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Effect of Heat Treatment on the Antioxidant Activity of Extracts from Citrus Peels

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The effect of heat treatment on the antioxidant activity of extracts from *Citrus unshiu* peels was evaluated. Citrus peels (CP) (5 g) were placed in Pyrex Petri dishes (8.0 cm diameter) and heat-treated at 50, 100, or 150 °C for 10, 20, 30, 40, 50, and 60 min in an electric muffle furnace. After heat treatment, 70% ethanol extract (EE) and water extract (WE) (0.1 g/10 mL) of CP were prepared, and total phenol contents (TPC), radical scavenging activity (RSA), and reducing power of the extracts were determined. The antioxidant activities of CP extracts increased as heating temperature increased. For example, heat treatment of CP at 150 °C for 60 min increased the TPC, RSA, and reducing power of EE from 71.8 to 171.0 μM, from 29.64 to 64.25%, and from 0.45 to 0.82, respectively, compared to non-heat-treated control. In the case of WE from CP heat-treated at the same conditions (150 °C for 60 min), the TPC, RSA, and reducing power also increased from 84.4 to 204.9 μM, from 15.81 to 58.26%, and from 0.27 to 0.96, respectively. Several low molecular weight phenolic compounds such as 2,3-diacetyl-1-phenylnaphthalene, ferulic acid, *p*-hydroxybenzaldoxime, 5-hydroxyvaleric acid, 2,3-diacetyl-1-phenylnaphthalene, and vanillic acid were newly formed in the CP heated at 150 °C for 30 min. These results indicated that the antioxidant activity of CP extracts was significantly affected by heating temperature and duration of treatment on CP and that the heating process can be used as a tool for increasing the antioxidant activity of CP.

KEYWORDS: Citrus peels; extracts; heat treatment; antioxidant activity

INTRODUCTION

Synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, and tertiary butylhydroquinone, have been widely used in foods for preventing oxidation. However, the use of these synthetic antioxidants in foods is discouraged because of their toxicity (*1*) and carcinogenicity (*2*). A few natural antioxidants have attracted special interest because they can remove free radicals, which may cause various diseases, carcinogenesis, and aging (*3*). Natural antioxidants such as flavonoids, tannins, coumarins, curcuminoids, xanthons, phenolics, and terpenoids are found in various plant products such as fruits, leaves, seeds, and oils (*4*), and some of these are as effective as synthetic antioxidants in model systems (*5*, *6*).

Citrus fruits contain sugar, organic acids, and a number of physiologically functional components such as citric acid, ascorbic acid, minerals, coumarins, and flavonoids such as naringin, hesperidin, neohesperidin, rutin, naringenin, hesperetin, nairutin, and tangeretin (*7*, *8*). Citrus peels (CPs) are the primary waste fraction of citrus fruits and have been used as a source for molasses, pectin, cold-pressed oils, and limonene (*9*). CPs have been also widely studied because they contain numerous biologically active compounds including natural antioxidants such as phenolic acids and flavonoids (*10*−*12*).

Many antioxidative phenolic compounds in plants, however, are most frequently present as a covalently bound form with insoluble polymer (*13*, *14*). To obtain natural antioxidants from plants, it is necessary to find an effective processing method to liberate them (*15*, *16*). Several methods such as heat treatment, far-infrared (FIR) radiation, fermentation, and protease treatment have been studied to liberate and activate low molecular weight natural antioxidants (*17*−*20*).

The objective of this research was to elucidate the relationship between heating temperature and time on the antioxidant activity of ethanol (EE) or water extracts (WE) prepared from CPs.

MATERIALS AND METHODS

Materials. *Citrus unshiu* fruits were purchased from a local market in Masan City, Korea, and divided into peel and edible parts. Peels were stored at 4 °C until used. Tannic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO), and Folin−Ciocalteu reagent was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were all of analytical grade and used as received.

