

Functional Ingredients Extraction from Citrus *Genkou* by Supercritical Carbon Dioxide

Yukihiro Kawamoto^{1,2}, Yuriko Suidou^{2,3}, Takuya Suetsugu², Arata Takamizu², Masahiro Tanaka², Munehiro Hoshino², Wahyudiono¹, Hideki Kanda¹ and Motonobu Goto^{1,*}

¹ Department of Chemical Engineering, Nagoya University
Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan

² Maruboshi Vinegar·Ascii, Food Technology and Biology of Technical Center (M. A. F. T)
2400 Tabara, Kawasaki-machi, Tagawagun, Fukuoka 827-0004, Japan

³ Department of Applied Chemistry, Chemical Engineering and Biomolecular Engineering
Tohoku University, Sendai, 980-8578, Japan

* Corresponding author's email: mgoto [AT] nuce.nagoya-u.ac.jp

ABSTRACT— *Citrus genkou* fruit was known as one of common sources for juice production. As residues, the citrus *genkou* peels has been discarded without any treatments. In this work, the citrus *genkou* peels were subjected as a starting material to extract flavonoids by using supercritical carbon dioxide (SC-CO₂). The extractions were carried out at temperatures of 40 – 80 °C and pressures of 10 – 30 MPa. The high-performance liquid chromatography analysis showed that hesperidin, nobiletin, and tangeretin were extracted from wet citrus *genkou* peels. The results showed that the extraction by using SC-CO₂ could be accomplished without compromising the organoleptic characteristics because SC-CO₂ could be directly applied into matrix without any treatment.

Keywords— Citrus *genkou*, Flavonoid, Supercritical carbon dioxide, Hesperidin, Nobiletin, Tangeretin

1. INTRODUCTION

Only three trees of citrus *genkou* are grown wild in Madara Island of Saga prefecture, Japan, and were found in October 2005. The local fruit, citrus *genkou* has sour flesh which has been used as seasoning agent and a lotion to get smooth skin and whitening effect by some islanders. Therefore, functional ingredients such as citrus flavonoids can be expected in citrus *genkou* as in many other citrus fruits. Most of the fruit was applied to juice processing and then its peel has been disposed without any usage. The juice processing waste contains abundant bioactive compounds such as flavonoids.

Flavonoids belong to the group of polyphenol compounds and are widely distributed in plants. Flavonoids have been shown to have a wide range of biological and pharmacological activities. For example, typical citrus flavonoids such as hesperidin and naringin were reported to prevent progress of hyperglycemia [1,2]. Nobiletin a citrus polymethoxylated flavone consisted in aged mandarin orange peel (a typical Chinese traditional drug), was found to treat Alzheimer's disease and vascular dementia [3,4]. Another polymethoxylated flavone, tangeretin, induces apoptosis in human promyelocytic leukaemia HL-60 cells, whereas the flavone showed no cytotoxicity against human peripheral blood mononuclear cells (PBMCs) [5,6]. Tangeretin also has been shown to inhibit the proliferation of a number of cancer cell lines [6,7].

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 [8]. 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 is harmless, and present widely in nature. Therefore, supercritical carbon dioxide (SC-CO₂) often has been applied to extract weakly polar and non-polar compounds from various plant materials [9,10]. In this study, peel of citrus *genkou* was used as a raw material to extract the functional ingredients using SC-CO₂.

2. EXPERIMENTAL SECTION

2.1 Materials

Citrus *genkou* fruits were harvested in Saga prefecture, Japan. The content of water in citrus *genkou* peels was around 80%. Prior to extraction, the citrus *genkou* peels were sliced into certain size. They were then refrigerated at < 278 K. Naringin (99.0%), hesperidin (95.0%), neohesperidin (98.0%), hesperetin (96.0%), phloretin (98.0%), nobiletin (95.0%), tangeretin (95.0%), methanol (99.7%), and dimethyl sulfoxide (DMSO, 99.0%) were purchased from Wako Pure Chem. Ind., Ltd (Osaka, Japan).

