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Excellence in Science

Nexera UC
Unified Chromatography

Development of online SFE-LC/MS system for analysis of metabolites in microbial cells

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Introduction

In bio-based fine chemical production, a short breeding cycle time of microbes is a key issue for improving productivity.

Metabolomics has been widely used as a quick and comprehensive analytical procedure due to its excellent features of dynamic monitoring and quick evaluation for bio-production.

On the other hand, sample pretreatment procedure is still generally tedious and time consuming while high-throughput analytical methods using LC/MS and GC/MS.

We are working on the development of online SFE-LC/MS system that provides COMPLETELY automated analytical process.

SFE is the abbreviation of “Supercrital Fluid Extraction”.

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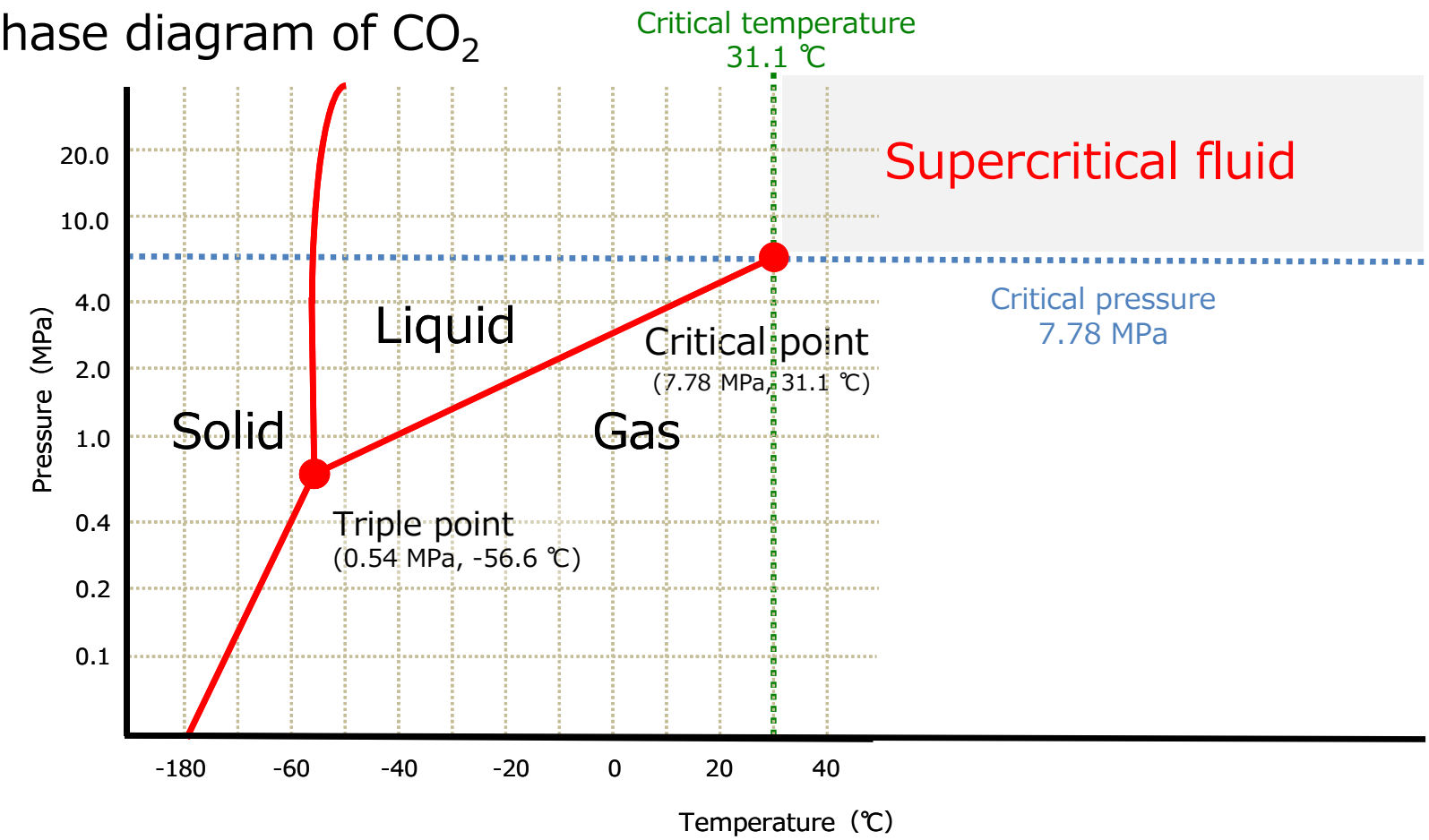
What is Supercritical Fluid?

Phase diagram of CO_2



Supercritical fluid is the one of states of material, NOT particular compounds

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Features of CO_2 as supercritical fluid

	Diffusivity (cm^2/s)	Density (g/cm^3)	Viscosity ($g/cm \cdot s$)
Liquid	10^{-6}	1	10^{-2}
Supercritical fluid	10^{-3}	0.2 - 0.8	10^{-3}
Gas	10^{-1}	10^{-3}	10^{-4}

- Compare to Gas;
  - Larger density
    → Work as solvent
- Compare to Liquid;
  - Lower viscosity
    → Easier to penetrate into the tissue
  - Higher diffusivity
    → Higher extraction efficiency

- The polarity of CO_2 is almost same as that of *n*-hexane, but miscible to methanol. Therefore methanol can be used as a modifier, then the range of polarity is wide.

