

## Characteristics of Flavonoids in Niihime Fruit - a New Sour Citrus Fruit

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Niihime fruit, produced in the coastal area of the Sea of Kumano in Mie prefecture, is a new sour citrus fruit. This is the first study to examine the characteristics of the flavonoids in niihime fruit. The content of flavonoids in the juice and peel of niihime fruit was determined by HPLC and their distribution was examined in comparison with seven other species of sour citrus fruits. Niihime fruit was found to contain a comparatively high quantity of bioactive flavonoids of the flavanone glycosides with rutinose sugar chain such as eriocitrin and hesperidin as well as the polymethoxyflavones such as nobiletin and tangeretin. The peel extract of niihime fruit exhibited comparatively high antioxidative activity among sour citrus fruits using the DPPH radical scavenging assay. Furthermore, the flavonoids eluted from niihime fruit by over time in hot and cold solutions of water, 5% ethanol, and 25% ethanol, which are commonly used in the field of food processing and cooking, were examined. The flavonoids eluted from niihime fruit in hot 25% ethanol solution was highest content in solutions, and the content of flavonoids eluted in 5 min was an approximate half of content eluted in 60 min. The flavanone glycosides, eriocitrin and hesperidin, were eluted in higher contents in hot solutions than in cold solutions. The polymethoxyflavones, nobiletin and tangeretin, were eluted to some extent in hot 5% ethanol but were found in low contents in cold solutions. The highest contents were eluted in hot 25% ethanol. The difference in the elution properties between flavanone glycosides and polymethoxyflavones is considered to be a result of their hydrophobic properties. The scavenging activity of DPPH radical for eluates was shown to increase over time, and the activity was suggested to be related to the elution content of eriocitrin, which is an antioxidant in niihime fruit. This study showed that niihime fruit contains a comparative abundance of bioactive flavonoids and these flavonoids are able to be eluted using hot solutions of water and ethanol.

Keywords: flavonoid, niihime fruit, sour citrus fruit

### Introduction

Citrus fruit contains many physiologically bioactive substances such as ascorbic acid, carotenoids, flavonoids, and coumarins, which have antimutagenic, antitumor, antioxidative, and anticarcinogenic activities (Attaway, 1994; Middleton and Kandaswami, 1994; Higashimoto *et al.*, 1998). Sour citrus fruit is a group of citrus fruit with the characteristics of high acidity and a strong citrus flavor, and includes citrus fruits such as lemon, lime, yuzu, sudachi, kabosu, daidai, and shiikuwasha. Sour citrus fruit is used widely in food seasoning and beverages, including alcohol beverages, because they possess a desirable citrus flavor and sour taste. Recently, the physiological activity of sour citrus fruit has been reported in experimental animals. A suppressive effect for high blood pressure is provided by the intake of lemon juice (Miyake *et al.*, 1998a), a hypocholesterolaemic effect is provided by the intake of kabosu juice (Ogawa and Mochizuki, 2003) and an increase in calcium bioavailability is provided by the intake of sudachi juice (Nii *et al.*, 2004). Flavonoids in citrus fruit have been reported to have many biological functions in antioxidative, anticarcinogenic, cardiovascular, and anti-inflammatory ac-

tivities (Attaway, 1994; Benavente-Garcia *et al.*, 1997). Eriocitrin, which is a flavanone glycoside and is abundant in lemon and lime fruits, has been reported to have antioxidative activity on lipid peroxidation and a suppressive effect on oxidative stress *in vivo* (Miyake *et al.*, 1998b; Minato *et al.*, 2003). Nobiletin and tangeretin, which are polymethoxyflavones abundant in shiikuwasha fruit, have been reported as having anti-tumor and anticarcinogenic activity (Kawaii *et al.*, 1999a; Murakami *et al.*, 2000; Suzuki *et al.*, 2004). Given these findings, citrus flavonoids have gained a lot of attention for their many physiology characteristics linked to the prevention of lifestyle diseases, and their distribution in citrus fruit has been widely examined (Kawaii *et al.*, 1999b).

Niihime fruit, which is produced in Mie prefecture along the coast of the Sea of Kumano, is a new sour citrus fruit. It is thought to be an autogenous citrus consisting of a mandarin and tachibana (*Citrus tachibana*) cross hybridization, and was registered as a new nursery plant in 1997 (Japan the plant variety protection, No. 5791, 11/14/1997). People of the region have eaten green niihime fruit and ripe niihime fruit and used it for food seasoning fried and roasted fish and for adding a sour flavor to distilled alcohol. However, the flavonoid characteristics of niihime fruit have yet to be reported. In this study, the distribution of flavanone glycosides and poly-

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methoxyflavones in niihime fruit was examined in comparison with other sour citrus fruits for first time. Furthermore, the elution properties of flavonoids from niihime fruit were examined using different solutions in order to evaluate the characteristics of flavonoids in niihime fruit for food processing and cooking.

