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Short Communication

EXTRACTION OF EPICUTICULAR WAX AND NONACOSAN-10-OL FROM *EPHEDRA* HERB UTILIZING SUPERCRITICAL CARBON DIOXIDE

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Abstract—Experimental results concerning the processing feasibility of using supercritical CO_2 were reported for the extraction of epicuticular wax from *Ephedra* herb. Subsequently the isolability of nonacosan-10-ol from the total extract of epicuticular wax was evaluated by TLC and gas chromatography. Also, Soxhlet extractions of *Ephedra* herb by n-hexane and chloroform were performed and these results were compared with those obtained by supercritical CO_2 extraction. As a result, we could demonstrate that supercritical CO_2 can be an economical alternative to the organic solvents in processing the medicinal plant.

Key words: *SFE, Ephedra Herb, Epicuticular Wax Layer, Nonacosan-10-ol, Carbon Dioxide*

INTRODUCTION

In recent years, a large body of experimental data has been accumulated on the solubility and extractability of chemical constituents from various natural products with supercritical fluids [McHugh and Krukonis, 1994; Stahl et al., 1988]. However, quantitative study relevant to process design on the extractability of chemical constituents from natural medicinal plants utilizing supercritical fluids has not been extensively reported yet.

In the orient, natural plants have been widely used for medicinal purposes. Traditionally concentrated liquid extracts are prepared by boiling and condensing raw mixtures of certain medicinal plants in water or alcohol. To date, however, only a limited information is available on a quantitative knowledge of oriental herb medicine in terms of modern pharmacy. On the other hand in the Western pharmacy, emphasis has been placed on the selective extraction and isolation of each pharmacologically active constituents by liquid-phase solvent extraction method. However, removal of trace amount of used solvents from extract remains as a very difficult problem. In these regards, when one uses extraction method with supercritical (SC)-CO_2, a viable option is possible for avoiding solvent contamination in pharmaceutical materials.

As a step toward the modernization of the traditional oriental medicine, we have been making a special effort to utilize solvent fluid extraction (SFE) techniques. Based on our effort, we report in this article a part of our results on the feasibility studies of replacing organic liquid solvents such as n-hexane or chloroform with SC-CO_2 for the extraction of phytochemical constituents from medicinal plants.

When one considers identification and/or isolation of specific compounds from a target plant, it is imperative to figure out the presence and the characteristics of their compositional distribution in the infrastructure of the plant body. In general, certain substances are not evenly distributed in a plant body but extremely localized among several layers. As shown in Fig. 1, typical outer epidermal wall of plants from the inside consists of several layers

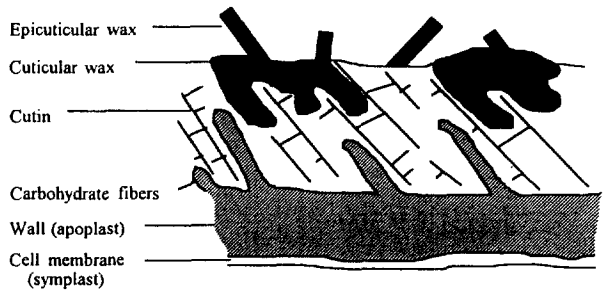

Fig. 1. Location of the epicuticular wax layer in plant tissue (Taken from ref. 3).

such as cell membrane, cell wall, cutin and epicuticular layer [Price, 1982]. The epicuticular wax layer plays an important role in restricting water vapor loss, regulating gas exchange and providing physical protection from pathogene and insect invasion. This layer also limits the intake of pesticides and reactive environmental pollutants. Thus, in several plants, it works as a storage layer of several bioactive compounds having antimicrobial and antifungal properties. The major constituents of the epicuticular wax layer are hydrocarbons, long chain esters, aliphatic alcohols, ketones, aldehydes, fatty acids, terpenes and flavonoids [Walton, 1990]. Among the constituents, nonacosan-10-ol is one of typical components of aliphatic alcohol [Prasad and Guelz, 1989; Guelz, 1994].

