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Subcritical Water Extraction of Nutraceutical Compounds from Citrus Pomaces

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Abstract: Subcritical water (SCW) extraction of citrus pomaces (CPs) was carried out, and antioxidant activity and nutraceutical compound levels of the SCW extracts were evaluated in detail. At first, CP samples were subjected to the SCW extraction under various conditions focusing on the extraction temperature and time. Consequently, the highest total phenol contents, radical scavenging activity, and reducing power were found when extraction was carried at 200°C and 1.4 MPa for 60 min. Furthermore, the amounts of three kinds of polymethoxylated flavones (sinensetin, nobiletin, and tangeretin) in the SCW extracts also showed the highest values under this condition. These results indicate that SCW is an effective medium for the fast and highly efficient extraction of the antioxidants and nutraceutical compounds from CPs.

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Keywords: Antioxidant activity, citrus pomaces, polymethoxylated flavones, subcritical water extraction

INTRODUCTION

Citrus is an important crop with production world-wide estimated at 80 million tons per years (1). The main uses of citrus in food industries include fresh juice or citrus-based drinks. Since the juice yield of citrus (mainly orange and grapefruit) is less than half of the fruit weight, numerous amounts of byproduct wastes such as peels and pomaces are obtained every year (2). Citrus byproducts have conventionally been valorized as molasses for animal feed (1), fiber (pectin) production (3,4), and fuel production (5). Recently, a number of studies have proposed that some fruit or vegetable byproducts could be a source of natural antioxidants (1,2,5,6).

In South Korea, more than 60 thousand tons of citrus pomaces (CPs) are annually produced after processing of citrus fruits. CPs have been used as a source for molasses, pectin, cold-pressed oils, and limonene (7). CPs have also been widely studied, because they contain a variety of biologically active compounds including natural antioxidants such as phenolic acids and flavonoids (1,8).

Many valuable natural materials have traditionally been extracted with organic solvents. However, the preparation methods are time-consuming and some of the solvents are toxic (9,10). The subcritical water (SCW) extraction is the technique carried out by using hot water at temperatures between 100 and 374°C under high pressure to maintain its liquid state (critical point of water, 22.4 MPa and 374°C). SCW has unique characteristics such as high density, high reactivity, and good solubility for a series of organic compounds having relatively low molecular weights. Based on these features, SCW can be used as an extractant instead of organic solvent for its environmentally friendly characteristics and high catalytic activity. Several attempts at extracting valuable materials with SCW from bio-resources have been reported. For example, SCW was used to extract nutraceuticals with antioxidant activities from oregano (11), flavor compounds from rosemary (12), and protein and amino acids from rice bran (13). In this study, we tried to find an effective and practical technique to extract antioxidants and bioactive compounds from CPs by using the SCW process. Concretely, we prepared SCW extracts of CPs at five different temperatures of 25, 100, 150, 200, and 250°C (0.1 to 5.0 MPa) for three different time periods of 10, 30, and 60 min, and evaluated the antioxidant activities and polymethoxylated flavones (PMFs) of the extracts.

EXPERIMENTAL

Materials and Reagents

Citrus pomaces (CPs), produced after pressing citrus (*Citrus unshiu*) fruit for citrus juice, were kindly supplied by Jeju Samdasoo Citrus Co. of Jeju Special Self-Governing Province Development Corporation (Jeju, Korea). CPs were freeze-dried, and finely ground using a homogenizer (MC-811C, Novita, Korea) to pass through a 48-mesh sieve. The CP powder sample was stored at 4°C for further experiments. Tannic acid, 2,2-dephenyl-1-picrylhydrazyl (DPPH), ferric chloride, potassium ferricyanide, trichloroacetic acid, methanol, acetonitrile (HPLC grade) were purchased from Sigma Chemical Co. (St. Louis, MO). Folin-Ciocalteau reagent and sodium carbonate were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sinensetin, nobiletin, and tangeretin were purchased from Extrasynthese Co. (Genay Cedex, France).

Extraction by Subcritical Water

Subcritical water (SCW) extraction was conducted by using a stainless steel vessel (Swagelok Co., Solon, OH, USA) with resistivity to high pressure and temperature. CP (0.1 g) and 10 ml of distilled water were placed in the vessel of an inner volume of 18.57 ml. After being tightly closed, the vessel was placed in an HP 5890 gas chromatograph oven (Hewlett-Packard, Wilmington, DE). The extraction was performed at the temperatures from 25 to 250°C (0.1 to 5.0 MPa) for 10, 30, or 60 min, respectively. After the desired conditions were achieved, the vessel was immediately taken out from the oven and cooled to room temperature. Then the extracts were centrifuged at 3,000 × *g* for 20 min, and the supernatants were filtered through a Whatman No. 1 filter paper. The extract was saturated with nitrogen gas and stored at 4°C until further experiments.