## Materials and Methods

**Samples and Reagents** Citrus fruits, consisting of lemon (produced in California), lime (produced in Mexico), yuzu (produced in Miyazaki), sudachi (produced in Tokushima), kabosu (produced in Ohita), and shiikuwasha (produced in Okinawa) were purchased from a supermarket. Fruits were obtained at approximately the same time and from the same store. Daidai and niihime (green and ripe fruits) were obtained courtesy of the Mie Prefectural Science and Technology Promotion Center, Agricultural Research Division. The green niihime fruit, which has a green peel, was collected on Nov. 1, 2004 and the ripe fruit, which has a yellow peel, was collected on Jan. 15, 2004. Eriocitrin was obtained by purification from lemon peel (Miyake *et al.*, 1997a). Nobiletin and tangeretin were received as a gift courtesy of Dr. M. Yano and Dr. M. Sugiura of the Department of Citriculture, Okitsu, National Institute of Fruit Tree Science, Shizuoka. Other flavonoids of neohesperidin, narirutin, naringin, hesperidin, and neohesperidin (HPLC grade) and reagents were purchased from Funakoshi, Ltd., Tokyo, Japan and Wako Pure Chemical Industries, Ltd., Osaka, Japan, respectively.

**Determination of flavonoids in sour citrus fruit** The content of flavonoids in the juice and peel of four fruits each of lemon, lime, yuzu, kabosu, sudachi, daidai, shiikuwasha, green niihime, and ripe niihime was determined by HPLC (JASCO Co., Ltd., Tokyo, Japan). The juice of sour citrus fruit was obtained by hand squeezing after cutting the fruit with a knife. The pulp was removed by filtration using gauze. After the flavonoids content in the juice was analyzed by HPLC, the juice of all four sour fruits was mixed. The pH and acidity levels of the juice were measured using a Metrohm Herisau apparatus (719S, Metrohm-Shibata Co., Ltd., Tokyo, Japan). The peel from the squeezed residue was crushed using a food processor, and a peel sample (1.0 g) was extracted with 10 mL of 75% ethanol, which has the highest level of extraction efficiency in water-ethanol solutions (Table 2), over night at room temperature. The flavonoids contents in the juice and the peel extract (N=4) were determined by HPLC using a YMC-Pack ODS column (YMC Co., Ltd., Kyoto, Japan, column size;  $\phi 4.6 \times 250$  mm, particle size; 5  $\mu$ m) and 280 nm UV detection. The mobile phase consisted of a linear gradient of methanol in distilled water with 5% acetic acid as follows: 15% (0-10 min), 15-50% (10-30 min), 50-90% (30-45 min), 100% (45-50 min). The column temperature was maintained at 40°C, and the flow rate was 1.0 mL/min. The retention time of standard flavonoids was 23.0 min (eriocitrin), 24.4 min (neohesperidin), 27.0 min (narirutin), 28.0 min (naringin), 29.2 min (hesperidin), 30.0 min (neohesperidin), 43.8 min (nobiletin), and 45.5 min (tangeretin). The identification of flavonoids

in sour citrus fruit was carried out in accordance with the retention time of standard flavonoids. Standard flavonoids were used for the determination of flavonoids in sour citrus fruit. The content of flavonoids in juice and peel was shown as mean  $\pm$  SD (N=4) in mg/100 mL juice and mg/100 g peel. The trace level (0.01-0.1 mg/100 mL juice or 100 g peel) was shown as trace, and the level of less than 0.01 mg/100 mL juice or 100 g peel was shown as nd (no detect).

**Extraction of niihime fruit with water-ethanol solution** The peel of ripe niihime fruit was extracted with water-ethanol solutions and the content of flavonoids in extract was determined by HPLC. The peel (1.0 g) of niihime fruit, which was crushed using a food processor, was extracted with 10 mL of water, 5% ethanol, 25% ethanol, 50% ethanol, 75% ethanol, and 100% ethanol over night at room temperature (N=3). The content of flavonoids in peel extract was determined by HPLC according to the described method. The extraction efficiency (%) of flavonoids in each water-ethanol solution was calculated based on the highest content of flavonoids in the solution. Data are presented as mean  $\pm$  SD (N=3).