Heat Treatment. CPs were dried under room temperature and finely ground using a blender (MC-811C, Novita). CP powder (5 g) was

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placed as a single layer in a Pyrex Petri dish (8.0 cm diameter) and heated in an electric muffle furnace (model DMF-802, Daeil Engineering) at 50, 100, and 150 °C for 10, 20, 30, 40, 50, and 60 min. After heating, CP was allowed to cool to ambient temperature before extraction.

Preparation of 70% Ethanol or Water Extracts from CP. Heat-treated or nontreated CP (0.1 g) was extracted overnight with 10 mL of 70% ethanol solution or distilled water, respectively, in a shaking incubator (100 rpm) at room temperature. Then the extracts were centrifuged at 1000*g* for 15 min, and the supernatants were filtered through a Whatman No. 1 filter paper. The 70% ethanol or distilled water extract of CP was diluted to the concentration of 2 mg/mL with 70% ethanol or distilled water, respectively, and used to determine antioxidant activity.

Total Phenolic Contents (TPC). The TPC of CP extracts were determined using the method of Gutfinger (*21*). The CP extract (1 mL) was mixed with 1 mL of the 50% Folin−Ciocalteu reagent and 1 mL of 2% Na_2CO_3 and centrifuged at 13400*g* for 5 min, and the absorbance was measured with a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) at 750 nm after 30 min of incubation at room temperature. TPC were expressed as tannic acid equivalents.

DPPH Radical Scavenging Activity. The DPPH radical scavenging activity of CP extracts was estimated according to the method of Blois (*22*). After 0.1 mL of CP extract had been mixed with 0.9 mL of 0.041 mM DPPH in ethanol for 10 min, the absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as percent inhibition and was calculated using the following formula:

$$\%\ \text{DPPH radical scavenging activity} = (1 - \text{sample OD/control OD}) \times 100$$

Reducing Power. The reducing power of CP extracts was determined according to the method of Oyaizu (*23*). CP extract (1 mL), phosphate buffer (1 mL, 0.2 M, pH 6.6), and potassium ferricyanide (1.0 mL, 10 mg/mL) were mixed and incubated at 50 °C for 20 min. Trichloroacetic acid (1.0 mL, 100 mg/mL) was added to the mixture and centrifuged at 13400*g* for 5 min. The supernatant (1.0 mL) was mixed with distilled water (1.0 mL) and ferric chloride (0.1 mL, 1.0 mg/mL), and then the absorbance was measured at 700 nm.

Gas Chromatography−Mass Spectrometry Analysis of CP Extracts. Extracts from untreated or heat-treated (150 °C for 30 min) CP were dissolved in ethanol (200 mg/mL) and centrifuged at 13400*g* for 5 min to precipitate undissolved materials. The supernatant was mixed with 4 volumes of BSA [*N*,*O*-bis(trimethylsilyl)acetamide] and derivatized in a water bath (70 °C) for 15 min (*24*). The compounds in CP extracts were identified using a gas chromatograph−mass spectrometer (GC6890/MS5973, Hewlett-Packard Co., Wilmington, DE). A split inlet (100:1) was used to inject sample (5 μL) into an HP-5 column (30 m, 0.32 mm i.d., 0.25 μm film; Hewlett-Packard Co.). A ramped oven temperature was used (100 °C for 2 min, increased to 270 °C at 10 °C/min, and held for 6 min). The inlet temperature was 250 °C, and the carrier gas was He at a constant flow of 1.5 mL/min. The ionization potential of the mass selective detector was 70 eV, and the scan range was *m*/*z* 19.1−400. Identification of compounds detected was achieved by comparing mass spectral data of samples with those of the Wiley library (Hewlett-Packard Co.).

Statistical Analysis. All measurements were done in triplicate, and analysis of variance was conducted according to the procedure of the General Linear Model using SAS software (*25*). Student−Newman−Keul's multiple-range tests were used to compare the significant differences of the mean values among treatments ($P < 0.05$).