Total Phenolic Contents (TPC)

TPC of the CP extract was determined according to the method of Gutfinger (14). One ml of the CP extract was mixed with 1 ml of 2% Na_2CO_3. After 3 min, 0.2 ml of a 50% Folin-Ciocalteu reagent was added. After 30 min of standing, the mixture was centrifuged at 13,400 × *g* for 5 min. The absorbance of the resulting supernatant was measured with a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) at 750 nm. The TPC were expressed as gallic acid equivalents.

DPPH Radical Scavenging Activity

Antioxidant activity of the CP extract was determined by radical scavenging activity (RSA) (15). After mixing 0.1 ml of the CP extract with 0.9 ml of 0.041 mM DPPH radical in ethanol for 10 min, the absorbance of the sample was measured at 517 nm. The RSA was expressed as percentage according to the following formula: % DPPH RSA = (1 – sample OD/control OD) $\times$ 100.

Reducing Power

The reducing power of the CP extract was determined according to the method of Meir et al. (16). The CP extract (1 ml, 1 mg/ml), phosphate buffer (1 ml, 0.2 M, pH 6.6), and potassium ferricyanide solution (1.0 ml, 10 mg/ml) were mixed and incubated at 50°C for 20 min. A trichloroacetic acid solution (1.0 ml, 100 mg/ml) was added to the mixture and centrifuged at 13,400 $\times$ *g* for 5 min. The resulting supernatant (1.0 ml) was mixed with distilled water (1.0 ml) and a ferric chloride solution (0.1 ml, 1.0 mg/ml), and then the absorbance was measured at 700 nm.

Analyses of Polymethoxylated Flavones

Polymethoxylated flavones (PMFs) in the CP extract were determined by HPLC. The HPLC system was consisted of Shimadzu LC-10ATVP pumps (Shimadzu Co. Ltd., Kyoto, Japan), a Shimadzu SCL–10AVP integrated system controller, a Shimadzu SPD-10AVP UV–Vis detector, and a Shimadzu CTO 10ASVP column oven. The column was a Shim-pack VP ODS column (5 µm, 250 $\times$ 4.6 mm, Shimadzu Co. Ltd.). Mobile phases were consisted of water (solvent A) and methanol (solvent B) and 0.1% acetonitrile (v/v) (solvent C). The solvent composition started at 100% solvent A. The gradient was as follows: 10 min, A-86%, B-7%, C-7%; 25 min, A-80%, B-10%, C-10%; 35 min, A-75%, B-10%, C-15%; 42 min, A-0%, B-30%, C-70%; 50 min, A-0%, B-0%, C-100%. Elution was performed at a solvent flow rate of 0.7 ml/min. Detection was accomplished with a UV–Vis detector, and chromatograms were recorded at 325 nm. The column was maintained at 60°C. The sample injection volume was 10 µl. Peaks were identified by comparing their retention times with authentic standards.

Statistical Analyses

All measurements were performed in triplicate, and analyses of variance were conducted by the General Linear Model procedure using SAS software (17). Student-Newman-Keul's multiple range tests were used to test for significant differences between the mean values for the treatments ($P < 0.05$).

RESULTS AND DISCUSSION

Total Phenolic Contents (TPC)

Phenolic compounds or polyphenols are ubiquitous in plants with more than 8,000 structures reported. It has been reported that phenolic compounds have antioxidant, antimutagenic, and free-radical scavenging activities. Antioxidant mechanisms of polyphenolic compounds are based on hydrogen donation abilities and chelating metal ions. After donating a hydrogen atom, phenolic compounds become resonance-stabilized radicals, which do not easily participate in other radical reactions (18).

Citrus fruit also contained a wide range of phenolic compounds such as cinnamic acid derivatives, coumarines, and flavonoids, which occur in the free form and/or as glycosides. The TPC in the SCW extracts of CP significantly increased with increasing temperature and time (Table 1). The maximum TPC (2974.7 μM) was achieved when the CP were extracted at 200°C and 1.4 MPa for 60 min. Our previous study also showed that phenolic compounds in citrus peel (19) and grape seeds (20) could be liberated by a simple heat treatment. For example, water extract from heat-treated citrus peels at 150°C for 60 min increased TPC from 84.4 to 204.9 μM (19). SCW extraction at high temperature also increased the phenolic contents of oregano leaves (11) and rosemary

Table 1. Effect of subcritical water on total phenolic contents from citrus pomaces (Unit: μM)

	Time (min)			
Temperature (°C)	0	10	30	60
25	830.4^{bw}	1077.8^{cz}	1046.4^{dy}	1007.3^{dx}
100	830.7^{bw}	1048.4^{dy}	956.3^{ex}	1252.2^{cz}
150	830.7^{bw}	1024.9^{ex}	1279.6^{cy}	2145.8^{bz}
200	831.9^{aw}	1134.6^{bx}	2590.6^{ay}	2974.7^{az}
250	828.7^{cw}	2841.5^{az}	1712.7^{by}	186.2^{ex}

$^{a\text{–}e}$Different letters within a row are significantly different ($p < 0.05$), $n = 3$.