In the present study, we have experimentally evaluated the economic and processing feasibility of SC-CO_2 as an alternative solvent for the extraction of chemical constituents from *Ephedra* herb, which has long been used in oriental medicine for its diaphoretic, antiasthmatic, and diuretic properties. In doing so, we performed first thin layer chromatography (TLC) test and gas chromatography (GC) for extracts obtained from SC-CO_2 screening apparatus. Thus, we could conformed that the extract consists mostly of lipophilic and volatile substances which are usually present in the epicuticular wax layer. Based on these results, we chose

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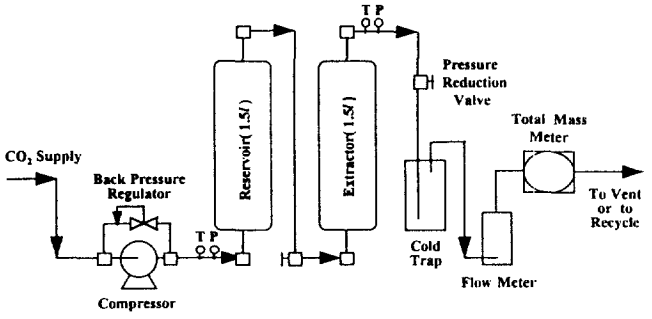

Fig. 2. Schematic diagram of large scale extractor.

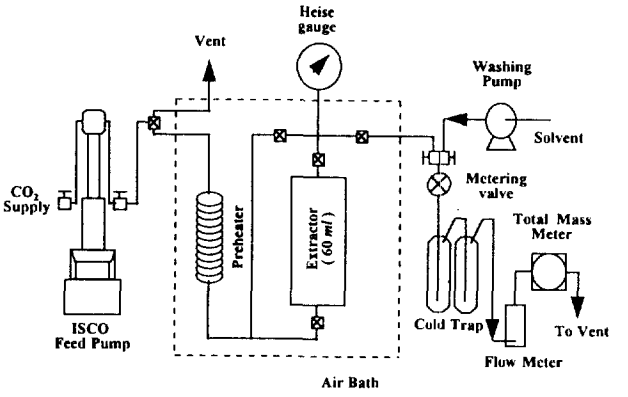

Fig. 3. Schematic diagram of microscale dynamic flow apparatus.

the optimal SFE conditions. Then, we obtained large amounts of total wax extract from *Ephedra* herb. Using the total extract, we performed spectral and chromatographic analysis for the identification and selective isolation of the target solutes. Based on these tests, we established the SFE characteristics and optimum conditions. For comparison, we also, performed Soxhlet extraction by n-hexane and chloroform, respectively. These results were then compared with those obtained from the microscale SFE apparatus.

EXPERIMENTAL

1. Extraction Apparatus

A schematic diagram of the large-scale flow-type SFE apparatus used in the present study is shown in Fig. 2. The internal volume of extraction cell was 1.5 L. The gas-phase CO_2 solvent was pressurized by a gas booster (HASKEL 75/15, USA) and was adjusted by a back pressure regulator (Tescom, 26-1700, USA). To minimize fluctuations in pressure, a surge tank line was installed between the booster and the extractor. The cell was heated by a heating tape and the temperature was controlled by a controller to ± 0.5 K. The effluent from the extractor was expanded to atmospheric pressure through the heated metering valve (HIP 60-11-HFV-V, USA). The flow rate and cumulative consumption of CO_2 at ambient state was measured by a mass flowmeter (Sierra 8810, USA) and wet testmeter (GCA/Precision Scientific, USA), respectively. To minimize measuremental error of using wet testmeter, the water in the testmeter is saturated by CO_2. The extract from the cell was collected in a cold trap in which entrainment was prevented by bubbling the expanded effluent flow into methanol. This equipment was used primarily for extracting large amount of total extract of epicuticular waxy substances from *Ephedra* herb.