RESULTS AND DISCUSSION

Effects of Heating Conditions on the Antioxidant Activities of CP Extracts. Phenolic compounds are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because of their stable radical intermediates, which prevent the oxidation of various food ingredients, particularly fatty acids and oils (*26*, *27*). Bocco et al. demonstrated that citrus peel and seed extracts have high levels of phenolics, which have strong antioxidant capability (*10*).

The TPC in EE or WE of CP significantly increased by heat treatments (**Table 1**). The TPC of EE was increased from 71.8 μM in nonheated control to 171.0 μM by heating at 150 °C for 40 min. The TPC of EE heated at 100 °C for 60 min was

Table 1. Effects of Heat Treatments on Total Phenolic Contents (Micromolar) of 70% Ethanol Extract (EE) and Water Extract (WE) from Citrus Peels[a]

temp (°C)	heating time 0 min	10 min	20 min	30 min	40 min	50 min	60 min	SEM[b]
EE								
50	71.8ax	69.8az	72.8az	69.1az	71.5ay	72.7az	73.3az	1.3
100	71.8dx	78.3cdy	86.6bcy	88.3bcy	91.6abcy	98.0aby	102.8ay	3.5
150	71.8cx	143.0bx	162.2ax	156.5abx	171.0ax	157.0abx	165.4ax	4.0
SEM	1.2	1.5	2.5	3.2	6.7	1.0	1.7	
WE								
50	84.4cx	89.3abz	88.7bz	91.4aby	92.6az	92.9az	93.0az	0.9
100	84.4dx	101.3cy	102.3cy	106.9y	110.3by	106.8by	119.0ay	1.0
150	84.4dx	153.8cx	199.0az	204.9ax	177.6bx	146.7cx	176.0bx	3.1
SEM	1.0	1.1	1.1	4.7	1.0	0.5	1.1	

[a] Different letters (a–d) within a row are significantly different ($P < 0.05$), $n = 3$. Different letters (w–z) within a column are significantly different ($P < 0.05$), $n = 3$. [b] Standard error of the means.

Table 2. Effect of Heat Treatments on Radical Scavenging Activity (Percent) of 70% Ethanol Extracts (EE) and Water Extracts (WE) from Citrus Peels[a]

temp (°C)	heating time 0 min	10 min	20 min	30 min	40 min	50 min	60 min	SEM[b]
EE								
50	29.64dx	33.53az	33.61ay	30.61cdz	31.21bcy	32.34aby	32.19aby	0.39
100	29.64cx	35.85ay	33.46by	32.48by	32.11by	33.83by	33.68by	0.42
150	29.64ex	50.60dx	58.83bcx	56.66cx	59.73bx	60.33bx	63.25ax	0.72
SEM	0.17	0.46	0.61	0.22	0.87	0.59	0.46	
WE								
50	15.81cx	18.30bcz	18.30bcz	18.30bcz	19.66abz	19.02abz	21.30az	0.64
100	15.81ex	19.94dw	22.44cy	24.29cy	28.13by	27.00by	33.33ay	0.66
150	15.81dx	44.80cx	55.34bx	58.26ax	54.84bx	44.16cx	54.70bx	0.51
SEM	1.06	0.34	0.48	0.87	0.41	0.4	0.2	

[a] Different letters (a–e) within a row are significantly different ($P < 0.05$), $n = 3$. Different letters (w–z) within a column are significantly different ($P < 0.05$), $n = 3$. [b] Standard error of the means.

Table 3. Effect of Heat Treatments on Reducing Power (Absorbance) of 70% Ethanol Extracts (EE) and Water Extracts (WE) from Citrus Peels[a]

temp (°C)	heating time 0 min	10 min	20 min	30 min	40 min	50 min	60 min	SEM[b]
EE								
50	0.451dex	0.484bcz	0.475cz	0.460dz	0.445ez	0.501az	0.489bz	0.003
100	0.451dx	0.676cy	0.745aby	0.761ay	0.726by	0.745aby	0.720by	0.007
150	0.451dx	0.762cx	0.811abx	0.814abx	0.814abx	0.803bx	0.820ax	0.003
SEM	0.002	0.004	0.005	0.004	0.002	0.007	0.005	
WE								
50	0.269fx	0.292ez	0.301dz	0.319cz	0.336bz	0.329bz	0.360ay	0.003
100	0.269cx	0.889aby	0.895dy	0.891aby	0.902ay	0.878aby	0.856by	0.009
150	0.269bx	0.950ax	0.934ax	0.928ax	0.935ax	0.956ax	0.936ax	0.014
SEM	0.003	0.012	0.002	0.006	0.005	0.017	0.013	