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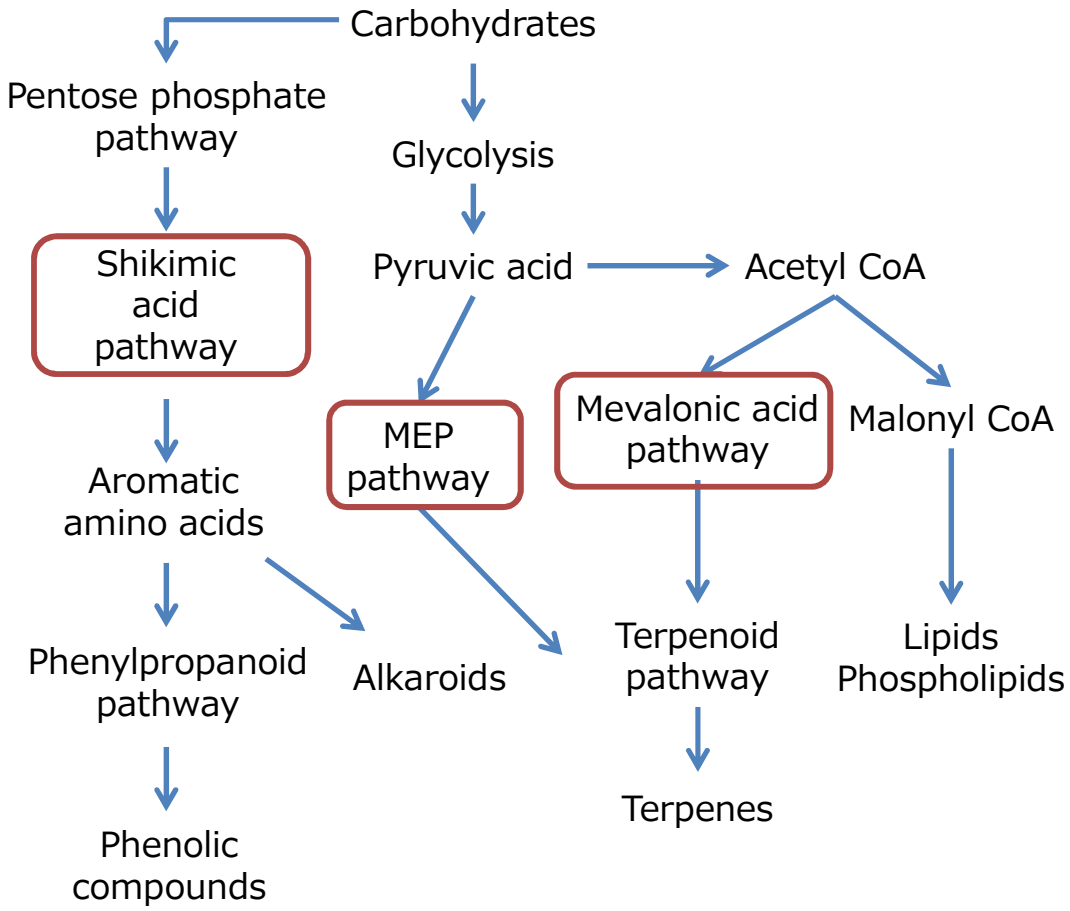
Samples & target compounds



- E.coli. and yeast cells were used as tested samples.
- Typical metabolic precursors for secondary metabolites on the shikimate, mevalonate, and MEP pathways were selected.

Shikimate	Mevalonate
Dehydroshikimate	Mevalonate-5-phosphate
Shikimate phosphate	Mevalonate-5-diphosphate
DHQ	DXP
Chorismate	MEP
Acetoacetyl-CoA	DOXP
HMG-CoA	Malonyl CoA

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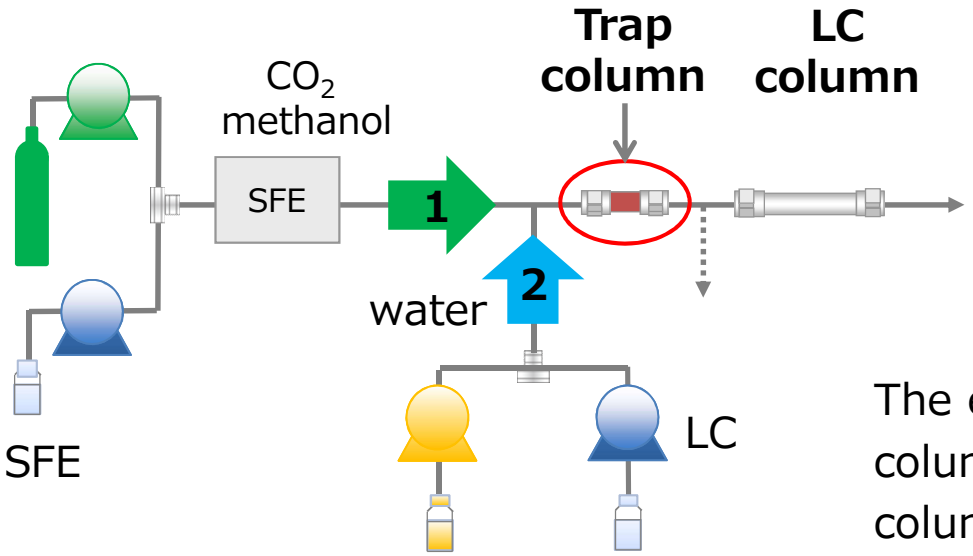
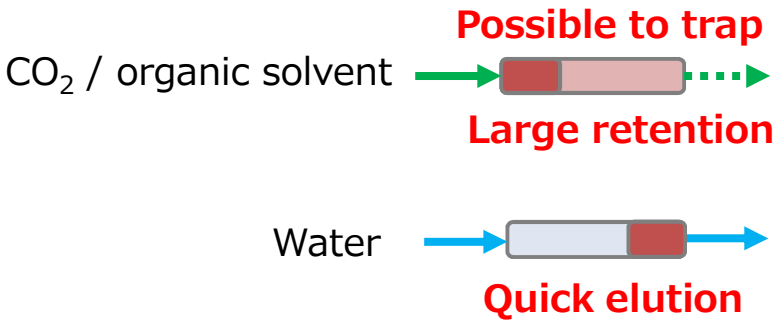
Component technologies for the Newly developed polymer-based column





The extract from SFE must stay in the trap column before introduction into the LC column for displacing SFE extractant with appropriate solvent due to poor miscibility of SFE and LC mobile phases.