As shown in Fig. 3, another microscale flow-type SFE apparatus was designed for establishing optimum extraction conditions, yield and selectivity for the target substances. The internal volume of this extractor was 60 mL. Pressurization and control were made by using a syringe pump (ISCO 260DM, USA). Pressure in the cell was accurately measured by Heise gauge (HEISE MM-43776, USA) and coil temperature was controlled by a bath to ± 0.1 K. The extract was collected by using two cold traps in series in which each trap contains methanol. After every 30 L of CO_2, the valve after the extractor was closed, and the line from the extractor was rinsed with solvents such as chloroform and methanol and, then, the solvent is removed from the extract by evaporation. Filter paper was installed over the outlets of the extractor to prevent entrainment.

Table 1. Identified spectral data for nonacosan-10-ol in *Ephedra herb*

OH CH_3-$(CH_2)_8$-CH-$(CH_2)_{18}$-CH_3 nonacosan-10-ol
m.p.: 83-84℃
IR: ν_{max} 3400, 2960 cm $^{-1}$
EI-mass (70 eV): (relative intensity) m/z 424 (5.2), 423 (12.2), 406 (9.8), 297 (43.4), 157 (49.8), 111 (35.3), 97 (80.2), 83 (100.0)
^{1}H-NMR (400 MHz, $CDCl_3$): 0.88 (CH_3), 1.17 (CH_2), 1.45 (CH_2), 3.57 (CH)
^{13}C-NMR (100 MHz, $CDCl_3$): 14.2 (CH_3), 22.7, 25.6, 29.4, 29.7, 31.9, 37.5, 72.0 (CH)

2. Instrumental Analysis

Total extracts and target substances were analyzed as follows: melting points was measured by a Gallenkamp apparatus. Molecular structure and extract composition was analyzed by FT-NMR (JEOL GSX 400 MHz), mass spectrometer (VG Trio-2), and FT-IR (Perkin Elmer 1710, USA). The quantitative information on extraction yields by both SFE and conventional Soxhlet extraction were obtained by GC using a FID detector (HP-5890 II with a HP-5 cross linked 5% phenyl silicone capillary column; 25 m$\times$ 0.32 mm$\times$0.17 μm film thickness).

3. Structure Elucidation

Analysis of IR, ^{1}H-NMR, and ^{13}C-NMR of the isolated compound suggested that it was an aliphatic alcohol. The molecular weight and the bonding position of OH group was determined by EI-mass spectrometry. As shown in Table 1, we found that this substance possessed a molecular weight of m/z 424 and the OH group was located in C-10 positions since the backbone carbon chain was fragmented at m/z 157 and 297 [Isono, 1976]. As a result, we concluded that the substance must be nonacosan-10-ol. The purity of this compound was determined as 97.0% by GC.

4. Extraction of Total Wax from *Ephedra* Herb

Dried raw authentic *Ephedra* herb was purchased from a domestic market. The sample was redried 24 hours at 313.15 K in an oven and the raw material cut into 5 to 10 mm size pieces for SFE experiments. The purity of the CO_2 gas was 99.9%. Organic solvents of HPLC-grade were used for cleaning the extrac-

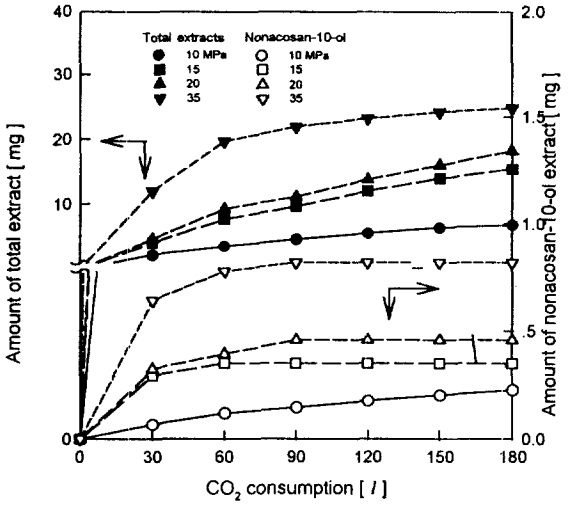

Fig. 4. Amounts of total extract and nonacosan-10-ol in 1 g *Ephedra* herb as a function of CO_2 consumption at 308.15 K.

tors and in the instrumental analysis.

