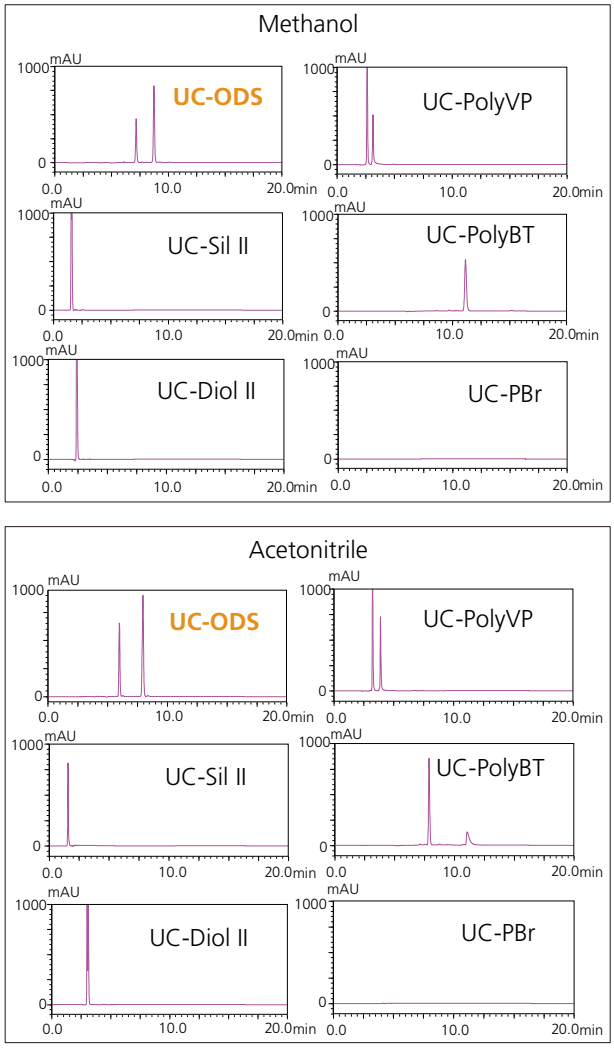


Fig. 3 Scouting Data from 6 columns set

Gradient conditions were optimized using the Shim-pack™ UC-ODS column, which is well-suited to lycopene and β-carotene separation. The analytical conditions used in gradient scouting are shown in Table 3. By adjusting gradient shapes with the aim to obtain the optimum peak separation, it was possible to identify the initial gradient concentration of 15 % as the best condition (Table 4).

Table 3 Analytical Conditions

Column	: Shim-pack UC-ODS (250 mm×4.6 mmI.D., 5 µm)
Mobile phase	: A; CO_2 B; Modifier: Acetonitrile
Flow rate	: 3 mL/min
Gradient	: 5 or 10 or 15 – 40 %(0 – 10 min) → 40 % (10-15 min) → Initial ratio (15 – 20 min)
Column temp.	: 40 ˚C
BPR pressure	: 15 MPa
BPR temp.	: 50 ˚C
Detection	: Photo diode array detector (wavelength = 190-800 nm) PDA Chromatogram at 460 nm
Cell	: High pressure cell for SFC (analytical)
Sample	: Lycopene, β-carotene 500 mg/L each (in $CHCl_3$)
Injection	: 3 µL

Table 4 Resolution of β-Carotene Separation Under Various Gradient Conditions

Initial Conc.	5 %	10 %	15 %
Resolution	10.3	10.7	11.0

4. Supercritical Fluid Extraction

Lycopene and β-carotene standards were used to investigate SFE conditions. Samples of each standard dissolved in solution were mixed with a special absorbent (1 g) and filled in a specific SFE extraction vessel (5 mL) with filter paper in place. The analytical conditions used are shown in Table 5. Adding the entire volume of the extract to an analytical column with its small inner diameter can overload the column and cause peak broadening. To address this issue, back pressure regulators (BPR) were used to split the flow path into two paths before the SFC column (one flow path to waste and one to the SFC column), which allowed just a portion of the extract to introduce the column by adjusting the pressure difference (differential pressure) at the outlet of the flow paths. Extraction solvent was fed into the extraction vessel, and after performing static extraction for 3 minutes and dynamic extraction for 6 minutes, the extract was analyzed by SFC. The lycopene and β-carotene standards were detected as separate peaks with very little peak broadening even after SFE. The chromatogram is shown in Fig. 4.