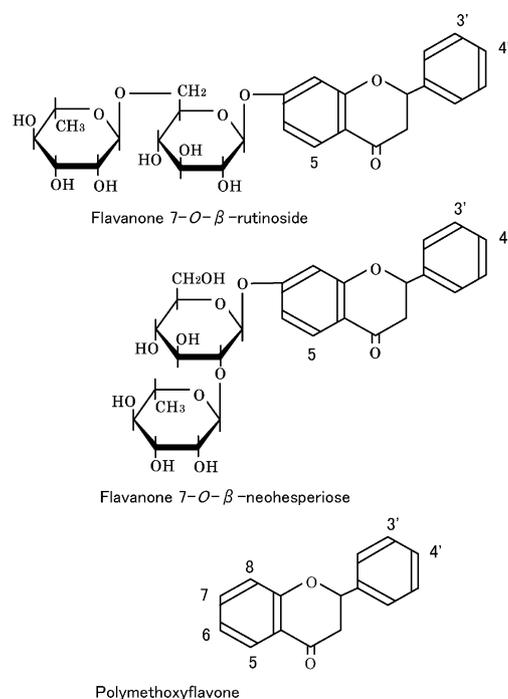
**DPPH radical scavenging activity** The ninety-six-well microplate was used for the assay of DPPH radical scavenging assay. Regarding the assay of peel extract of sour citrus fruit (N=4), the reaction mixture contained 100  $\mu$ L of 0.13 mg/mL DPPH (1,1-diphenyl-2-picrylhydrazyl) dissolved in ethanol, 90  $\mu$ L of 0.1 M Tris  $\cdot$  HCl buffer (pH 7.4), 4.0  $\mu$ L of test sample for peel extract, and 6.0  $\mu$ L water. For the assay of niihime fruit elution (N=4), the reaction mixture contained 100  $\mu$ L of 0.13 mg/mL DPPH dissolved in ethanol, 16  $\mu$ L of 0.5 M Tris  $\cdot$  HCl buffer (pH 7.4), and 84.0  $\mu$ L of eluate. After 1-h incubation at room temperature, the absorbance was recorded at 517 nm using a plate reader (Sunrise Rainbow, Tecan, Ltd.). Results were expressed as percent decrease with respect to control values (Chen and Ho *et al.*, 1995; Kikuzaki *et al.*, 2002). The control sample contained solvent (ethanol or water of sample solvent) in place of the test sample. Ascorbic acid was used as the reference sample. Data are presented as mean  $\pm$  SD (N=4).

**Elution of flavonoids from niihime fruit with solution** Niihime green fruit (18.4-20.6 g, 19.6  $\pm$  0.65 mean  $\pm$  SD) was cut in half using a knife. All cut fruit was soaked in 200 mL of a cold solution at 4°C and a hot solution of 90°C, which consisted of water, 5% ethanol solution, and 25% ethanol solution (N=4). The fruit was left to stand at room temperature and stirred occasionally with a grass rod. The eluate was taken from 1 mL of solution at 1, 3, 5, 10, 20, 40, and 60 min. The initial temperatures of the cold solution (4°C) and hot solution (90°C) were changed to 15°C and 35 °C, respectively, after examination for 60 min at room temperature (22°C). The quantity of flavonoids in the sampling solutions was determined by HPLC in accordance with the above method, and the elution content from niihime fruit (g) was calculated. The sampling solutions were assayed using the DPPH radical scavenging assay in accordance with the above method. Data are presented as mean  $\pm$  SD (N=4).

## Results and Discussion

*Distribution of Flavonoids in sour citrus fruits* The pH level of juice from sour citrus fruits including niihime was between 2.4 to 2.8, and the acidity level was between 3.6 to 6.1 (Table 1). Niihime juice from green and ripe fruits was shown to have characteristics including low pH and high acidity, identical to other sour citrus fruits. Flavonoids are found in a variety of vegetables and fruits. In particular, flavanone glycosides and polymethoxyflavones are reported to be particularly abundant in citrus fruit (Miyake *et al.*, 1998c; Kawaii *et al.*, 1999b). Flavonones in citrus fruits exist mainly as glycosides with rutinose sugar chain (6-*O*- $\alpha$ -L-rhamnosyl- $\beta$ -D-glucose) such as eriocitrin, narirutin, and hesperidin, and with neohesperidose sugar chain (2-*O*- $\alpha$ -L-rhamnosyl- $\beta$ -D-glucose) such as neoeriocitrin, naringin, and neohesperidin (Fig. 1). Polymethoxyflavones, such as nobiletin and tangeretin, are also particularly abundant in citrus fruits (Fig. 1). In this study, the quantity of these flavonoids in the juice and peel of sour citrus fruits, including niihime, was determined for the purpose of examining the distribution characteristics of flavonoids in niihime fruit (Table 1). The content of flavonoids in the peel of all sour citrus fruits was shown to be higher than that in the juice.