Newly developed polymer-based column that showed large retention under SFE condition whereas quick elution under LC condition was employed.

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Component technologies for the Newly developed polymer-based column

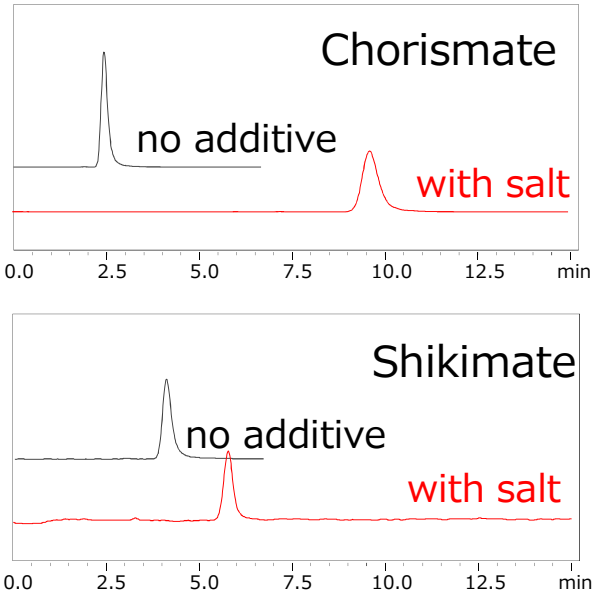
Retention behaviours of metabolites in new polymer-based column

Under SFE condition

Possible to trap

CO_2 / organic solvent

Large retention

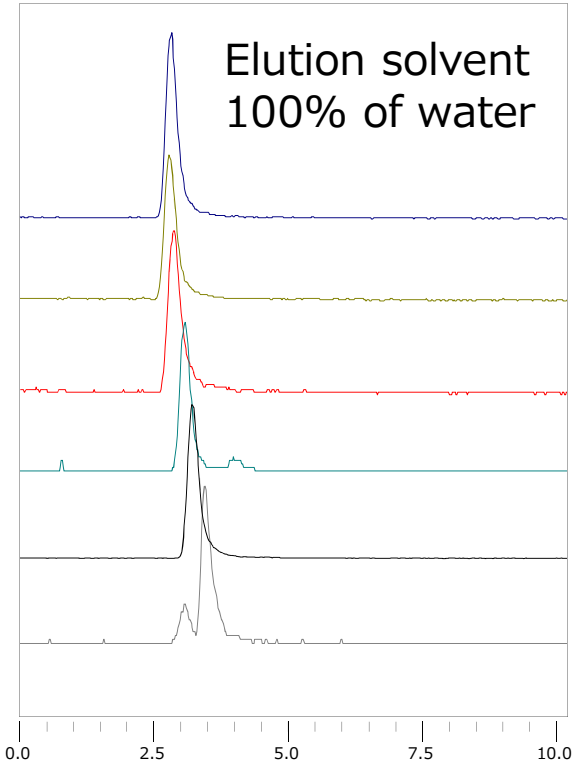


Chromatograms of typical metabolites under SFE condition

Under LC condition

Water

Quick elution



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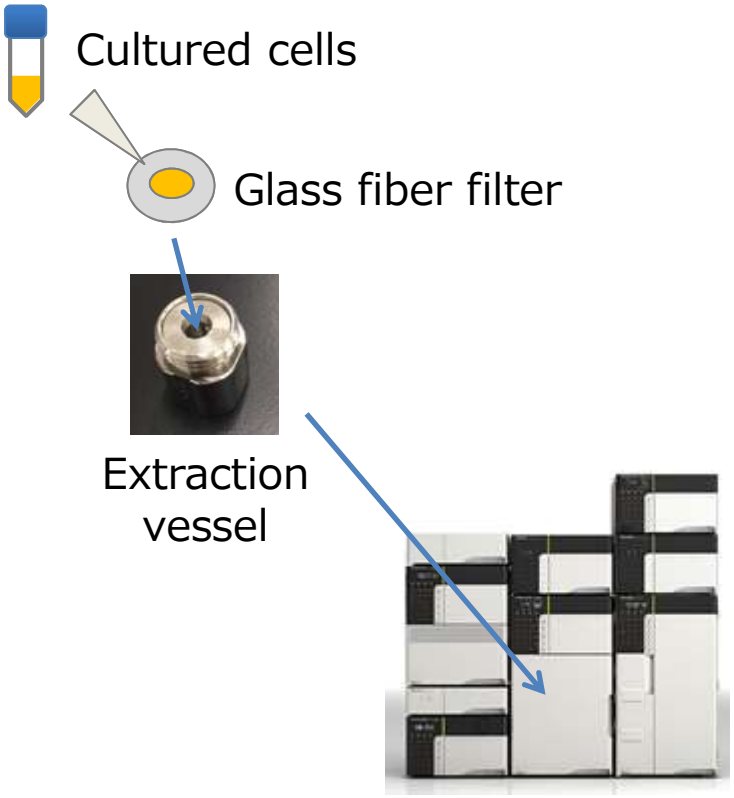
Methods and Materials
Instrumentation



SFE conditions

Modifier	0.1% ammonium formate-methanol
Flow rate	1.0 mL/min
Extraction	Static extraction : 3 min. Dynamic extraction : 2 min.
BPR	15 MPa
Vessel	0.2 mL

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Methods and Materials
Instrumentation

LC/MS conditions

Column	SUPELCO Discovery HS F5-3 (4.6 x150 mm, 3 µm)
Mobile Phase	0.1% formic acid-water / 0.1% formic acid acetonitrile
Gradient program	0%B (0-2min) => 25%B (5min) => 35% B (11min) => 95%B (15-20min) => 0%B (20.01-25min)
Flow rate	0.8 mL/min
Oven temperature	40℃
Ionization	ESI positive, negative
Mode	MRM

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with Triple quadrupole mass spectrometer (LCMS-8060)

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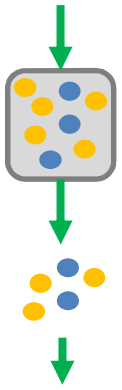
Component technologies for the online SFE-LC/MS

However hydrophobic compounds like lipids made back ground to be noisy and/or peak shape to be poor.
Therefore, multi-step extraction method was considered.