2.2 Solvent Extraction

In this extraction, 10 g of citrus *genkou* peels was introduced with mixtures of methanol-DMSO solution (methanol/DMSO = 1/1, v/v) to determine the total amount of flavonoids. The citrus *genkou* peels were ground in a coffee grinder and dried at 70 °C for 24 h. Then, they were loaded in the methanol-DMSO solution for 24 h at room temperature [11]. After extraction process, the extract was transferred to bottle that covered with aluminium foil and refrigerated until analysis. The amount of flavonoids in the extract sample was calculated as follows:

$$\text{The total amount of flavonoids [-]} = \frac{\text{Amount of extracted flavonoids}}{\text{Amount of loaded citrus genkou fruit}} \quad (1)$$

By using this extraction technique, the total amount of hesperidin, nobiletin, and tangeretin could recovered at around 792.29, 64.71, and 50.09 mg/100 g sample, respectively.

2.3 Supercritical Carbon Dioxide Extraction

Figure 1 shows schematic diagram of SC-CO₂ extraction apparatus. In the flavonoids extraction, a 50 g portion of raw material was directly charged in the extractor (100 mL) without drying treatment. Liquid CO₂ from a cylinder with siphon attachment was passed through a chiller at -5 °C, and compressed CO₂ was flowed through a heating coil before entering the extractor placed in an oven that was maintained at the operating temperature. The pressure of extractor was controlled by back-pressure regulator (BPR) 1, while the pressure in the separator was controlled by BPR 2. After reach to the desired conditions, the supply of the carbon dioxide was stopped, and then the system was immediately depressurized to ambient condition. This process was repeated in five times, next, the extracts were collected in vials via separator. The SC-CO₂ extraction of flavonoid was carried out at the pressures of 10 – 30 MPa and temperatures of 40 – 80 °C.

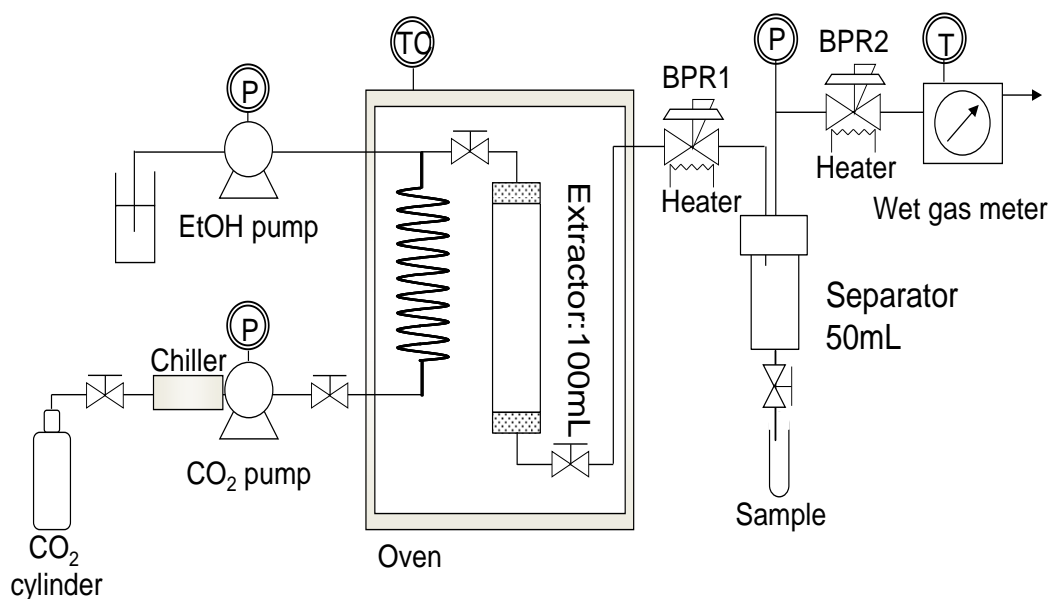


Figure 1: Schematic diagram of SCCO₂ extraction system.