Regarding green and ripe niihime fruit, eriocitrin, narirutin, naringin, hesperidin, nobiletin, and tangeretin were detected in the juice and peel; however, no neoeriocitrin or neohesperidin were detected. Niihime fruit was shown to have flavanone glycosides with rutinose sugar chain, such as eriocitrin, narirutin, and hesperidin. The content of flavanone glycosides with rutinose sugar chain was found to be higher than that with neohesperidose sugar chain in sour citrus fruits, with the exception of daidai. Naringin, neoeriocitrin, and neohesperidin with neohesperidose sugar chain are reported to taste bitterer than narirutin, eriocitrin, and hesperidin, which have rutinose sugar chain (Rouseff, 1980; Miyake *et al.*, 1998c). Daidai juice was shown to taste very bitter because of a high content of neoeriocitrin, naringin, and neohesperidin of flavanone glycoside with neohesperidose sugar chain. However, niihime juice was shown to taste less bitter because it contained only a low content of naringin, which is a flavanone glycoside with a neohesperidose sugar chain. In Table 1, green niihime (green peel) and ripe niihime (yellow peel) were shown to contain the bioactive flavonoids hesperidin and eriocitrin. The juice of green niihime had a higher content of eriocitrin, narirutin, and hesperidin than that of ripe niihime. The



	C3'	C4'	C5	C6	C7	C8
Flavanone 7- <i>O</i> - $\beta$ -rutinoside						
Eriocitrin	OH	OH	OH	H	rutinose	H
Narirutin	H	OH	OH	H	rutinose	H
Hesperidin	OH	OCH <sub>3</sub>	OH	H	rutinose	H
Flavanone 7- <i>O</i> - $\beta$ -neohesperiose						
Neohesperidin	OH	OH	OH	H	neohesperidose	H
Naringin	H	OH	OH	H	neohesperidose	H
Neoeriocitrin	OH	OCH <sub>3</sub>	OH	H	neohesperidose	H
Polymethoxyflavone						
Nobiletin	OCH <sub>3</sub>					
Tangeretin	H	OCH <sub>3</sub>				

Fig. 1. Chemical formula of flavonoids in sour citrus fruit.

**Table 1.** Determination of flavonoids in juice and peel of sour citrus fruit.

**A. Content of flavonoids in juice of sour citrus juice**

mean ± SD (n=4), trace: 0.01–0.1 mg/100 mL juice, nd; less than 0.01 mg/100 mL juice

conventional name	scientific name	Flavonoid (mg/100mL juice)								pH	acidity
		Flavanone glycoside						Polymethoxyflavone			
		Sugar chain of rutinose			Sugar chain of neohesperidiose			Nobiletin	Tangeretin		
		Eriocitrin	Narirutin	Hesperidin	Neoeriocitrin	Naringin	Neohesperidin				
Lemon	<i>Citrus limon</i>	6.1±0.6	nd	6.0±0.45	nd	nd	nd	nd	nd	2.4	5.5
Lime	<i>Citrus latifolia</i>	8.3±0.5	2.2±0.28	17.2±1.8	nd	nd	nd	trace	trace	2.4	6.1
Yuzu	<i>Citrus junos</i>	nd	6.6±2.1	4.9±1.1	nd	2.5±0.9	1.3±0.3	nd	nd	2.6	5.7
Kabosu	<i>Citrus sphaerocarp</i>	nd	3.8±0.1	2.7±0.07	nd	1.2±0.04	0.7±0.02	nd	nd	2.6	4.9
Sudachi	<i>Citrus sudachi</i>	12.0±2.5	20.1±3.4	9.8±2.0	nd	11.1±1.8	6.8±1.5	nd	nd	2.5	5.5
Daidai	<i>Citrus aurantium</i>	3.7±0.06	3.5±0.03	nd	39.6±1.8	98.3±7.0	49.3±8.0	nd	nd	2.7	4.6
Shiikuwasha	<i>Citrus depressa</i>	trace	3.0±0.4	16.4±1.4	nd	nd	nd	1.4±0.6	0.5±0.1	2.8	4.8
Niihime (green fruit)	–	2.7±0.2	4.1±0.2	20.2±0.9	nd	1.1±0.2	nd	0.4±0.1	0.1±0.03	2.8	3.6
Niihime (ripe fruit)	–	0.9±0.5	2.5±2.7	6.8±5.01	nd	0.12±0.1	nd	0.8±0.1	0.3±0.03	2.6	4.3