Single-step extraction

CO_2 / modifier=80 / 20

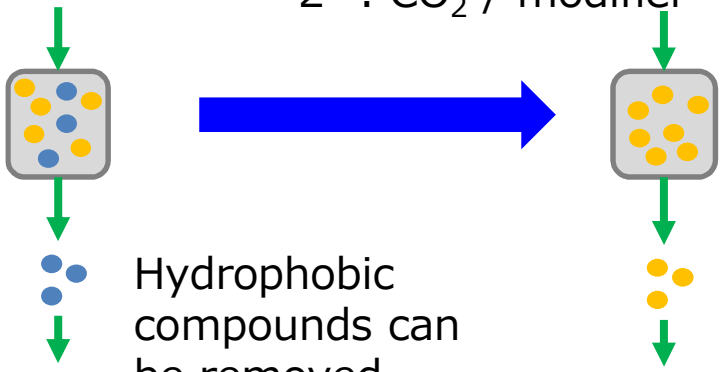


collection

Multi-step extraction

1st.CO_2 / modifier=100 / 0 (clean-up)

2nd. CO_2 / modifier=80 / 20



drain

Hydrophobic compounds can be removed.

collection

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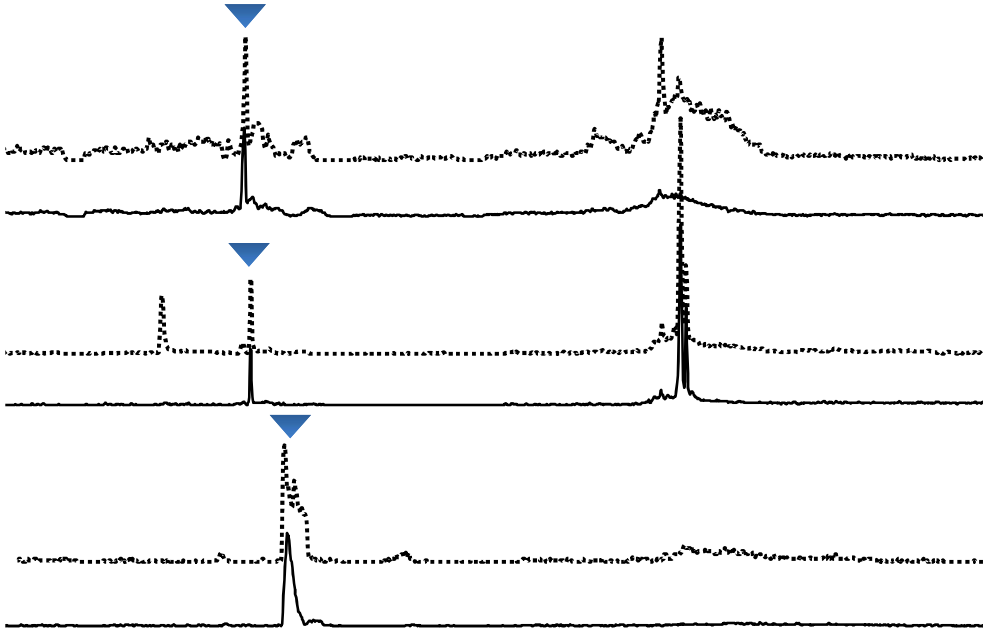
Chromatograms of sample extracts (E.coli.) by each extraction



Multi-step extraction method afforded

- Automatic extraction of typical metabolites from microbial cells without any additional pretreatment.
- Low back ground noise in MS detection due to clean-up step (i.e. 1st extraction with 0% of modifier).

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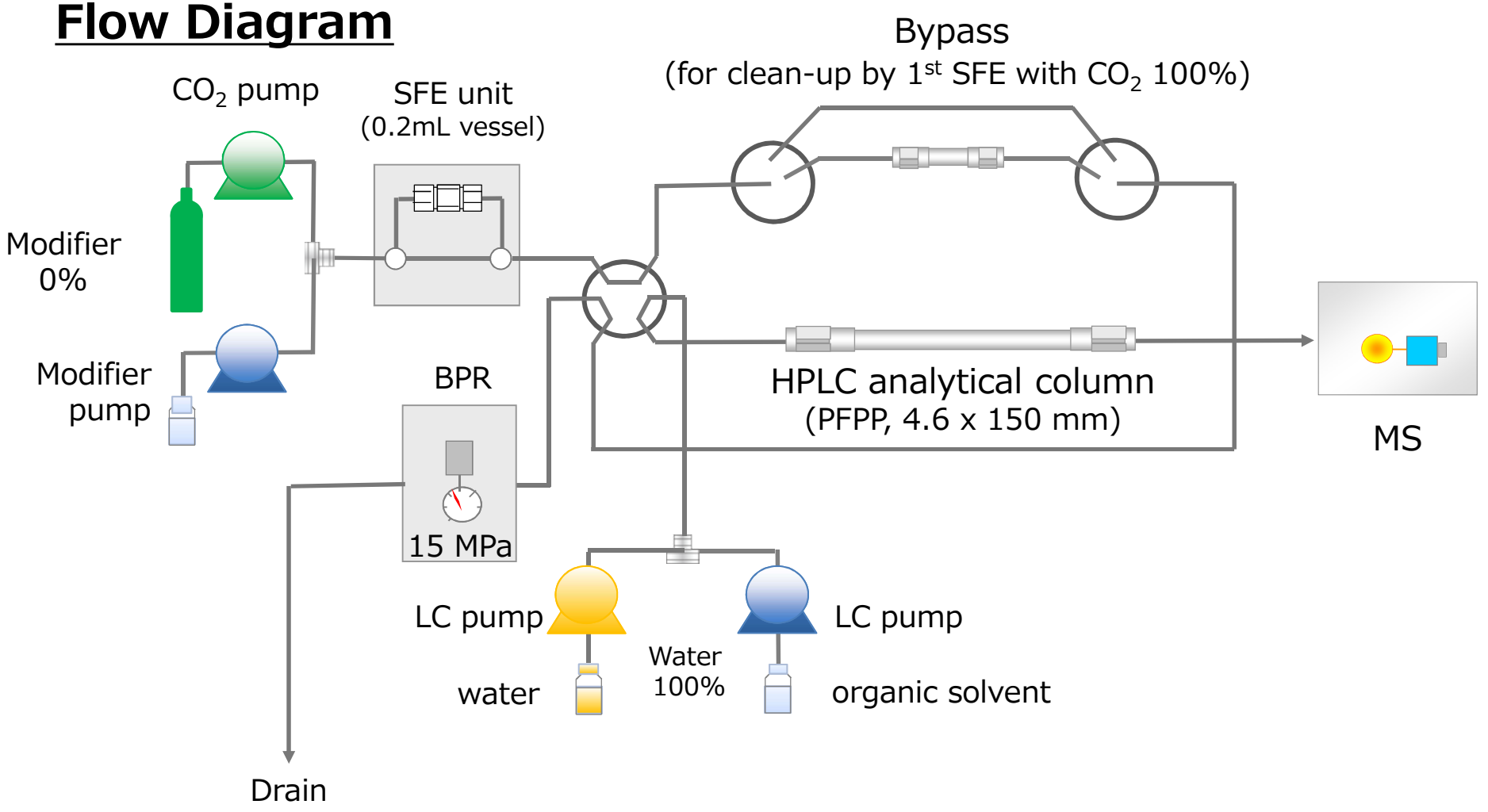
Development of online SFE-LC/MS system