The amount of extract was determined as follows:

$$\text{Recovery of extract [\%]} = \frac{\text{Weight of extract}}{\text{Weight of raw material}} \times 100 \quad (2)$$

$$\text{Yield of flavonoid [\%]} = \frac{\text{Extracted flavonoid}}{\text{Total extracted flavonoids}} \times 100 \quad (3)$$

2.4 Analytical Methods

The extracted flavonoids were analyzed using a high-performance liquid chromatography (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. 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). To determine the content of flavonoids, the wavelength was set 285 nm.

3. RESULTS AND DISCUSSION

In principle, extraction could be defined as the removal of soluble substances from an insoluble materials, either liquid or solid, by treatment with a liquid solvent. This might occur when the solvent is passed through the plants matter at high pressure and high temperature. Accordingly, the extraction could occur when the carbon dioxide was introduced into the plant materials at high temperatures and high pressures. Figure 2 showed the amount of extracts from citrus *genkou* peels at various extraction conditions. At these conditions, CO₂ possesses high diffusivity and low viscosity and therefore has ability to penetrate into citrus *genkou* peels to access the inner cells containing flavonoid substances. The high densities of CO₂ under supercritical conditions would contribute to greater solubilisation of citrus *genkou* peels substances [12]. Consequently, the amount of extracts increase clearly with increasing temperatures or pressures at a constant pressure or temperature. The amount of extract was around 7 % at 40 °C and 10 MPa, then it increased significantly to 24 % when the CO₂ pressure was elevated to 30 MPa at the same extraction temperature. This result is in agreement with those of other workers for plants matter components extraction by using SC-CO₂ [13–16].

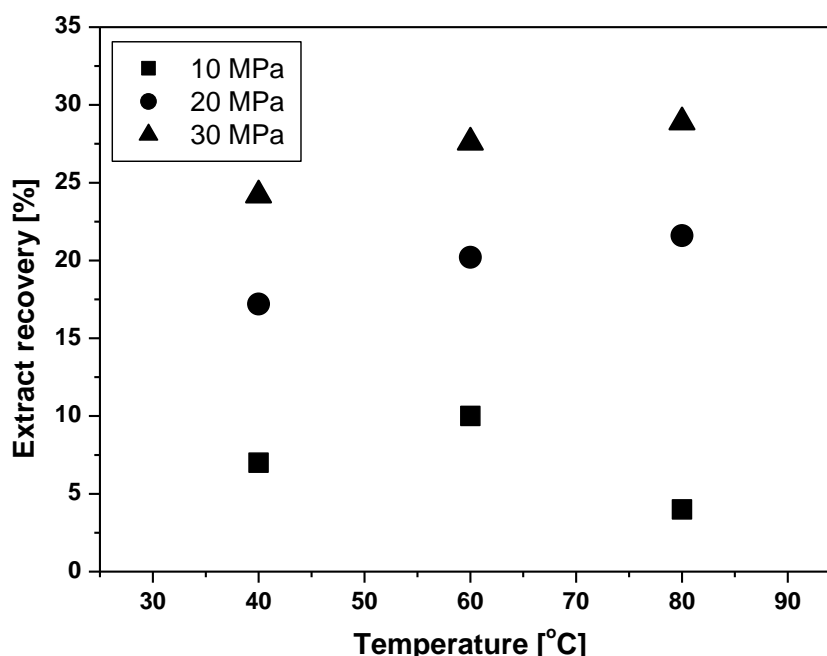


Figure 2: Extracts obtained from wet citrus *genkou* peels by SC-CO₂.

