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Development of an online SFE-SFC method to analyze alkaloids and triglycerides in lotus seeds

1. Introduction

Lotus seed (LS) is an important component of the lotus plant due to its nutritional and bioactive properties. Many bioactive compounds, including flavonoids, alkaloids, lipids and glycosides, have been identified in various lotus organs. In particular, alkaloids in LS have been highlighted for their antioxidant and anti-inflammatory activities. Many alkaloids have been isolated from LS, which is particularly rich in liensinine, isoliensinine, and neferine. Figure 1 shows the chemical structures of these three alkaloids.
Supercritical fluid chromatography (SFC) is a separation method that mostly uses supercritical carbon dioxide as the mobile phase. Online SFE-SFC is an extraction and separation technology used to extract components by supercritical fluid extraction (SFE) and then transfer the extract directly into a column for separation by SFC.
In this study, ground LS were firstly extracted using neat CO_2, which allowed extracting mostly triglycerides and alkaloids. Then, UHPLC and SFC methods were developed to analyze the extracts. Finally, an online SFE-SFC analysis of ground LS was conducted to achieve continuous extraction and separation of target compounds. The separation performance of online SFE-SFC was compared to offline SFE-SFC.

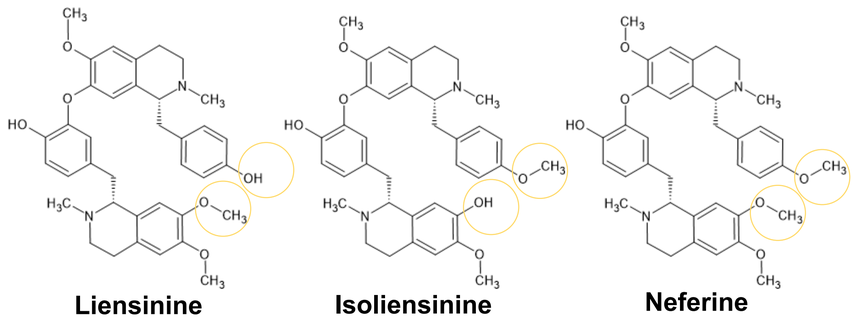

Figure 1 Chemical structures of targeted alkaloids

2.Supercritical Fluid Extraction of lotus seeds

In this study, SFE was used for the extraction of lotus seeds. Figure 2 shows the flow diagram of offline SFE system. Table 1 shows the offline SFE conditions. Before the extraction, LS shell was first removed with a tool, then the remaining plumule, cotyledon, and membrane were manually ground using a mortar and a pestle. To prepare the sample for extraction, 1 g of ground LS was mixed with 0.84 g of dehydrating reagent and then placed in a 5 mL extraction vessel. After the extraction, the extracts were automatically collected by a fraction collector, then they were dried by nitrogen gas for further analysis.

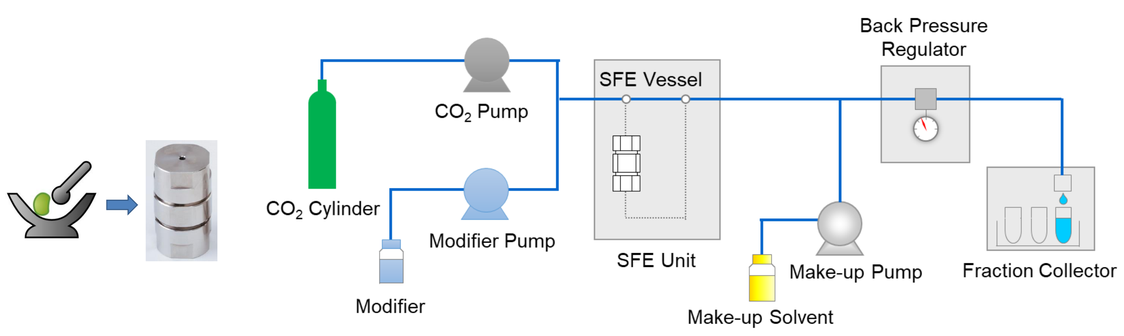

Figure 2 Flow diagram of offline SFE system

Table 1 Offline SFE conditions

Extraction vessel	: 5 mL
Extraction time	: 62 min (static 2 min → dynamic 60 min)
Extraction solvent	: Neat CO_2
Flow rate of extraction solvent	: 3 mL/min
BPR pressure (B)	: 150 bar
Extraction temperature	: 40 ℃
Make-up solvent	: Ethanol
Flow rate of make-up solvent	: 0.15 mL/min