**B. Content of flavonoids in sour citrus peel**

mean ± SD (n=4), trace: 0.01–0.1 mg/100 g peel, nd; less than 0.01 mg/100 g peel

conventional name	scientific name	Flavonoid (mg/100g peel)							
		Flavanone glycoside						Polymethoxyflavone	
		Sugar chain of rutinose			Sugar chain of neohesperidiose			Nobiletin	Tangeretin
		Eriocitrin	Narirutin	Hesperidin	Neoeriocitrin	Naringin	Neohesperidin		
Lemon	<i>Citrus limon</i>	127.0±35.4	8.4±0.3	123.0±23.5	nd	nd	nd	0.7±0.4	nd
Lime	<i>Citrus latifolia</i>	52.7±11.7	41.1±5.9	101.3±25.8	nd	nd	nd	2.1±0.3	nd
Yuzu	<i>Citrus junos</i>	nd	85.1±11.4	115.9±12.6	nd	45.2±7.1	35.2±3.9	0.6±0.02	nd
Kabosu	<i>Citrus sphaerocarp</i>	nd	159.7±8.6	167.1±7.0	nd	52.3±2.8	44.7±4.6	1.4±0.8	2.6±0.4
Sudachi	<i>Citrus sudachi</i>	65.6±13.1	136.4±22.3	216.4±85.2	16.6±4.1	78.0±14.9	101.0±31.6	nd	nd
Daidai	<i>Citrus aurantium</i>	nd	15.5±5.9	nd	194.9±23.4	988.5±129.1	480.7±103.2	13.2±2.0	7.0±0.8
Shiikuwasha	<i>Citrus depressa</i>	nd	26.4±1.2	227.9±18.5	nd	nd	nd	149.4±17.2	81.8±13.8
Niihime (green fruit)	–	76.1±5.5	69.5±7.3	256.1±19.8	nd	30.3±2.0	nd	225.4±35.2	45.9±6.6
Niihime (ripe fruit)	–	43.0±0.7	54.5±1.8	113.0±12.5	nd	12.7±0.3	nd	87.2±3.7	21.2±1.0

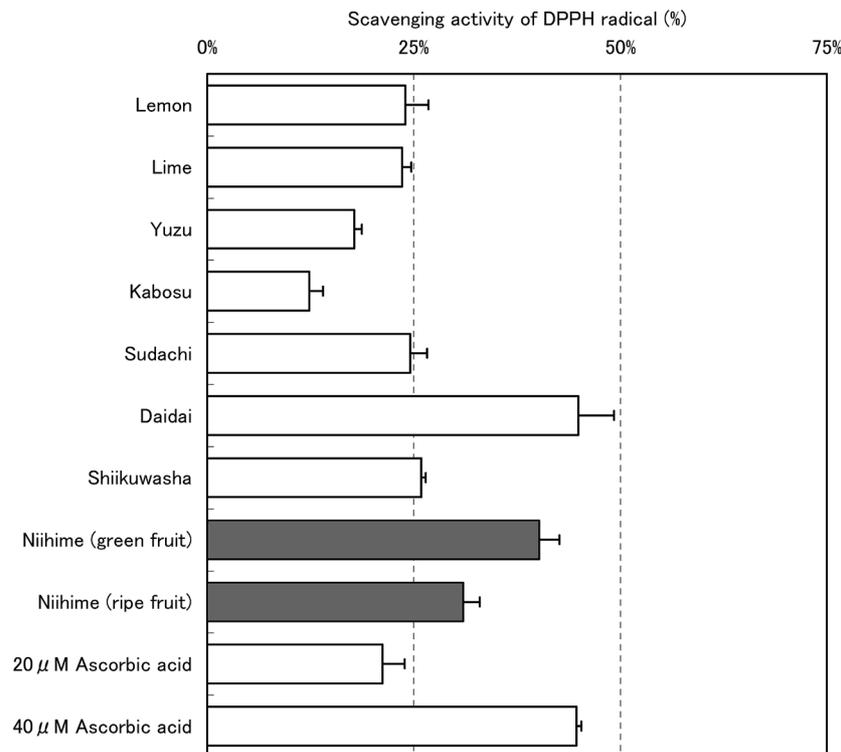
**Table 2.** Flavonoid content in extract of niihime peel extracted by ethanol-water solution.