The same phenomenon was also obtained when the extraction processes were conducted at 60 and 80 °C. The maximum amount of extract was found when the extraction process was carried out at 80 °C and 30 MPa. This figure also described the effect of extraction temperature on the amount of citrus *genkou* peels extract. It increased with an increase in temperature at the same extraction pressures. This indicated that the solute vapor pressure had high influence on the extraction process which increased with increasing extraction temperature. It was well known that the water solubility in CO₂ increases with temperatures due to the increase in vapor pressure [17]. Hence, the high moisture content of citrus

genkou peels (around 80%) seemed to have an effect on the amount of citrus *genkou* peels extract. It should be noted that the various moisture content of citrus *genkou* peels was not modified during the course of an experiment. In addition, the elevating extraction temperature may assist to the disruption of cell walls, and as a result the removable of citrus *genkou* peels substances availability for extraction was increased [13,14].

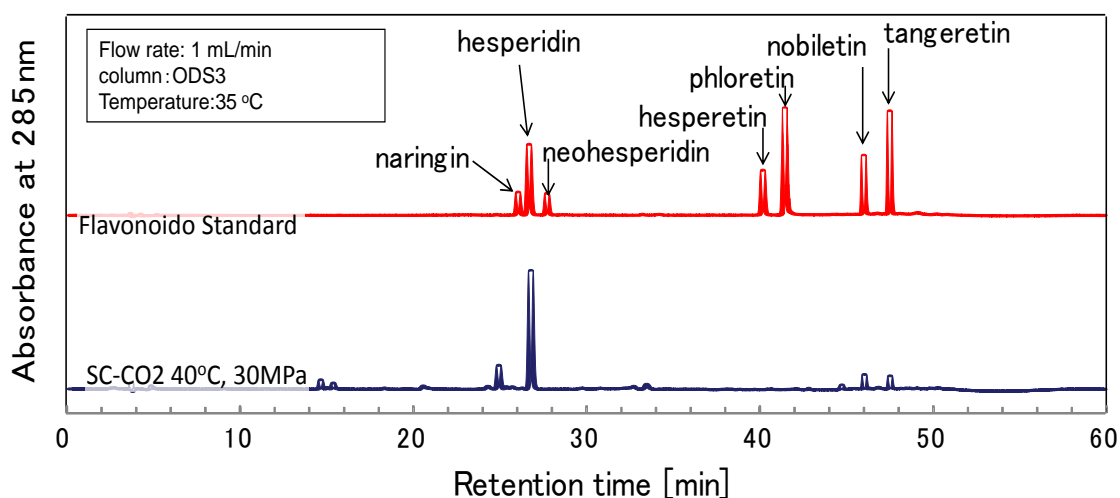


Figure 3: HPLC chromatograms of extracted compounds.

As informed before that during SC–CO₂ extraction process, the penetration of CO₂ into cell wall of citrus *genkou* peels occurred, allowing the removal of its constituents in through solubilisation. Figure 3 showed the HPLC chromatograms of extract citrus *genkou* peels by SC–CO₂ and standard substances. As shown in Fig. 3, there are many flavonoid compounds could be extracted from citrus *genkou* peels. However, in this work, hesperidin, nobiletin, and tangeretin were subjected as the target of flavonoid compounds recovery from citrus *genkou* peels, and determined quantitatively by using HPLC. The citrus *genkou* peels components in extracts were recovered with 2 mL of ethanol, and all the solutions were filtered using a disposable filter of 0.45 µm pore size prior to HPLC analysis. Initially, the pure compound of hesperidin or nobiletin or tangeretin diluted in ethanol as a standard was introduced in the HPLC system to create calibration curve in 5 point. Next, the amount of each standard leaving the HPLC column will assign the intensity of the signal generated in the detector. By comparing the time it takes for the peak to show up (the retention time) with the retention times for the standard, the amount of hesperidin or nobiletin or tangeretin in the extract can be quantified. This analysis can be conducted with good precision; therefore, other techniques analysis was not performed.