3. Offline UHPLC and SFC analysis of SFE extracts

Dried extracts were resolubilized by 0.2 mL of methanol for UHPLC and SFC analysis. First, a UHPLC method was developed using a Shimadzu Nexera UHPLC system equipped with both PDA and ELSD detector, with the analytical conditions described in Table 2. A reversed-phase core-shell column Shim-pack Velox C18 was selected for separation. The chromatograms are shown in Figure 3A.
Then a SFC method was developed using Shimadzu Nexera UC system equipped with PDA detector and single quadrupole mass spectrometer LCMS-2050, with the analytical conditions described in Table 3. Shim-pack UC-RP column, which is a polar-embedded octadecyl phase column, was used for SFC. The chromatograms are shown in Figure 3B.

Table 2 UHPLC analytical conditions

System	: Nexera XS
Column	: Shim-pack Velox C18 (150 mm L., 4.6 mm I.D., 2.7 µm)
Mobile phase	: A) 0.1% Formic acid aqueous solution B) Acetonitrile/ IPA = 50/50
Time program	: B Conc. 70% (0 min) - 100% (10 min) - 100% (10.01-20 min) - 70% (20.01 - 25 min)
Flow rate	: 1 mL/min
Column temperature	: 25 ℃
Injection volume	: 1 µL
Detection	: UV 220 nm, 260 nm and ELSD

Table 3 SFC analytical conditions

System	: Nexera UC
Column	: Shim-pack UC-RP (250 mm L., 4.6 mm I.D., 5 µm)
Modifier (Mobile phase B)	: 20 mM Ammonium hydroxide methanol solution
Time program	: B Conc. 5% (0 min - 20 min)
Flow rate	: 3 mL/min
Column temperature	: 25 ℃
BPR pressure	: 150 bar
Injection volume	: 3 µL
Detection	: UV 220 nm, 260 nm
MS ionization source	: DUIS
Make-up solvent	: 0.1% Formic acid methanol solution
Flow rate of make-up solvent	: 0.1 mL/min

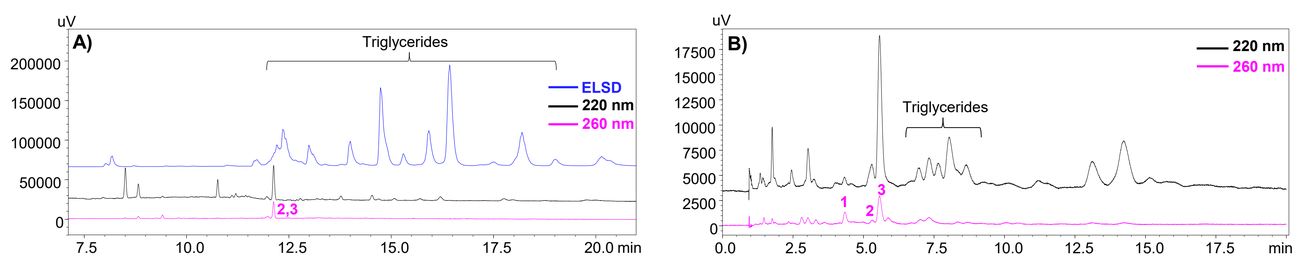

Figure 3 Chromatograms of A) offline UHPLC, B) offline SFC
Peak 1: neferine, peak 2 and 3: liensinine and isoliensinine

Based on MS spectra, peak1 was identified as neferine, and peaks 2 and 3 were identified as liensinine and isoliensinine. In UHPLC analysis, neferine could not be found, while alkaloids and triglycerides were both strongly retained and eluted only when the mobile phase was **100% organic solvent.** In addition, in UHPLC analysis, alkaloids were eluted in the same elution window as triglycerides. On the other hand, with an isocratic elution of **5% of 20 mM ammonium hydroxide methanol solution in CO_2**, alkaloids were well separated from triglycerides with the SFC method.

