

## Extraction of citrus flavonoids from peel of *Citrus junos* using supercritical carbon dioxide with polar solvent

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### ABSTRACT

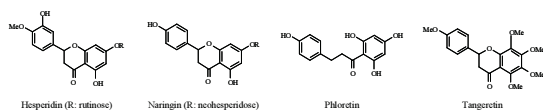
Citrus juice processing residues are mainly composed of peel, juice sack and seed. The peel, especially, consists of bioactive compounds such as flavones. Supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction of flavonoid was carried out at a pressure of 30 MPa and temperature ranging from 80 to 160°C. Ratio of water and ethanol as a co-solvent was varied from 0 to 100%. Flavonoids such as naringin and hesperidin have glycoside group, thus water was a good solvent for extraction of these compounds. Extraction behavior of tangeretin was quite different from those of flavonoid glycosides, and the yield of tangeretin tended to increase with ethanol concentration.

Key words: *Citrus junos*, Flavones, Supercritical carbon dioxide, Sub-critical water

### 1. INTRODUCTION

Most of Japanese sour citrus have been used for juice processing. One of them is *Citrus junos*, called yuzu, which is harvested mainly in Kochi Prefecture in Japan. Yuzu is a well-known citrus fruit in the southern part of Japan. Juice processing residue contains valuable compounds such as essential oil, pectin and flavonoids. The essential oil of yuzu is very expensive due to its unique characteristic odor and low yield. Recently, Hoshino et al. succeeded in effectively extracting essential oil from yuzu peel using a semi-continuous flow supercritical carbon dioxide (SC-CO<sub>2</sub>) extractor in our laboratory.[1] The peel residue of SC-CO<sub>2</sub> extraction still contains polar and non-polar bioactive compounds such as flavonoids and pectic substances.

Almost all citrus flavonoids are contained in all parts of fruit except the juice. Fig.1 shows some kinds of flavonoids and chalcones of yuzu peel.



**Fig.1.** Structural illustrations of some flavonoids of Yuzu peel

Hesperidin and naringin are found in citrus genus, and their concentrations are much higher than many other

flavonoids. These two flavanones have bioactivities such as hypolipidemic effect and apoptosis inducing effect in cancer cell [2]. It has been thought that hydroxylated chalcone, like phloretin, has anti-allergenic activity and induced apoptosis for human cancer cell [3, 4]. Polymethoxyflavone tangeretin was also reported to have anti-proliferative and apoptotic effects on human cancer cell lines [5].

Extraction of flavonoids from plant materials has been accomplished by traditional extraction processes, such as solid-liquid extraction, using organic solvent such as methanol, ethanol, and acetone.[6] Even though high yield of target compounds can be obtained by organic solvent extraction, the use of organic solvent is not recommended because residual organic solvents have potential adverse effect on human health. Carbon dioxide and water are harmless, and present widely in nature. Therefore, subcritical water and SC-CO<sub>2</sub> have been applied individually to extract polar and non-polar compounds from various plant materials.[7] Flavonoids are weakly polar, so it is difficult to effectively extract these compounds using SC-CO<sub>2</sub> or subcritical water separately.

In this work, extraction of various flavonoids from citrus junos peel using green solvents, e. g. CO<sub>2</sub> and water, simultaneously were investigated by varying their operating conditions. SC-CO<sub>2</sub> is suitable for extracting weakly polar substances such as flavonoids by simply

adding small amount of polar co-solvent such as ethanol and water.

## 2. MATERIALS AND METHODS

### 2.1. Materials and chemicals

Using yuzu flavedo as the starting material, the residue from supercritical CO<sub>2</sub> extraction of essential oil at 60°C and 20 MPa was supplied by ASCII Co. Ltd. (Fukuoka, Japan). The residue was freeze-dried, ground, and sieved. Flavedo with a particle size of 170–450 µm was obtained as raw materials. Standard compounds of hesperidin, naringin, phloretin and tangeretin for use in High performance liquid chromatography (HPLC) analyses were purchased from Wako Pure Chem. Ind., Ltd (Osaka, Japan).

### 2.2. Solvent extraction

A 0.1 g of raw material was extracted with 50 % of methanol solution (methanol/Dimethyl sulfoxide (DMSO)=1/1, v/v) to investigate the total amount of flavonoids. The yield of each flavonoid was determined based on the amount of recovery obtained by organic solvent extraction.