Flow Diagram



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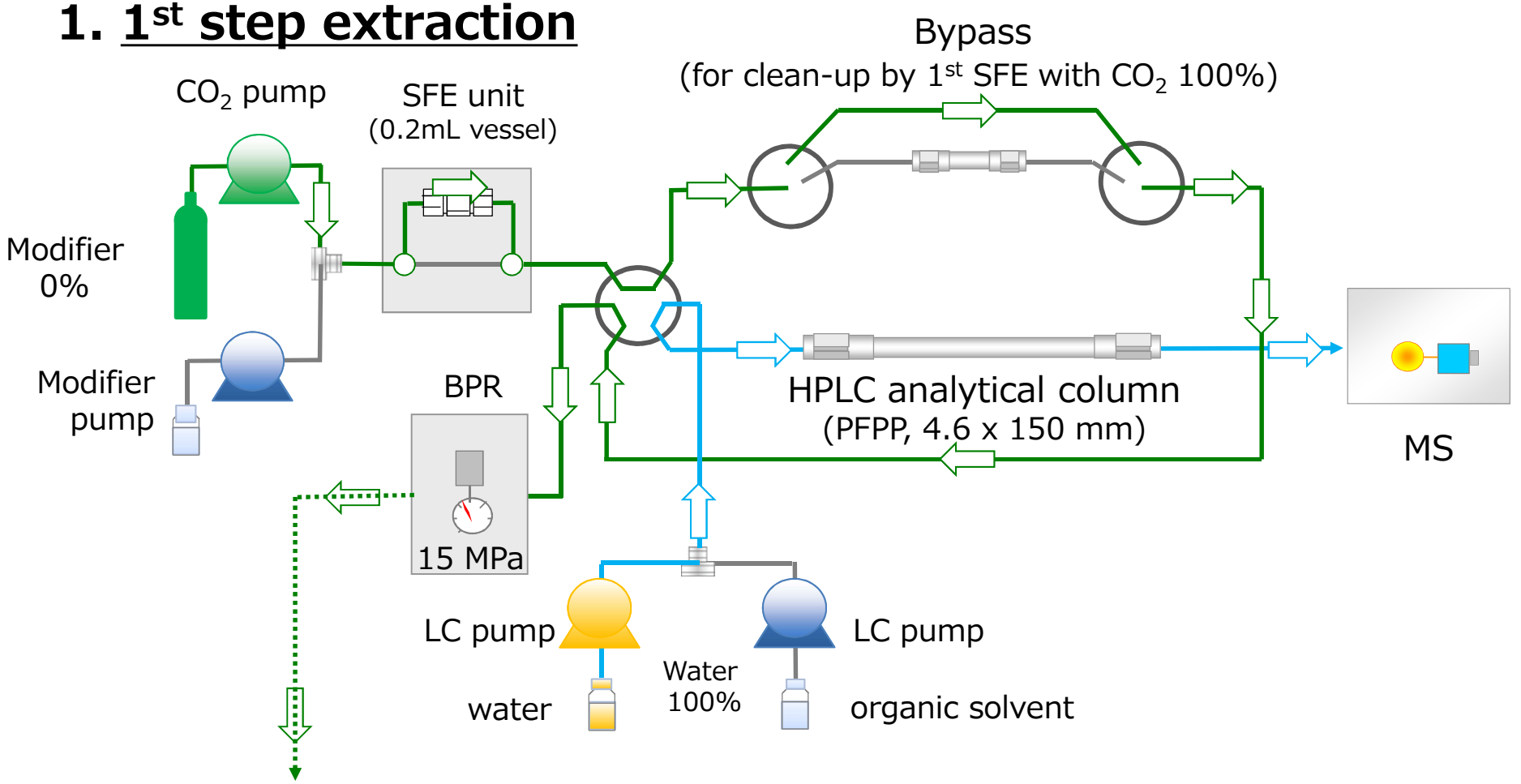
Development of online SFE-LC/MS system

1. 1st step extraction



Hydrophobic compounds (lipids etc.) are extracted and removed.