4. Online SFE-SFC analysis of lotus seeds

Finally, an online SFE-SFC method was developed and the analytical conditions are shown in Table 4. Figure 4 shows the flow diagram of online SFE-SFC system. To avoid sample overload and peak deterioration, two BPRs were used for this analysis. This way, the sample introduction rate can be controlled by the pressure difference of the two BPRs. Figure 5 shows the flow chart of online SFE-SFC analysis.
Figure 6 shows the chromatograms of online SFE-SFC. Resolution between the critical pair (peaks 2 and 3) was 1.04 for online SFE-SFC, and 1.10 for offline SFE-SFC, respectively. It demonstrated that in this case, **the extracts of SFE can be trapped at the column head during the extraction so the separation performance could be maintained when using online SFE-SFC**.

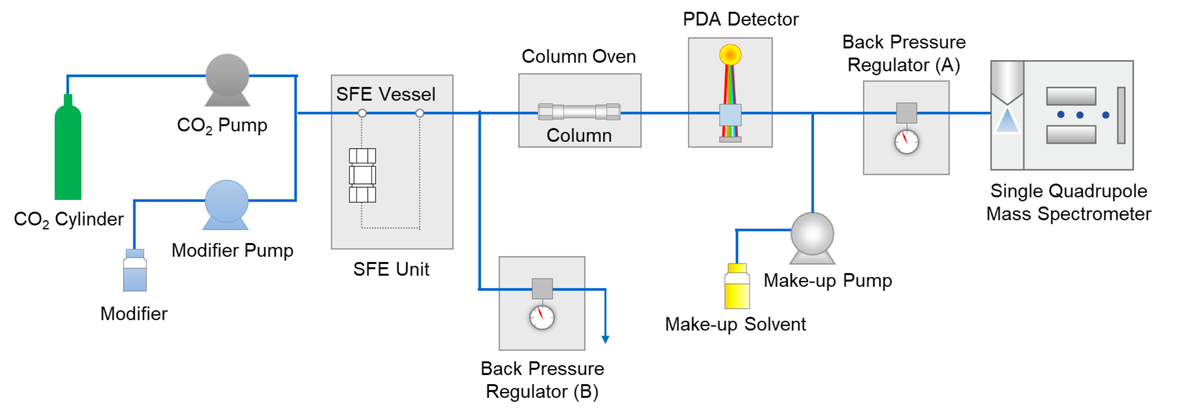

Figure 4 Flow diagram of Shimadzu Nexera UC system in online SFE-SFC-MS mode

Table 4 Analytical conditions of online SFE-SFC

SFE	
Extraction time	: 7 min (static 2 min → dynamic 5 min)
Extraction solvent	: Neat CO_2
Flow rate	: 3 mL/min
BPR pressure (A)	: 148 bar
BPR pressure (B)	: 150 bar
Extraction temperature	: 40 ℃

SFC	
Analytical Column	: Shim-pack UC-RP (250 mm L., 4.6 mm I.D., 5 µm)
Modifier (Mobile phase B)	: 20 mM Ammonium hydroxide methanol solution
Time program	: B Conc. 5% (9 min - 29 min)
Flow rate	: 3 mL/min
BPR pressure (A)	: 150 bar
Column temperature	: 25 ℃

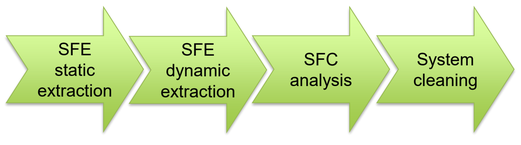

Figure 5 Flow chart of online SFE-SFC analysis

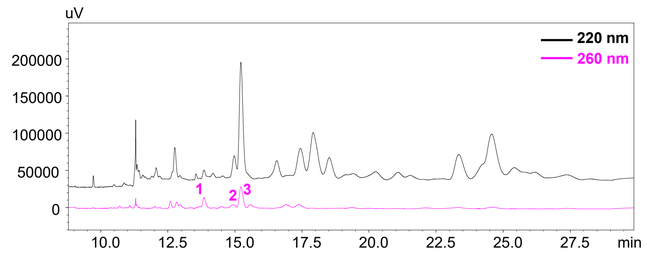

Figure 6 Chromatograms of online SFE-SFC-PDA

5. Summary

- An online SFE-SFC method was developed to achieve continuous extraction and analysis of alkaloids and triglycerides in lotus seeds.
- Results of online SFE-SFC were compared to offline SFE-SFC, demonstrating that the separation performance of target compounds was maintained when using online SFE-SFC.