Table 5 Analytical Conditions

[SFE]	
Extraction vessel	: 5 mL
Flow rate	: 3.0 mL/min
B. Conc.	: 10 %
Extraction temperature	: 40 ˚C
Extraction time	: Static (0-3 min) → Dynamic (3-9 min)
BPR pressure	: A: 14.8 MPa, B: 15 MPa
[SFC]	
Column	: Shim-pack UC-ODS (250 mm x 4.6 mmI.D., 5 µm)
Mobile phase	: A: CO_2, B: Acetonitrile
Flow rate	: 3.0 mL/min
Gradient program	: B. Conc. 15- 40 % (0-10 min) → 40 % (10 -15 min)
Column temp.	: 40 ˚C
BPR pressure	: A: 15.0 MPa, B: 40.0 MPa
BPR temp.	: 50 ˚C
Detection	: Photo diode array detector (wavelength = 190-800 nm) PDA Chromatogram at 460 nm
Cell	: High pressure cell for SFC (analytical)
Sample	: Lycopene, β-carotene 56 µg each

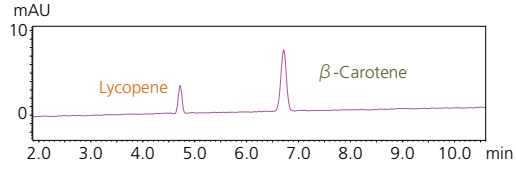


Fig. 4 Chromatogram of Carotenoid Standards after SFE

5. Extracting Lycopene from a Processed Tomato Product

0.1 g of a processed tomato product was weighed, mixed with a special absorbent (1 g), and filled in a specific SFE extraction vessel (5 mL) with filter paper in place. The processed tomato product was weighed and filled in the extraction vessel quickly to prevent carotenoid oxidation. The extraction vessel was then placed in the SFE unit and analysis was performed under the same conditions as shown in Table 5. The resulting chromatogram is shown in Fig. 5. The chromatogram shows that lycopene was fully separated from unwanted β-carotene and eluted as a single peak. The peak shape of lycopene is also as good as that of the lycopene standard, confirming the system can extract and isolate lycopene from a real sample.

Fig. 6 shows the comparison between the conventional sample pretreatment (which includes mixing, liquid separation, and evaporation to dryness) and the online SFE-SFC system analytical workflow (which includes sample loading and setting up the SFC system).

The online SFE-SFC system can greatly shortened the pretreatment time. (from 1 hour of manual labor to only 5 minutes).

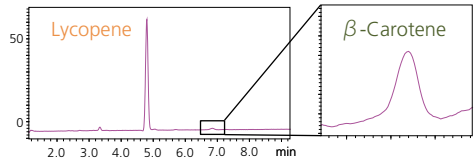


Fig. 5 Chromatogram from 4.6 mm I.D. Column after SFE of Processed Tomato Product

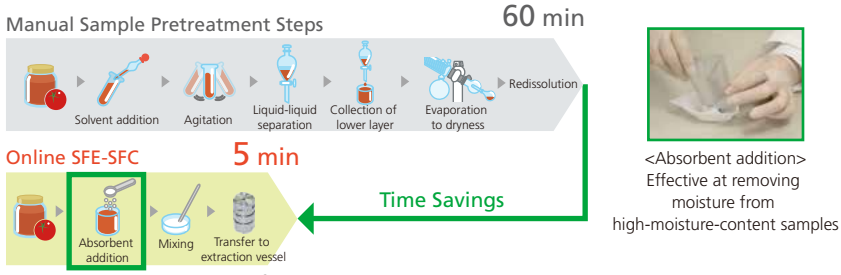


Fig. 6 Simplified Sample Pretreatment with Online SFE-SFC

6. Scale-up and Preparative Isolation of Functional Ingredients

Once extraction and separation conditions were investigated and selected, the process was scaled up to the preparative scale. Column inner diameter, flowrates, column loading, and the amount added to the column were adjusted and investigated for extraction and recovery of lycopene from 0.5 g of processed tomato product. Extraction and fractionation were performed using the conditions shown in Table 6. Unlike 4.6 mm I.D. column for the analytical scale process, back pressure regulators were not used to split the flow before the SFC column; instead, a larger I.D. column allowed the entire extract to be added to the SFC column.