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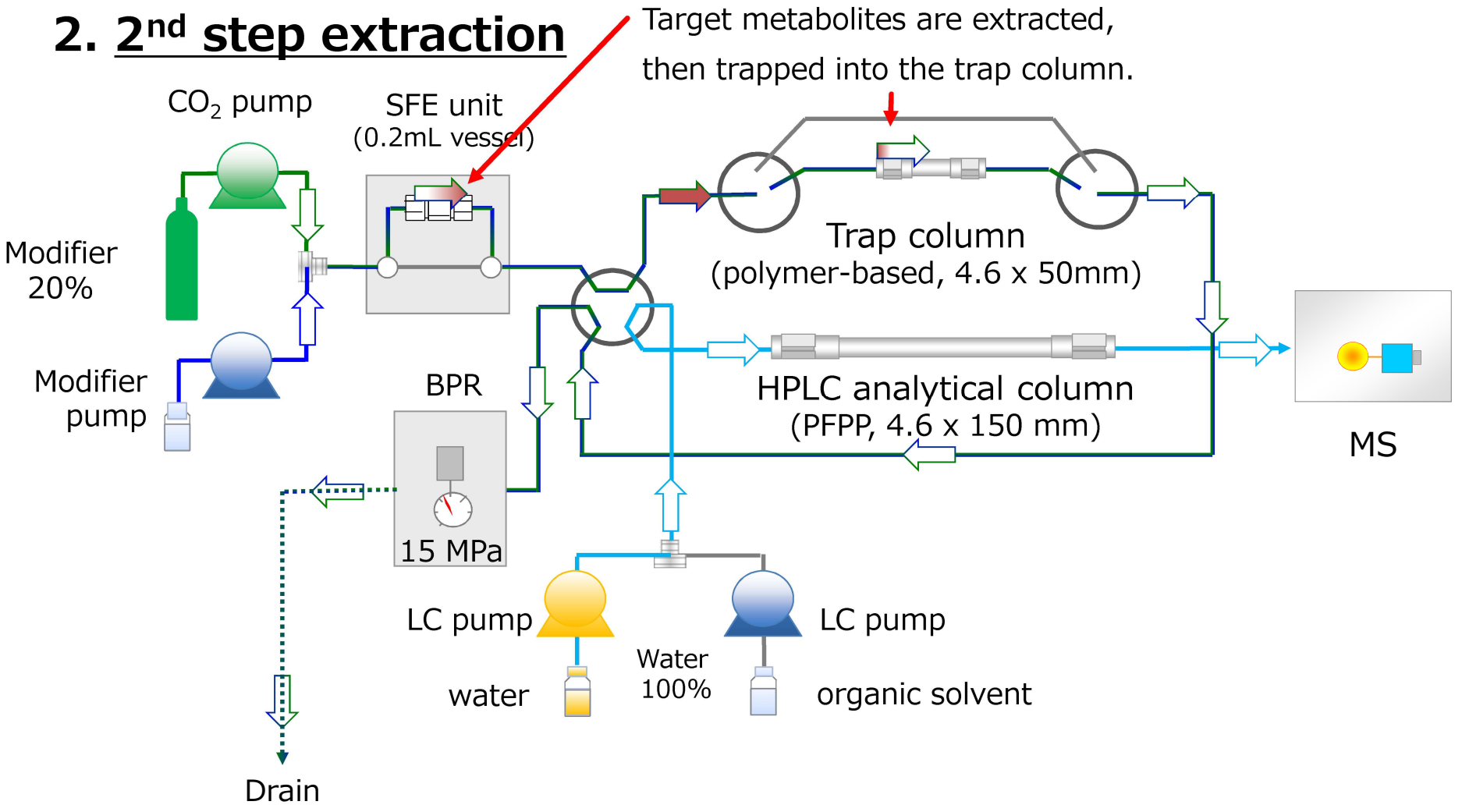
2. 2nd step extraction



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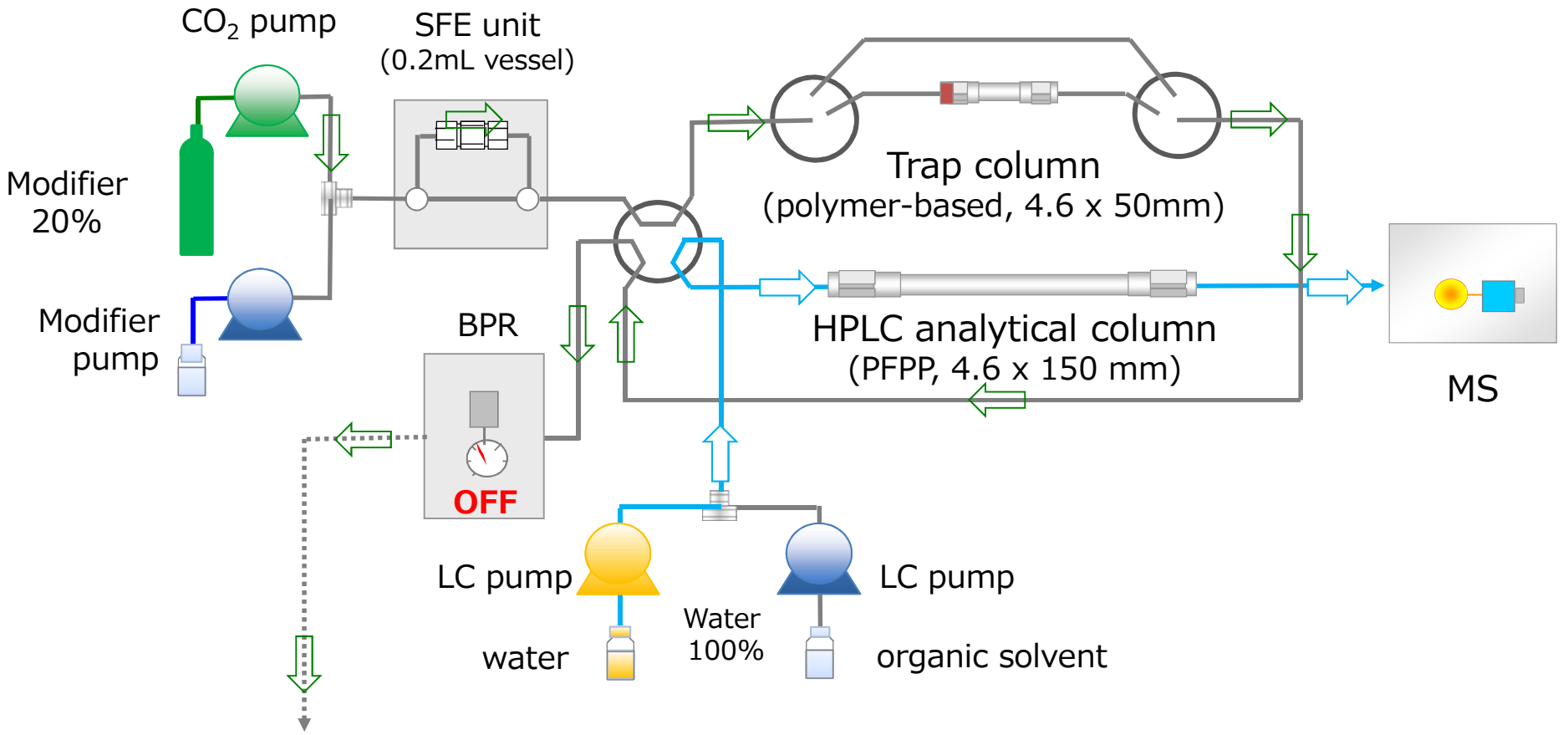
3. Releasing back pressure