[a] Different letters (a–f) within a row are significantly different ($P < 0.05$), $n = 3$. Different letters (x–z) within a column are significantly different ($P < 0.05$), $n = 3$. [b] Standard error of the means.

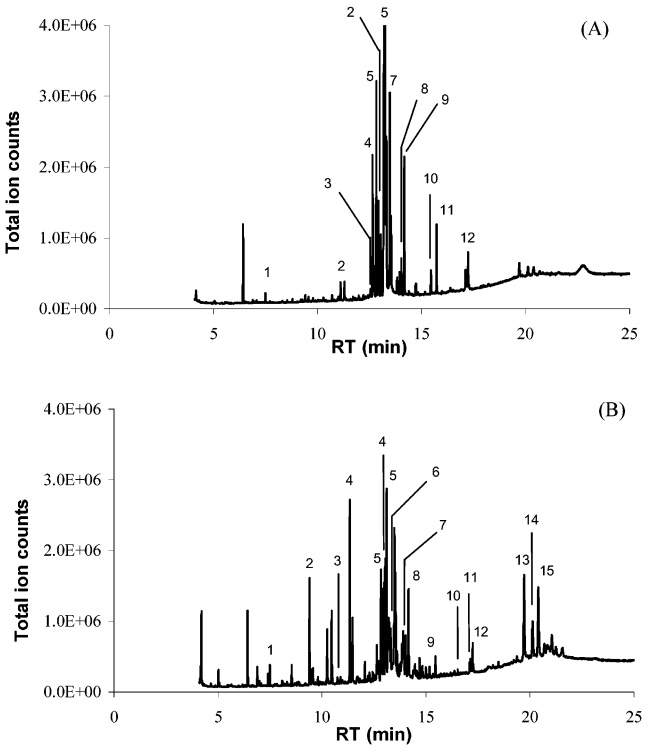

Figure 1. Typical gas chromatography of 70% ethanol extracts from citrus peels nonheated (**A**) and heated (**B**) at 150 °C for 30 min. Peaks in (**A**): 1, bishydroxybutanedioic acid; 2, 2-azathianthrene; 3, 2-oxybenzoic acid; 4, 1*H*-indole-3-carboxaldehyde; 5, arabinofuranose; 6, α-DL-arabinofuranoside; 7, β-L-arabinopyranose; 8, glucopyranose; 9, palmitic acid; 10, 2,4-bishydroxybenzaldehyde; 11, stearic acid. Peaks in (**B**): 1, bishydroxybutanedioic acid; 2, 2,3-diacetyl-1-phenylnaphthalene; 3, 1,2-benzenedicarboxylic acid ethyl ester; 4, 2-azathianthrene; 5, arabinofuranose; 6, β-D-galactofuranose; 7, β-L-arabinopyranose; 8, glucopyranose; 9, palmitic acid; 10, ferulic acid; 11, *p*-hydroxybenzaldoxime; 12, stearic acid; 13, D-ribofuranose; 14, glucofuranoside; 15, β-D-galactofuranoside.