Figure 4 showed the yields of hesperidin, nobiletin, and tangeretin at various extraction conditions. Generally, increasing temperature and/or pressure in SC–CO₂ extraction system gave a significant effect on the extraction yield of substances. Apparently, the evolution of hesperidin, nobiletin or tangeretin also increases gradually or remains constant or decreases slightly, depending on extraction conditions. This indicated that the change of extraction temperature and/or pressure had influence on the removal of hesperidin, nobiletin, and tangeretin from citrus *genkou* peels. The highest yields of them were reached when the extraction processes were conducted at higher pressure (30 MPa). However, as shown in Figure 4(a), the accumulated yield of hesperidin was still less than 2%. It seemed hard to extract hesperidin from citrus *genkou* peels by using SC–CO₂ due to its polarity which resulted in a low solubility in SC–CO₂. The change of extraction parameters did not give effects on the extraction process, in other words it could be said that pure CO₂ was not good as a solvent to extract hesperidin. Usually, solvent modifiers including water were applied as entrainer in the SC–CO₂ extraction system to enhance the solubility of polar compounds in SC–CO₂ [18–21]. This is, of course, a positive effect in terms of extraction substances from plant matters by using SC–CO₂ because water may have a beneficial effect by swelling the plant matters, thus making it easier for the SC–CO₂ to penetrate and diffuse into the matrix. Jinap *et al.* [20] informed that the water addition might improve the supercritical extraction of caffeine from coffee and moisture content between 3 % and 12 % has an effect on extractability of oils from seeds with SC–CO₂. Nevertheless, in this work, the extraction result did not show this beneficial effect on the hesperidin extraction from citrus *genkou* peels with high moisture content. Even, Durante *et al.* [21] reported that the presence of water in plant matters can make difficult the extraction of certain compounds. They explained that the high water content of many plant matters is detrimental to the efficient extraction of nutraceuticals with SC–CO₂. Water may hinder the fluid flow through the plant matters decreasing the contact surface between solutes and solvent and increasing the diffusion resistance of solutes.

In the case of nobiletin and tangeretin yields, the high water content in citrus *genkou* peels looks like to have positive effect in this extraction technique. The yields of nobiletin and tangeretin could approach to 18% and 12% when the

extraction processes were conducted at pressure of 30 MPa with extraction temperatures of 60 and 80 °C, respectively. At these conditions, there was possibility that the higher water content in citrus *genkou* peels could enhance the polarity of SC-CO₂ via incorporating of water-CO₂. Lee *et al.* [22] reported that the yield of nobiletin and tangeretin from *Citrus depressa* Hayata increased with the increasing percentage of water in ethanol up to 15% when they conducted SC-CO₂ extraction with ethanol as a modifier. They explained that the higher water in modifier led excessive polarity of the supercritical solvent which was not suitable for nobiletin and tangeretin extraction; next, the yield decreased. Liu *et al.* [19] also performed SC-CO₂ extraction of flavonoids from *Maydis stigma* with various water content. They informed that the higher water content is associated with increased polarity of the modifier, which enables the polar flavonoids in the *Maydis stigma* to be extracted more readily. Similar to Lee *et al.* [22], they also explained that the excessive polarity of the SC-CO₂ was not suitable for extraction when the water content in the co-solvent was more than 20%. Besides, the low yields of hesperidin, nobiletin, and tangeretin at high water content in citrus *genkou* peels may be partially due to the high water content resulting in transport difficulties from matrix (plugging of matrix pores) or the limit of solubility of water in the SC-CO₂ leading in the increase of mass transfer limitation [19,21,22].