	water	5%EtOH	25%EtOH	50%EtOH	75%EtOH	100%EtOH
Eriocitrin (mg/100g of peel) (extraction efficiency, %)	26.9±0.7 (63)	29.0±0.2 (67)	34.6±1.0 (80)	40.5±4.8 (94)	43.0±0.7 (100)	35.4±2.0 (82)
Narirutin (mg/100g of peel) (extraction efficiency, %)	27.2±0.6 (50)	30.1±0.4 (55)	37.7±1.3 (69)	53.2±4.6 (98)	54.5±1.8 (100)	44.0±3.2 (81)
Naringin (mg/100g of peel) (extraction efficiency, %)	4.9±4.4 (37)	5.5±4.4 (42)	9.1±2.3 (69)	13.1±1.7 (100)	12.7±0.3 (97)	6.5±0.5 (50)
Hesperidin (mg/100g of peel) (extraction efficiency, %)	21.5±0.5 (19)	24.1±0.6 (21)	37.8±0.8 (33)	90.7±6.3 (80)	113.0±12.5 (100)	67.9±2.5 (60)
Nobiletin (mg/100g of peel) (extraction efficiency, %)	11.6±0.4 (13)	18.6±1.3 (21)	52.7±5.0 (60)	79.9±9.5 (92)	87.2±3.7 (100)	85.2±5.3 (98)
Tangeretin (mg/100g of peel) (extraction efficiency, %)	1.7±0.1 (8)	3.2±0.3 (15)	11.3±1.3 (53)	19.4±2.4 (92)	21.2±1.0 (100)	21.1±1.5 (100)

peel of green niihime contained a higher content of eriocitrin, hesperidin, nobiletin, and tangeretin than ripe niihime fruit. In addition, the green niihime fruit was found to contain an abundance of flavonoids in comparison with the ripe niihime fruit. Hesperidin is reported to have antihypertensive activity (Galati *et al.*, 1996) and is widely distributed in citrus fruits (Kawaii *et al.*, 1999b). Eriocitrin is reported to exhibit the highest antioxidative activity among all citrus flavonoids and is especially abundant in lemon and lime (Miyake *et al.*, 1998c). Eriocitrin has also been identified in sudachi fruit (Kawaii *et al.*, 1999b). In the present study, eriocitrin was shown to also be found in niihime fruit, although the content in niihime fruit was lower than that in lemon fruit. Polymethoxyflavones, nobiletin and tangeretin, were shown to be more abundant in shiikuwasha and niihime fruits than in other sour citrus fruits (Table 1). The content of nobiletin and tangeretin in shiikuwasha fruit was re-

ported to be the highest among several citrus fruits (Kawaii *et al.*, 1999b). In this study, it was shown that the bioactive flavonoids nobiletin and tangeretin contain in the juice and peel of niihime fruit. The content of nobiletin in the peel of green niihime was shown to be higher than that of shiikuwasha.

*Extraction of flavonoids from niihime fruit in water-ethanol solution* The extraction efficiency of flavonoids from niihime peel (ripe fruit) was examined as shown in Table 2. In this study, water and ethanol were used for extraction solutions, as they are common solutions in the field of food processing and cooking. Solutions of water, 5% ethanol, 25% ethanol, 50% ethanol, 75% ethanol, and 100% ethanol were used for the extraction of flavonoids from niihime peel. The content of flavonoids in water and low-content ethanol solutions was generally lower than in high-content ethanol solutions. The extraction efficiency of eriocitrin, narirutin, hesperidin, nobiletin, and



**Fig. 2.** Antioxidative activity of peel extract of sour citrus fruits. The DPPH radical scavenging activity of the peel extract of sour citrus fruits was measured.