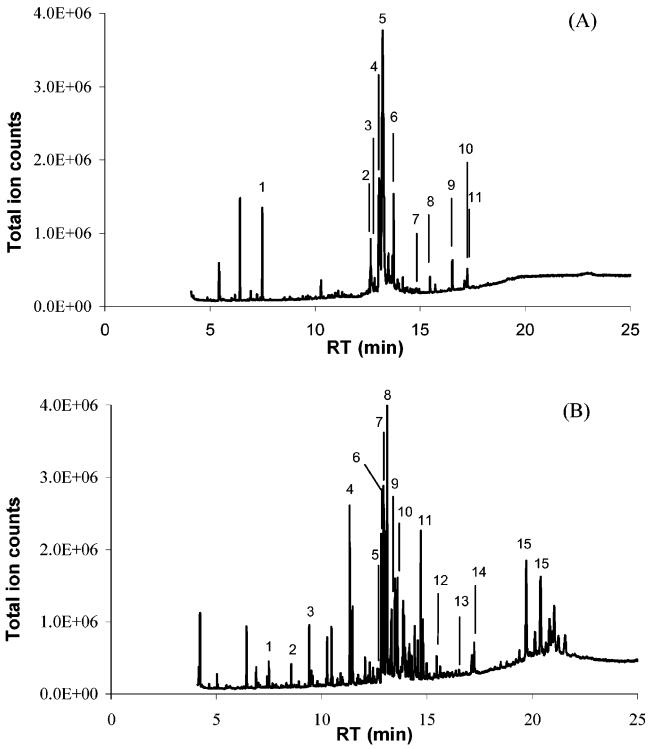

Figure 2. Typical gas chromatography of water extracts from citrus peels nonheated (**A**) and heated (**B**) at 150 °C for 30 min. Peaks in (**A**): 1, bishydroxybutanedioic acid; 2, 1*H*-indole-3-carboxaldehyde; 3, 9-phenylcarbazole; 4, 2-azathianthrene; 5, arabinofuranose; 6, 1*H*-indole-2-carboxylic acid; 7, *p*-cinnamic acid; 8, palmitic acid; 9, isoferulic acid; 10, 1*H*-indole-1-acetic acid; 11, stearic acid. Peaks in (**B**): 1, bishydroxybutanedioic acid; 2, 5-hydroxyvaleric acid; 3, 2,3-diacetyl-1-phenylnaphthalene; 4, 9-phenyl-9*H*-carbazole; 5, 9-phenylcarbazole; 6, 2-azathianthrene; 7, arabionfuranose; 8, arabinose; 9, vanillic acid; 10, β-D-glucopyranose; 11, glucose; 12, palmitic acid; 13, ferulic acid; 14, stearic acid; 15, galactofuranoside.

increased from 71.8 to 102.8 μM, but the TPC of EE heated at 50 °C was not significantly changed. Also, the TPC of WE heated at 150 °C for 30 min increased from 84.4 to 204.9 mM. These results indicate that phenolic compounds in CP can be liberated by heat treatment. However, our previous study (*20*) showed that simple heat treatment could not cleave covalently bound phenolic compounds from rice hull but that far-infrared treatment could. This indicates that phenolic compounds of plants should be present in different bound status depending on species. Thus, effective processing steps for liberating phenolic compounds from various plants may be different.

Radical scavengers were evaluated by their reactivity toward a stable free radical, DPPH. The radical scavenging activity of CP extract was also significantly increased by heat treatment (**Table 2**). The maximum radical scavenging activity values of EE or WE were found after heat treatment of 150 °C for 60 min. However, as in TPC, the effect of heat treatment at lower temperature on radical scavenging activity was minimal.

The power of certain antioxidants is associated with their reducing power (*28*), which is associated with the presence of reductones (*29*). The reducing power of EE or WE was increased significantly by heat treatment (**Table 3**). The reducing power of EE was increased from 0.451 to 0.761 by heating CP at 100 °C for 30 min and to 0.814 by heating at 150 °C for 30 min. Also, that of WE was increased from 0.269 to 0.889 by heating CP at 100 °C for 10 min and to 0.950 by heating at 150 °C for 10 min. Heating of CP at 50 °C did not increase the reducing power of WE significantly.