To prevent direct mixing of SFE and LC mobile phases, CO_2 is evaporated and eliminated to outside.

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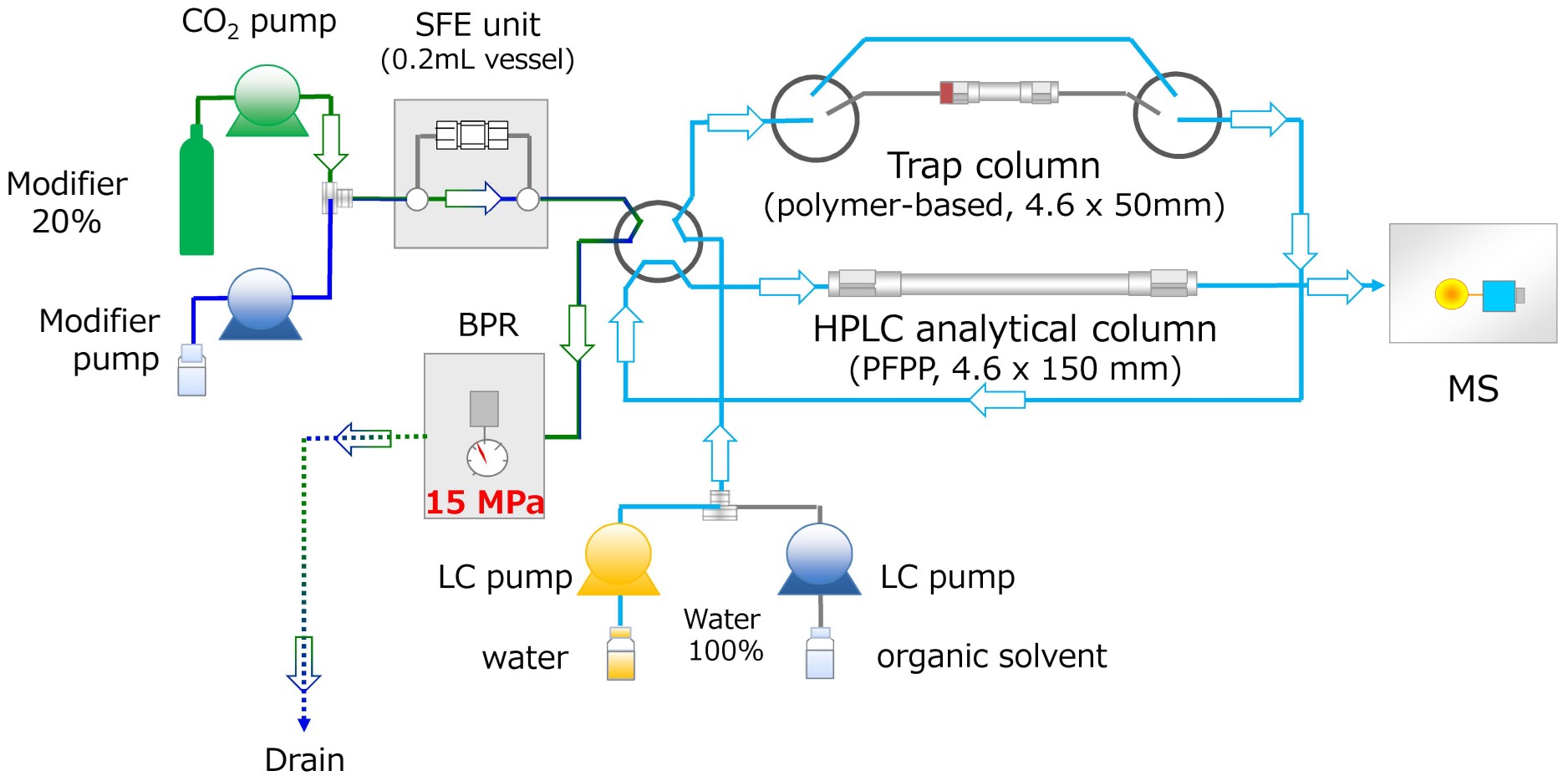
Development of online SFE-LC/MS system