### 2.3. HPLC analysis of flavonoid

Flavonoid extracts were analyzed using a HPLC LC-10AD gradient system, equipped with Diode Array Detector SDP-M10A. Inertsil ODS-3 column was used for separation at 35°C. The mobile phase consisted of solvent A, 0.1 % acetic acid in water, and solvent B, 0.1 % acetic acid in acetonitrile (acetonitrile/water = 75/25, v/v). The flow rate was 1.0 mL/min. As shown in Table 1, the gradient elution was carried out according to the following steps: time 0 min A-B (88:12); time 18 min A-B (78:22); time 28 min A-B (72:28); time 35 min A-B (62:38), time 48 min A-B (52:48), time 58 min A-B (0:100); time 70 min A-B (88:12).

Table 1. Gradient condition of HPLC analysis

Ratio of solvent B	Time [min]
12%	0
22%	5
28%	15
38%	22
48%	32
68%	37
100%	42
100%	45
12%	50
12%	60

Peaks were measured at a wavelength of 285 nm to quantify flavonoids.

### 2.4. SC-CO<sub>2</sub> extraction

Fig.2 shows schematic diagram of SC-CO<sub>2</sub> extraction apparatus with an option of adding a co-solvent. The maximum working conditions of the apparatus are 450°C and 45 MPa. The pressure in the extractor was controlled by a back-pressure regulator (HBP-450; Akico Co., Ltd.). The extraction temperature was monitored by the thermocouples at the inlet and outlet of the extractor. In the flavonoids extraction, a 2.0 g portion of raw material was charged in the extractor (10

mL). SC-CO<sub>2</sub> extraction of flavonoid was carried out at a pressure of 30 MPa and temperatures from 80 to 160°C. The percent ratio of water in ethanol as a co-solvent was varied from 0 to 100%. Flow rates of CO<sub>2</sub> and co-solvent were also fixed at 3.0 and 0.2 mL/min, respectively.

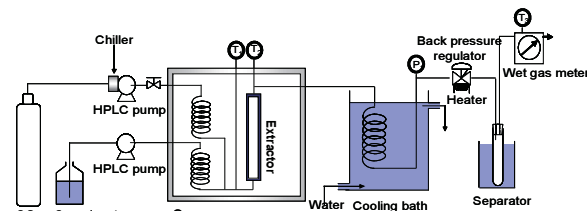


Fig. 2. Schematic diagram of SC-CO<sub>2</sub> extraction apparatus with line for co-solvent

### 2.5. Statistical analysis

All extraction experiments were carried out in duplicated. HPLC analysis of each fraction obtained in each run was also carried out in duplicated. Analysis of variance (ANOVA) was carried out using Excel Statistics 2004 to analyze the effect of temperature and pressure on the total yield of extract and composition of essential oil extract. The significance level was stated 95% with p-value <0.05.

## 3. RESULT AND DISCUSSION

### 3.1. Identification and quantitation

The flavonoids in raw material were identified by comparison with commercial standards. Fig.3 shows HPLC chromatogram of flavonoids obtained by typical SC-CO<sub>2</sub> with co-solvent extract and conventional organic solvent extract.

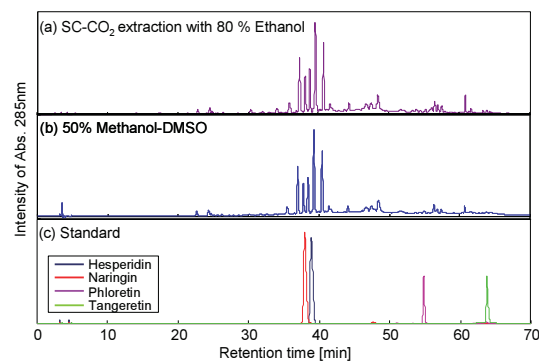


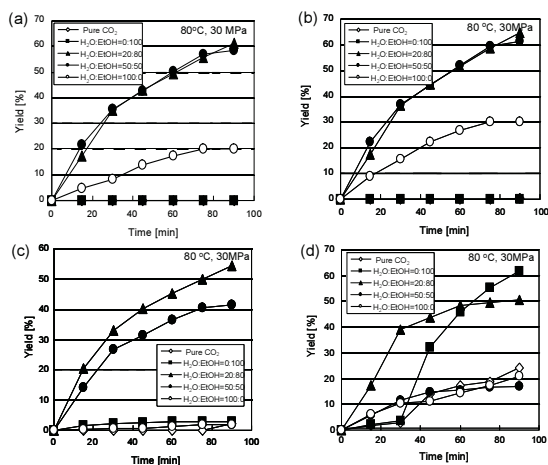
Fig.3. HPLC chromatograms of extract for SC-CO<sub>2</sub> with co-solvent (a) and conventional organic solvent (b) and standard substances (c)

The closely matched spectra and retention times confirmed naringin, hesperidin, phloretin and tangeretin as important components in raw material, and application of the peak purity software to the diode array data indicated no impurities present in any chromatographic peaks of interest.