Identification of Compounds in CP Extracts. Phenolic compounds are the most active antioxidant derivatives in plants (*30*). Generally, the outer layers of plants such as peels, shells, and hulls contain large amount of polyphenolic compounds to protect inner materials. A number of phenolic acids are linked to various cell wall components such as arabinoxylans and proteins (*31*, *32*). Phenolic compounds such as narirutin, naringin, hesperidin, and neohesperisin that have antioxidant activity were found in the peel and seed of citrus (*10*). Manthey and Grohmann (*11*) also reported that polyphenol compounds such as *p*-coumaric, ferulic, and sinapic acids and narirutin were present in CP.

A number of phenolic compounds were identified in both heat-treated and nonheated control extracts from CP (**Figures 1** and **2**). Phenolic compounds such as *p*-cinnamic acid, ferulic acid, isoferulic acid, 5-hydroxyvaleric acid, vanillic acid, and 2-oxybenzoic acid were detected in CP extracts. There are slight differences in the kinds of phenolic compounds detected in nonheated and heated CP extracts. In EE, 2-oxybenzoic acid and 2,4-bishydroxybenzaldehyde were detected in nonheated extract, whereas 2,3-diacetyl-1-phenylnaphthalene, ferulic acid, and *p*-hydroxybenzaldoxime were detected when CP was heated at 150 °C for 30 min (**Table 4**). In WE, *p*-cinnamic acid and isoferulic acid were detected in nonheated extract, whereas 5-hydroxyvaleric acid, 2,3-diacetyl-1-phenylnaphthalene, vanillic

Table 4. Detected Compounds in a Gas Chromatography of 70% Ethanol Extracts from Citrus Peels Heat-Treated at 150 °C for 30 min (EE) and from Nonheated Citrus Peels

compound	total ion counts × 10^4	
	nonheated	EE
bishydroxybutanedioic acid	201	407
2,3-diacetyl-1-phenylnaphthalene	0	2219
1,2-benzenedicarboxylic acid ethyl ester	0	120
2-azathianthrene	412	3777
2-oxybenzoic acid	101	0
1*H*-indole-3-carboxaldehyde	3377	0
arabinofuranose	4381	2820
α-DL-arabinofuranoside	4514	0
β-D-galactofuranose	0	1342
β-L-arabinopyranose	781	1391
glucopyranose	3112	2496
palmitic acid	501	476
2,4-bishydroxybenzaldehyde	1360	0
ferulic acid	0	103
p-hydroxybenzaldoxime	0	432
stearic acid	856	759
D-ribofuranose	0	4257
glucofuranodize	0	2224
β-D-galactofuranoside	0	4181
total	19596	26004

Table 5. Detected Compounds in a Gas Chromatography of Water Extracts from Citrus Peels Heat-Treated at 150 °C for 30 min (WE) and from Nonheated Citrus Peels

compound	total ion counts × 10^4	
	nonheated	WE
bishydroxybutanedioic acid	1804	530
5-hydroxyvaleric acid	0	494
1*H*-indole-3-carboxaldehyde	1092	0
2,3-diacetyl-1-phenylnaphthalene	0	1417
9-phenyl-9*H*-carbazole	0	3626
9-phenylcarbazole	465	388
2-azathianthrene	412	3239
arabionfuranose	194	4104
arabinose	0	4606
1*H*-indole-2-carboxylic acid	1385	0
p-cinnamic acid	109	0
vanillic acid	0	921
β-D-glucopyranose	0	2184
glucose	0	2803
palmitic acid	327	510
isoferulic acid	655	0
ferulic acid	0	120
stearic acid	465	866
galactofuranoside	0	5514
total	6908	31322

acid, and ferulic acid were newly detected when CP was heated at 150 °C for 30 min (**Table 5**).

It has been reported that far-infrared radiation and/or fermentation of natural plant products could liberate low molecular antioxidant compounds from the repeating subunits of high molecular weight polymers (*14*, *19*, *20*). However, this study indicated that phenolic compounds could be released by simple heat treatment from CP. This means that phenolic compounds with antioxidant activity in plants present several kinds of bound states, and a simple heating process can be used as a tool for increasing the antioxidant activity of CP.

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