The fraction collector is equipped with a LotusStream™ gas-liquid separator (patented). Eluate from preparative SFC is typically at risk of scattering due to volumetric expansion associated with the sudden vaporization of supercritical carbon dioxide into a gas. The LotusStream gas-liquid separator prevents this scattering by effectively separating gaseous carbon dioxide from the liquid eluate and ensuring good eluate recovery. Fig. 7 shows an illustrative representation of the LotusStream gas-liquid separator and the LotusStream separator during actual fractionation.

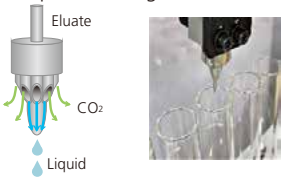


Fig. 7 Illustrative Representation of LotusStream Separator (Left) and Fractionation with the LotusStream Separator (Right)

The chromatogram obtained after extraction and separation from the processed tomato product is shown in Fig. 8. The chromatogram shows the system was successful in automatically extracting and purifying lycopene from β-carotene and other contaminants at the preparative scale.

Table 6 Extraction and Fractionation Conditions

[SFE]	
Extraction vessel	: 5 mL
Flow rate	: 5.0 mL/min
B. Conc.	: 10 %
Extraction time	: Static (0-3 min) → Dynamic (3-9 min)
Extraction temperature	: 40 ˚C
BPR pressure	: A: 15 MPa, B: 40 MPa
[SFC]	
Column	: Shim-pack UC-ODS (250 mm x 10 mmI.D., 5 µm)
Mobile phase	: A: CO_2, B: Acetonitrile
Flow rate	: 5.0 mL/min
Gradient program	: B. Conc. 15 - 40 % (0-10 min) → 40 % (10 - 25 min)
Column Temperature	: 40 ˚C
BPR pressure	: A: 15.0 MPa, B: 40.0 MPa
BPR temp.	: 50 ˚C
Detection	: Photo diode array detector (wavelength = 190-800 nm) PDA Chromatogram at 460 nm
Cell	: High pressure cell for SFC (analytical)
Make up pump	: 0.5 mL/min (Acetone)

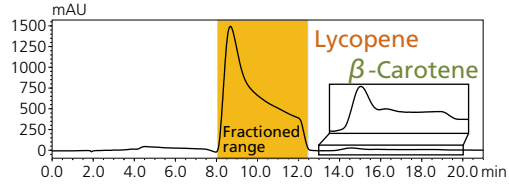


Fig. 8 Preparative Chromatogram after SFE of Processed Tomato Product

The lycopene fraction was then diluted up to 20 mL in a measuring cylinder and reanalyzed. The conditions used in this analysis are shown in Table 7 and the resulting chromatogram is shown in Fig. 9. The process of extracting and recovering lycopene from the processed tomato product was also performed three times to check repeatability. Table 8 shows the results obtained from analyzing the three lycopene fractions by SFC. The lycopene peak areas detected in the extract and the lycopene fractions show good repeatability.

Table 7 Lycopene Fraction Analytical Conditions

Column	: Shim-pack UC-ODS (250 mm x 4.6 mmI.D., 5 µm)
Mobile phase	: A: CO_2, B: Acetonitrile
Flow rate	: 5.0 mL/min
Gradient program	: B. Conc. 15 - 40 % (0-10 min) → 40 %(10 - 15 min) → 15 %(15 - 20 min)
Column Temperature	: 40 ˚C
BPR pressure	: A: 15.0 MPa, B: 40.0 MPa
BPR temp.	: 50 ˚C
Injection	: 20 µL
Detection	: Photo diode array detector (wavelength = 190-800 nm) PDA Chromatogram at 460 nm
Cell	: High pressure cell for SFC (analytical)

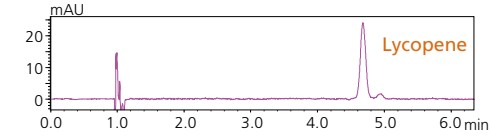


Fig. 9 Chromatogram of Preparative Lycopene Fraction

Table 8 Repeatability of Lycopene Extraction and Recovery

	Peak Area after Extraction	Peak Area of Lycopene Fraction
No.1	179520263	141354
No.2	174978002	139289
No.3	177317967	139527
Mean	**177272077**	**140057**
%RSD	**1.05**	**0.659**

7. Conclusion

This article describes a preparative online SFE-SFC system that seamlessly extracts and recovers target ingredients from samples. This automated and seamless extraction, analysis, and isolation of preparative-scale amounts of functional ingredients from food ensures substantial time savings for users.

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