4. Conditioning



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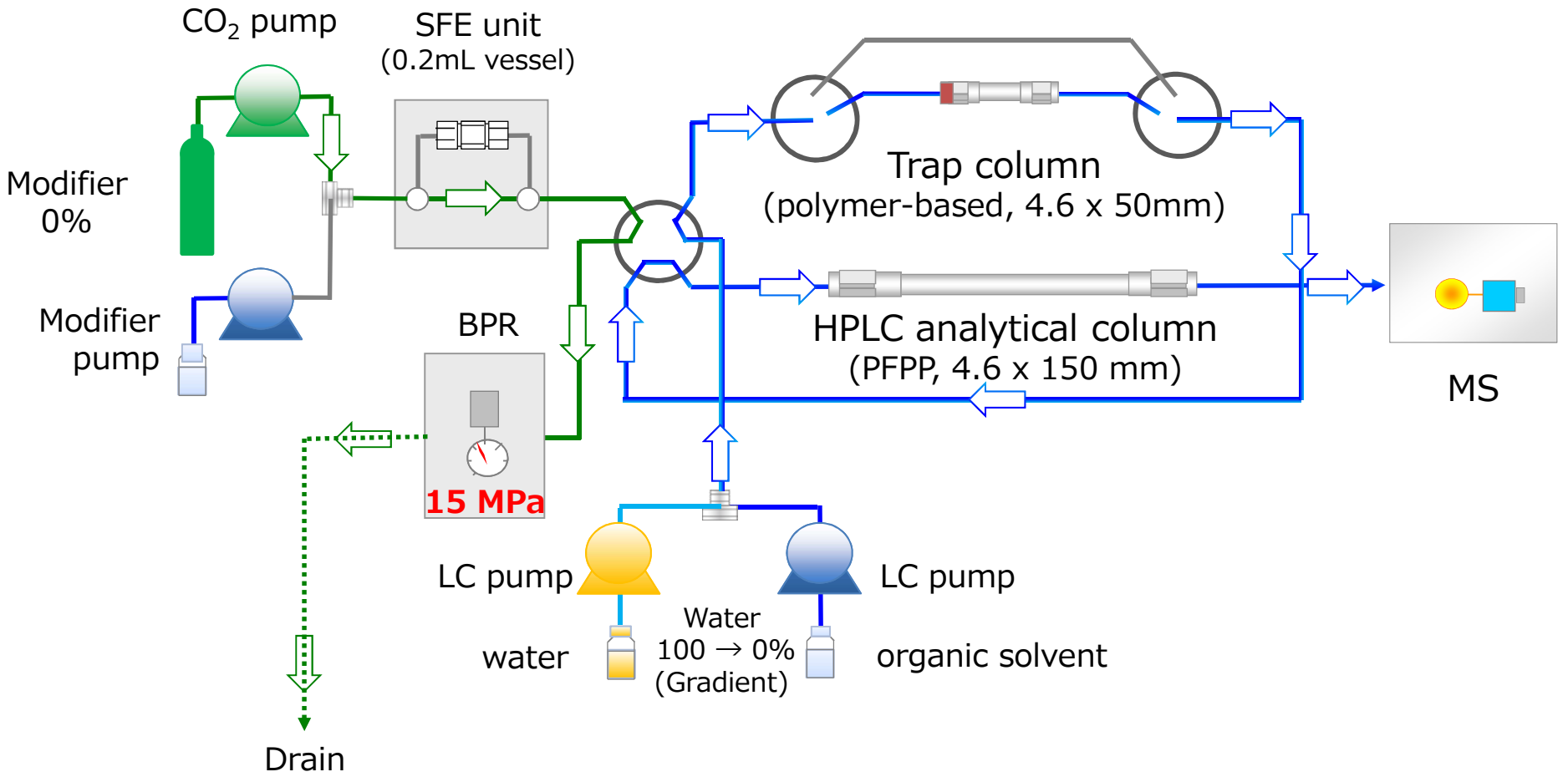
Development of online SFE-LC/MS system

5. LC/MS analysis



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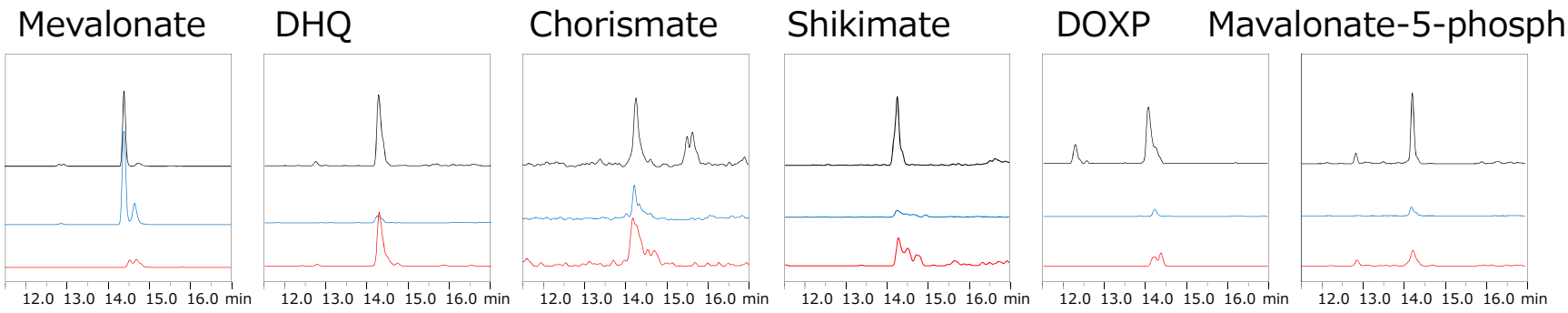
Metabolites analysis in real samples using SFE-LC/MS



- E.coli. and yeast cells collected from their culture mediums by centrifugation were used.
- Some metabolites were successfully extracted from E.coli. and yeast cells without any pretreatment.

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Conclusions

An online SFE-LC/MS system using newly developed polymer-based trap column for analysis of metabolites in microbial cells has been successfully developed.

- Multi-step extraction method that afforded automatic extraction of typical metabolites from microbial cells and low background noise in MS detection due to clean-up step.
- An online SFE-LC/MS system using newly developed polymer-based trap column for analysis of metabolites in microbial cells.

This system provides COMPLETELY automated analytical process resulting labor-saving.