$^{w\text{–}z}$Different letters within each extract are significantly different ($p < 0.05$), $n = 3$.

(12). This indicates that phenolic compounds in plants can be liberated by heating processes, and that SCW is an promising processing method for the extraction of phenolics from CP.

DPPH Radical Scavenging Activity (RSA)

It is generally agreed that the oxidation is initiated by free radical attack; therefore, assays to evaluate the radical scavenging activity are representative of the potential of a compound to retard oxidation. DPPH is a stable free radical that can accept an electron and hydrogen radical to become a stable diamagnetic molecule. Among the radical scavenging assays, the utilization of DPPH radical was chosen due to its simplicity and worldwide acceptance for comparative purpose. The free RSA of the CP extracts were investigated by DPPH RSA assay. The DPPH RSA of the SCW extracts from the CP also significantly increased with increasing the temperature and the treatment period (Table 2). The CP extracts at 200°C for more than 10 min showed over 95% of DPPH RSA. SCW extraction was able to obtain antioxidant compounds from rosemary (12) and licorice roots (21). In the case of licorice roots, DPPH RSA of SCW extract at 200°C for 60 min dramatically increased from 12.36 to 93.08% compared to that at 50°C for 10 min (21). Our results suggest that antioxidant compounds in CPs are also able to be effectively extracted with SCW.

Table 2. Effect of subcritical water on DPPH radical scavenging activity from citrus pomaces (Unit: %)

	Time (min)			
Temperature (°C)	0	10	30	60
25	80.8^{bw}	78.3^{ey}	76.1^{ex}	79.2^{dz}
100	80.2^{bw}	85.0^{cx}	90.1^{cy}	92.3^{bz}
150	81.0^{bw}	81.9^{dy}	80.0^{dx}	90.1^{cz}
200	81.7^{aw}	97.5^{ay}	98.0^{az}	95.2^{ax}
250	79.0^{cw}	85.6^{by}	96.1^{bz}	74.1^{ex}

$^{a–e}$Different letters within a row are significantly different ($p < 0.05$), $n = 3$.
$^{w–z}$Different letters within each extract are significantly different ($p < 0.05$), $n = 3$.

DPPH radical scavenging activity of vitamin C (a positive control) at concentration of 100 μg/ml was 85.1%.

Reducing Power

The reducing power of the SCW extracts was evaluated by measuring the reduction of Fe^{3+} as a form of ferricyanide complexes to ferrous ion. The reducing powers of the SCW extracts shown in Table 3 exhibit almost the same trend as that of the TPC, which increased with increasing extraction temperature and time. The highest RP of 0.311 was found in the SCW extracts carried out at 200°C for 60 min, which was more than 2 times that (0.145) of the extract generated at 25°C for 60 min. The above results of the TPC, DPPH, RSA, and RP analyses indicate that SCW is highly effective for increasing the antioxidant activities of CP extracts.

HPLC Analysis for PMFs

Citrus fruits are a rich source of flavonoids, which are secondary plant metabolites. Polymethoxylated flavones (PMFs) such as nobiletin, sinensetin, and tangeratin are the major active flavonoid compounds in citrus fruits (Fig. 1). Nobiletin and sinensetin are reported as a novel promising immunomodulatory and anti-inflammatory drug (22) and as an inhibitor to human mammary cancer cells (23). Tangeretin is a more potent inhibitor of tumor cell growth than free hydroxylated flavonoids, and also possesses potent anti-invasive and anti-metastatic activities (23,24). In addition to its hypoglycemic effect, PMFs has been shown to possess antioxidant properties such as scavenging reactive oxygen species, increasing superoxide dismutase (SOD) activity.