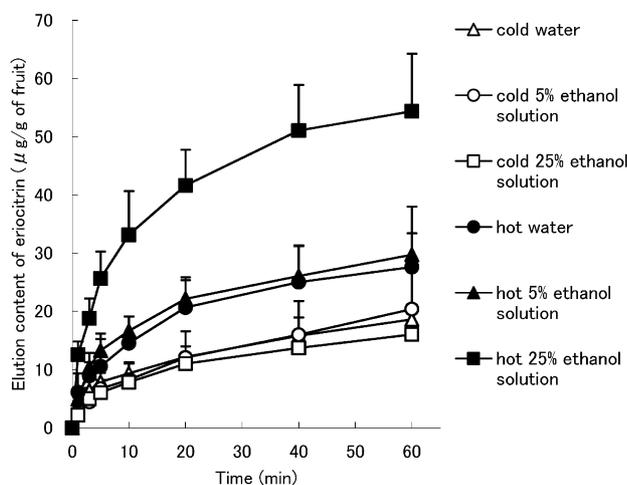
tangeretin was to be highest with 75% ethanol solution. That of naringin was highest with 50% ethanol solution but was at 97% with 75% ethanol solution. Based on these results, 75% ethanol solution was shown to be a suitable extraction solution using water and ethanol. A 75% ethanol solution was used for the extraction solution in the determination of flavonoids in sour citrus peel in Table 1. In Table 2, the flavanone glycosides eriocitrin, narirutin, naringin, and hesperidin were eluted to some extent in water or low-content ethanol solutions, and their contents in 100% ethanol were lower than that in 50% and 75% ethanol solutions. However, the content of polymethoxyflavones, nobiletin and tangeretin, was very low in water and was high in 75% and 100% ethanol solutions. The difference in the elution properties of flavonoids was suggested to be related to the hydrophobic nature of flavonoids.

**Antioxidative activity of sour citrus fruits** Aging and carcinogenesis is suspected to be strongly associated with excess oxidative stress in the body. Antioxidants in food, which have been used for the prevention of lipid peroxidation in food storage, have been shown to have a suppressive effect on oxidative stress *in vivo*, and are thought to play a role in the prevention of atherosclerosis and complications from diabetes (Yagi, 1987; Osawa *et al.*, 1990). Citrus flavonoid is also thought to play a role in the prevention of disease because it has been reported to have antioxidative activity and a suppressive effect on oxidative stress *in vivo* (Middleton and Kandaswami, 1994; Miyake *et al.*, 1998b). Based on this background, the present study evaluated the antioxidative activity of sour

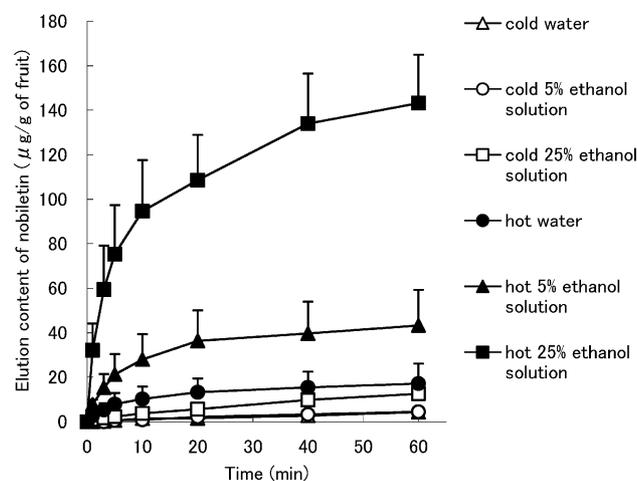
citrus fruits, including niihime fruit. The peel extract of sour citrus fruit was examined for antioxidative activity using DPPH radical scavenging (Fig. 2). Niihime exhibited high antioxidative activity in the assay of sour citrus fruits. Among citrus flavonoids, eriocitrin and neoeriocitrin are reported to exhibit high antioxidative activity because of their structure containing a catechol group at the 3' and 4' position, which has radical-scavenging ability (Miyake *et al.*, 1997b). The antioxidant in the peel extract of lemon is reported to have been isolated as eriocitrin (Miyake, *et al.*, 1997a). Furthermore, we attempted to fractionate the antioxidative substances from the peel extract of daidai, and one was identified as neoeriocitrin (data not shown). Eriocitrin and neoeriocitrin were identified in the lemon, daidai, lime, sudachi, and niihime (Table 1). The antioxidative activity of these fruits was suggested to be related to the presence of eriocitrin and neoeriocitrin being antioxidative flavonoids. The green niihime was suggested to exhibit a higher activity than ripe niihime because of its high content of eriocitrin in comparison with ripe niihime. However, the high antioxidative activity of shiikuwasha could not be explained by these flavonoids. Shiikuwasha fruit was suggested to contain antioxidants other than these, which will require further research.