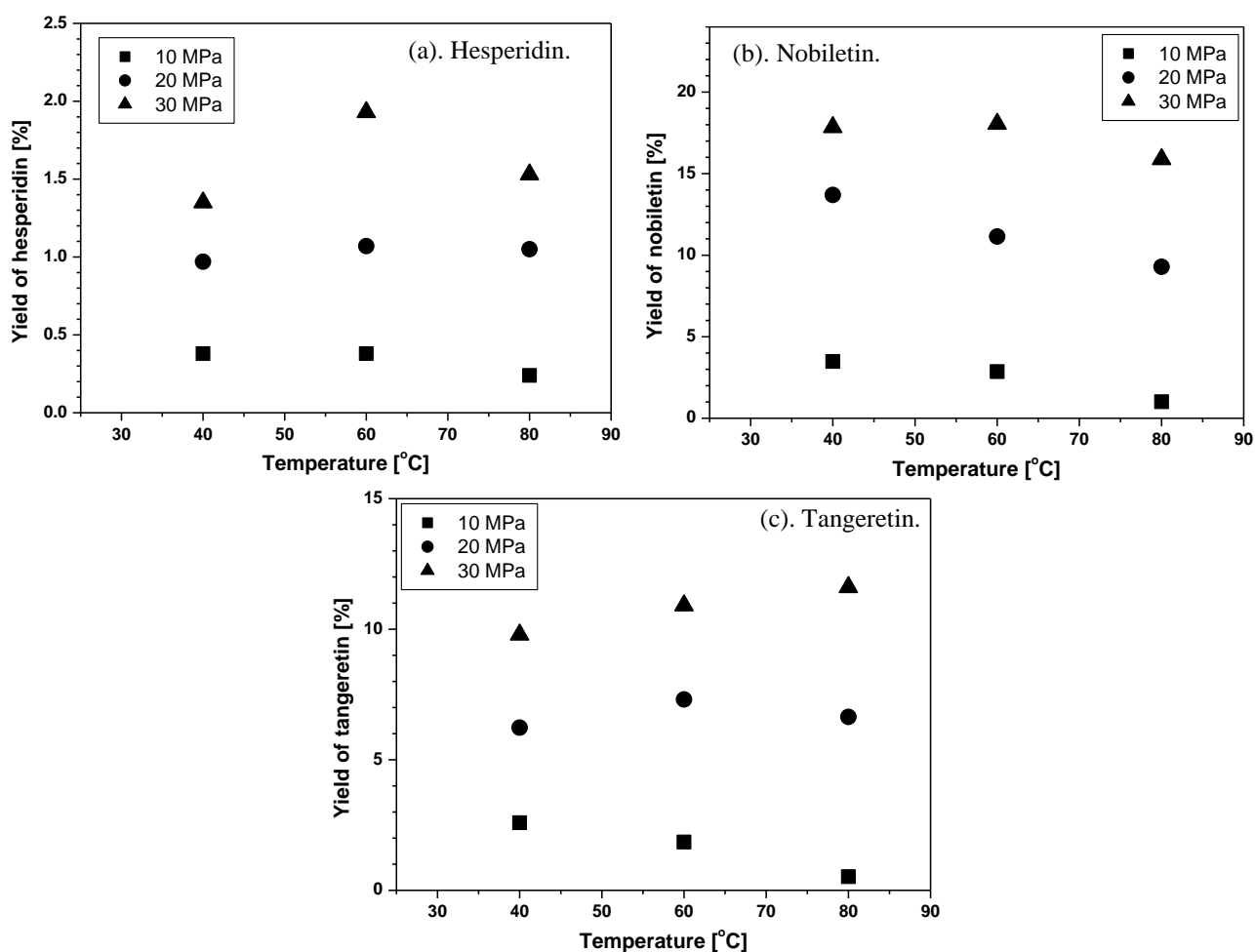


Figure 4: Yields of hesperidin (a), nobiletin (b), and tangeretin (c), respectively.

4. CONCLUSIONS

Supercritical CO₂ extraction of flavonoids from citrus *genkou* peels has been performed at temperatures of 40 – 80 °C and pressures of 10 – 30 MPa. The wet citrus *genkou* peels was contacted with SC-CO₂ directly without any pre-treatment and immediately depressurized. At supercritical conditions, CO₂ has ability to penetrate into citrus *genkou* peels to access flavonoid substances. The result showed that the moisture content seemed to have an effect on the amount of citrus *genkou* peels extract. As flavonoid extracted compounds, the maximum yields of hesperidin, nobiletin, and tangeretin were around 2, 18, and 12 %, respectively. Based on the results, it could be said that the SC-CO₂ was successful applied on high moisture plant matters where extraction could be accomplished without compromising the organoleptic characteristics.

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