For experiment in the large scale SFE system, a 150 g sample was put into the apparatus. Extraction at 15 MPa and 313.15 K yields approximately 700 mg total extract of epicuticular waxy substance. Each experiment took about 10 hours and the total consumption of CO_2 was about 800 L at ambient condition.

5. Analysis of Total Extract

After obtaining the TLC and GC patterns of the extract from the preliminary step, the total extract from the large scale SFE apparatus was fractionated by silica gel column chromatography (Silica gel 60, 400 mesh, Merck Art. 9385, Germany). Using an elution solvent system consisting n-hexane and chloroform (10 : 1-3 : 1), the total extract sample was fractionated. Among the fractions separated from the extract, nonacosan-10-ol was identified by spectral analysis.

6. Analysis of Nonacosan-10-ol

To set optimal extraction conditions for nonacosan-10-ol, GC with FID detector was used. GC analysis was performed at column temperature 523.15 K. The injection temperature was held at 553.15 K and the FID detector temperature was 563.15 K. Helium was used as the carrier gas at 2.0 mL/min. The injected volume was 2.0 μL. The retention time of nonacosan-10-ol was recorded as 18.5 min.

To obtain the optimum extraction conditions and yields for nonacosan-10-ol, further extraction experiments in the microscale SFE were carried out at temperatures 308.15, 313.15, and 323.15 K and pressures 10, 15, 20, and 35 MPa, respectively. In this experiment, the extracts were collected for every 30 L of CO_2 at ambient condition until six cumulatively fractionated extracts were obtained. The amount of nonacosan-10-ol in each extraction collected was analyzed by GC.

To compare these results with those of conventional liquid extraction technique, a Soxhlet extraction was carried out for 6 hours with a 5 g sample, using 100 mL of n-hexane and chloroform as solvents, respectively.

RESULTS AND DISCUSSION

1. Soxhlet Extraction

Table 2. Amounts of total extract and nonacosan-10-ol per 1 g of *Ephedra* herb sample

Pressure (MPa)	$SC-CO_2$ extraction						Soxhlet extraction			
	Temperature (K)						n-Hexane		Chloroform	
	308.15		313.15		323.15					
	A¶	B	A	B	A	B	A	B	A	B
10	8.58	0.43	12.22	0.46	6.84	0.23	11.43	0.50	20.12	0.41
15	18.52	0.48	14.94	0.54	15.40	0.35				
20	14.42	0.35	14.82	0.40	18.22	0.46				
35	14.28	0.46	9.72	0.49	24.88	0.82				

¶ A: Total amount of total extract [mg]
B: Amount of nonacosan-10-ol extract [mg]

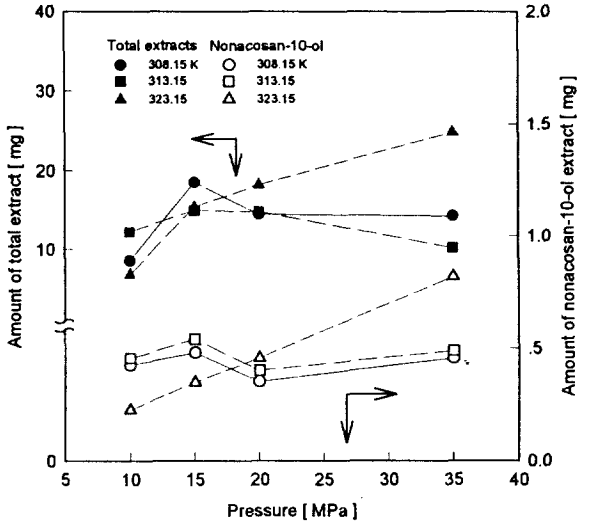

Fig. 5. Effect of pressure on the amount of total extract and nonacosan-10-ol in 1 g *Ephedra* herb.

Conventionally, n-hexane or chloroform have been widely used for the extraction of lipophilic compounds from natural plants [Jetter and Riederer, 1995]. Thus, with these two solvents, we carried out Soxhlet extractions for 6 hours. From hexane extraction, we obtained 56.6 mg of total extract from a 5 g sample, and subsequently, 2.49 mg nonacosan-10-ol and with chloroform, 100.6 mg total extract and 2.06 mg nonacosan-10-ol, respectively.