### 3.2. Effect of water content on flavonoid extraction

To study the effect of changing polarity of extracting solvent, SC-CO<sub>2</sub> extraction of flavonoid was carried out using co-solvent with various water contents in ethanol. Fig.4 shows the effect of water content on SC-CO<sub>2</sub>

extraction of various flavonoids.

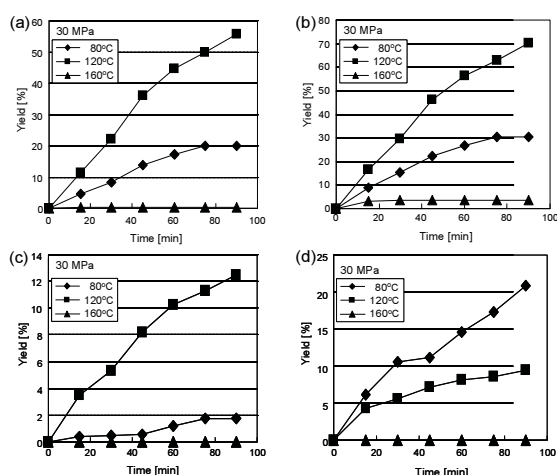


**Fig.4.** Effect of water content on SC-CO<sub>2</sub> extraction of various flavonoids. (a)Naringin, (b)Hesperidin, (c)Phloretin and (d)Tangeretin.

Naringin and hesperidin were well extracted when water was added in ethanol. It was thought that naringin and hesperidin has higher polarity than other flavonoid due to having sugars in its structure. The yield of phloretin also increased with the addition of water. However, the increase of water content from 20 to 50% decreased the yield by about 15%. The yield of tangeretin tended to increase with ethanol concentration. It was thought that the yield of tangeretin was related to low polarity derived from having many methyl groups. These results showed selective extraction of flavonoids was possible by manipulating the ratio of water and ethanol.

### 3.3. Effect of temperature on flavonoid extraction

Fig.5 shows the effect of temperature on extraction behavior of flavonoids using SC-CO<sub>2</sub> with only water as co-solvent.



**Fig. 5.** Effect of temperature on the yield of various flavonoids in SC-CO<sub>2</sub> extraction with water. (a)Naringin, (b)Hesperidin, (c)Phloretin, (d)Tangeretin

The extraction of naringin and hesperidin was enhanced by rise in temperature. It was indicated that flavonoid glycosides such as naringin and hesperidin extracted by

using only green solvent. However extraction efficiency of flavonoids dramatically decreased possibly due to partial degradation of flavonoid structure at the highest temperature of 160°C

## 4. CONCLUSIONS

Citrus peel includes many valuable resources. However, most of that have been disposed of without being used. In particular, flavonoids contained in the peel are known as a high value-added component. SC-CO<sub>2</sub> was used as a solvent to extract the flavonoids from *citrus junos* peel. Flavonoids such as naringin and hesperidin have glycoside group, thus water was a good co-solvent for extraction of these compounds. This method can be an effective means to recover the solvent free extracts.

These results showed that it is possible to extract flavonoids selectively by changing the ratio of water and ethanol.

## Acknowledgement

This work was supported by Kumamoto University Global COE Program "Global Initiative Center for Pulsed Power Engineering".

## References

- [1] M. Hoshino, Utilization of Japanese citrus resource and development of high concentration vinegar fermentation method. PhD Thesis, Kumamoto university (2007)
- [2] S. Kanno, A. Shouji, K. Asou, and M. Ishikawa, *J. Pharm. Sci.*, **92**, 166–170 (2003)
- [3] T. Kanda, H. Akiyama, A. Yanagida, M. Tanabe, Y. Goda, M. Toyoda, R. Teshima, and Y. Saito, *Biosci. Biotechnol. Biochem.*, **62**(7), 1248-1289 (1998)
- [4] K. Masuko, H. Shinmoto, T. Tsushida, K. Shinohara, Phloretin-induced apoptosis in B 16 melanoma 4A5 cells by inhibition of glucose transmembrane transport, *Cancer Letters*, **119**, 207-212 (1997)
- [5] J. Rodriguez, J. Yanez, V. Vincent, M. Alcaraz, O. Benavente-Garcia, J. Castillo, J. Lorente, and J.A. Lozano, *Melanoma Res.*, **12**, 99-107 (2002)
- [6] R.J. Braddock, Handbook of Citrus By-products and Processing Technology, Willy Inter Science, New York, 1999.
- [7] H. Ueno, M. Tanaka, M. Hoshino, M. Sasaki, and M. Goto, Extraction of valuable compounds from the flavedo of *Citrus junos* using subcritical water, *Sep. Pur. Tech.* **62**, 513-516 (2008)

(Received May 29, 2014; Accepted July 8, 2014)