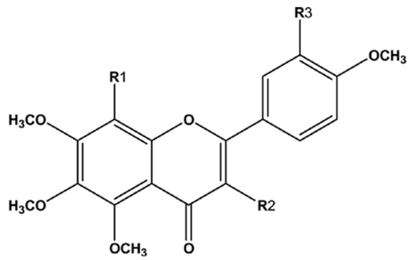
Table 3. Effect of subcritical water on reducing power from citrus pomaces (Unit: OD)

	Time (min)			
Temperature (°C)	0	10	30	60
25	0.113^{bw}	0.108^{ex}	0.124^{ey}	0.145^{ez}
100	0.114^{bw}	0.148^{cx}	0.153^{cy}	0.167^{cz}
150	0.114^{bw}	0.154^{by}	0.150^{dx}	0.260^{bz}
200	0.120^{aw}	0.110^{dx}	0.203^{ay}	0.311^{az}
250	0.117^{bw}	0.165^{az}	0.153^{by}	0.150^{dx}

$^{a\text{–}e}$Different letters within a row are significantly different ($p < 0.05$), $n = 3$.

$^{w\text{–}z}$Different letters within each extract are significantly different ($p < 0.05$), $n = 3$.

Reducing power of vitamin C (a positive control) at concentration of 100 µg/ml was 0.911 OD.



	R1	R2	R3
Nobiletin	OCH_3	H	OCH_3
Sinensetin	H	H	OCH_3
Tangeretin	OCH_3	H	H

Figure 1. Structure of main polymethoxylated flavones in citrus fruit.

The contents of PMFs in SCW extract of CPs are listed in Table 4. PMFs of the extracts were significantly affected by SCW extraction temperature and time. Sinensetin of SCW extract increased from 133.1 ppm in the extract generated at 25°C for 60 min to 1897.1 ppm in the extract generated at 200°C and 1.4 MPa for 60 min, which is greater than a 14-fold increase. Nobiletin and tangeretin of the extract also increased from 5381.5 and 1724.6 ppm at 25°C for 60 min to 8187.7 and 2317.9 ppm, respectively, in the extract at 200°C for 60 minm which are more than 1.5- and 1.3-fold increase, respectively. However, more vigorous condition such as 250°C decreased the amount of PMFs. A SCW extraction of antioxidant compounds from rosemary showed that flavonoids that were selectively extracted depend on SCW temperature (12), and active compounds such as glycyrrhetic acid, glycyrrhizin, and liquiritin of SCW extracts of licorice roots showed increasing independent behavior (21). These results strongly support that polyphenolic compounds including flavonoids in plants present in different bound forms depend on species and compounds, and could be liberated by appropriate treatment. Thus, effective processing methods for increasing specific bioactive compounds from bioresouces might be specified.

CONCLUSION

Subcritical water has become of great interest as an alternative solvent for the extraction of natural active compounds. In this study, subcritical water extraction was applied to fast recovery of phenolic compounds having antioxidant activities from citrus pomaces. At first, the extraction

Table 4. Effect of subcritical water on polymethoxylated flavones contents of methanol extracts from citrus pomaces (Unit: ppm)

	Time (min)		
Temperature (°C)	10	30	60
Sinensetin			
25	143.5^{bz}	70.2^{ex}	133.1^{ey}
100	143.2^{by}	110.0^{dx}	1184.9^{cz}
150	190.0^{ay}	149.6^{cx}	1770.3^{bz}
200	190.4^{ay}	467.0^{ay}	1897.1^{az}
250	41.4^{ex}	183.3^{by}	864.9^{dz}
Nobiletin			
25	391.9^{bx}	445.0^{ey}	5381.5^{dz}
100	386.0^{bx}	772.4^{dy}	6072.5^{cz}
150	650.7^{ax}	1100.1^{cy}	7681.1^{bz}
200	657.0^{ax}	3306.3^{ay}	8187.7^{az}
250	659.5^{ax}	2762.8^{by}	4618.8^{ez}
Tangeretin			
25	93.6^{bx}	191.0^{by}	1724.6^{cz}
100	94.1^{bx}	172.8^{cy}	1502.7^{dz}
150	122.7^{ax}	155.1^{ay}	2094.9^{az}
200	124.0^{ax}	436.2^{ay}	2317.9^{bz}
250	13.8^{ex}	38.8^{ey}	736.4^{ez}

$^{a–e}$Different letters within a row are significantly different ($p < 0.05$), $n = 3$.

$^{x–z}$Different letters within each extract are significantly different ($p < 0.05$), $n = 3$.

conditions such as temperature and time were examined in detail. As a result, the extraction procedure at 200°C for 60 min under 1.4 MPa yielded the polymethoxylated flavones in the highest concentrations from citrus pomaces. Furthermore, the extracts obtained under this condition showed the highest total phenolic contents, DPPH radical scavenging activity, and reducing power. These results indicated that subcritical water process could be used as a method for extracting a large amount of nutraceutical compounds having high antioxidant activities from citrus pomaces.

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