2. SFE by $SC-CO_2$

Two experimental apparatuses are used. The large scale extractor shown in Fig. 2 is used for obtaining total extract from the herb and the flow type equilibrium cell is used for fractional extraction of biochemicals from the total extract. The amounts of total extract of epicuticular wax from *Ephedra* herb and nonacosan-10-ol per every 30 L CO_2 at 323.15 K are shown in Fig. 4 as a function of pressure (10, 15, 20, and 35 MPa). Other results were summarized in Table 2. From Table 2, it would appear that most of the waxy substances and nonacosan-10-ol were extracted in the first 30 L CO_2 fraction. These results are similar that found for other plant materials extracted with $SC-CO_2$. At 15 MPa, maximum yields of extraction could be accomplished at both temperatures 308.15 and 313.15 K. However, at 323.15 K, both extraction yields of wax and nonacosan-10-ol tend to increase with increasing pressure up to 35 MPa. As a result, the yield of extraction at high temperatures tends to increase with increasing extraction pressure as shown in Fig. 5.

Table 3. Percent yields of nonacosan-10-ol in total wax extract

Pressure (MPa)	$SC\text{-}CO_2$ extraction			Soxhlet extraction	
	Temperature (K)			n-Hexane	Chloroform
	308.15	313.15	323.15		
10	4.97	3.73	3.33	4.39	2.04
15	2.59	3.61	2.27		
20	2.45	2.71	2.51		
35	3.21	5.04	3.30		

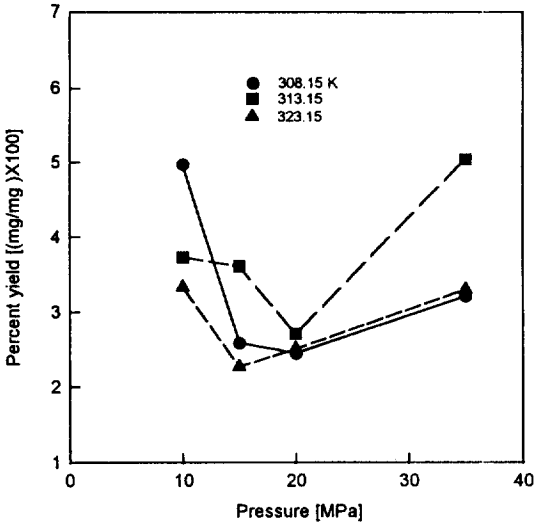

Fig. 6. Percent yield of nonacosan-10-ol in the total extract as a function of extraction conditions.

Finally, the results from the Soxhlet extraction by n-hexane and chloroform are summarized in Table 2 together with the result from $SC\text{-}CO_2$ extraction. From identical amounts of sample, approximately twice the amount of the total extract and nonacosan-10-ol can be obtained by the supercritical extraction (323.15 K, 35 MPa). Also, in Table 3, we compared the variation of the contents of nonacosan-10-ol in the total extracts obtained by both the Soxhlet extraction and $SC\text{-}CO_2$ extraction. We can conclude that even within the mild conditions of $SC\text{-}CO_2$ extraction (308.15 K and 10 MPa), the selectivity of isolation of nonacosan-10-ol can be easily controlled when we use SFE method. Such selectivity is not obtained in the case of Soxhlet extraction as shown in Fig. 6.

CONCLUSION

Two results can be drawn from the present study. First, by applying the supercritical extraction technique for the processing of *Ephedra* herb, we could obtain a significant amount of nonacosan-10-ol from the epicuticular wax layer of *Ephedra* herb. Secondly, the maximum yield of extraction can be accomplished within a temperature range of 308.15 to 313.15 K and pressure of 10 to 15 MPa.

In summary, we can regard the SFE technique with CO_2 as a viable alternative for practical replacement of the traditional toxic organic solvent extraction in the pharmaceutical industry. Although we limited our main attention only to a target substance, nonacosan-10-ol in the present article, we believe that SFE method is obviously an attractive tool for the extraction and isolation of other substances such as triterpene and steroids present in the epicuticular wax layers of numerous other oriental medicinal plants.

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