**Elution property flavonoids from niihime fruit to solution** Niihime fruit was shown to contain bioactive flavonoids such as eriocitrin, hesperidin, nobiletin, and tangeretin (Table 1). In this study, the elution properties of these flavonoids from niihime fruit were investigated using hot and cold solutions of water, 5% ethanol, and

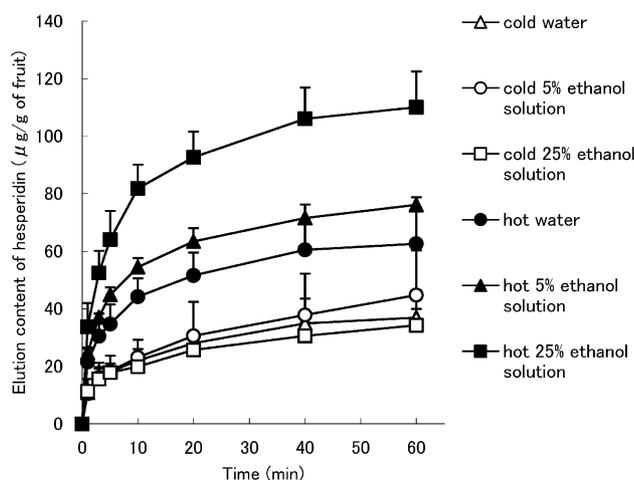
A. Elution of eriocitrin in solutions from niihime fruit



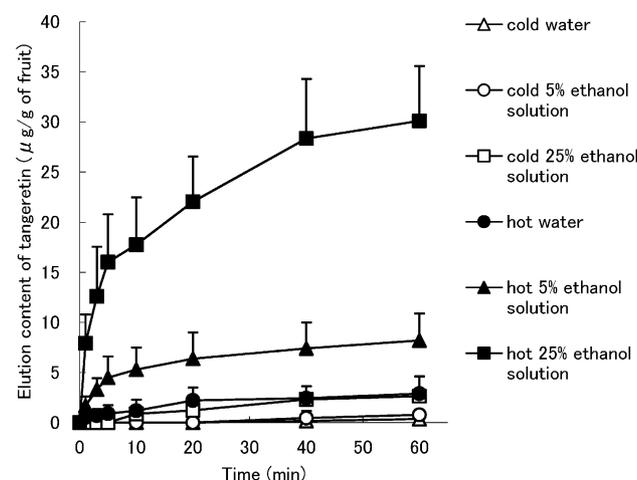
A. Elution of nobiletin in solutions from niihime fruit



B. Elution of hesperidin in solutions from niihime fruit



B. Elution of tangeretin in solutions from niihime fruit



**Fig. 3.** Elution content of flavanone glycosides from niihime fruit in hot and cold solutions of water and ethanol. (A) is elution content of eriocitrin from niihime fruit. (B) is elution content of hesperidin from niihime fruit.

**Fig. 4.** Elution content of polymethoxyflavones from niihime fruit in hot and cold solutions of water and ethanol. (A) is elution content of nobiletin from niihime fruit. (B) is elution content of tangeretin from niihime fruit.

25% ethanol solutions, which are commonly used in food processing and cooking. In Table 2, the extraction efficiency of 75% ethanol solution was the highest in water-ethanol solutions; however, this solution is seldom used in food processing or cooking because of the high ethanol content. 5% and 25% ethanol solutions are used commonly because these solutions are available in commercial products, such as alcoholic beverages. Based on this background, the water, 5% ethanol, and 25% ethanol solutions were used for determining elution properties in this study. Moreover, this study attempted to gain basic data for the application of the bioactive flavonoids in niihime fruit to food science. Niihime fruits were cut using a knife and the cut fruit was soaked in hot or cold solutions of water and ethanol for up to 60 min. The content of flavonoids in the eluate was determined by HPLC (Fig. 3, Fig. 4). In comparison with other solutions, hot 25% ethanol solution exhibited the highest elution content of the flavanone glycosides eriocitrin and hes-

peridin. The contents of eriocitrin and hesperidin were higher in hot solutions than in cold solutions (Fig. 3). The content of flavonoids eluted in 5 min was an approximate half of content eluted in 60 min. The dissolution property of purified eriocitrin to solution is different from that of purified hesperidin showing that eriocitrin dissolves easily in water while hesperidin does not dissolve easily in water. However, the changes over time in eriocitrin contents eluted from niihime fruit were shown to be similar to that of hesperidin. Based on the present results, hot 25% ethanol solution is suitable for eluting the bioactive flavanone glycosides eriocitrin and hesperidin from niihime fruit. Eriocitrin and hesperidin were shown to be eluted to some extent from niihime fruit in a hot solution of water and 5% ethanol.

As for the polymethoxyflavones nobiletin and tangeretin, the changes over time in the contents of nobiletin eluted from niihime fruit were similar to that of tangeretin (Fig. 4). The eluted contents of nobiletin and

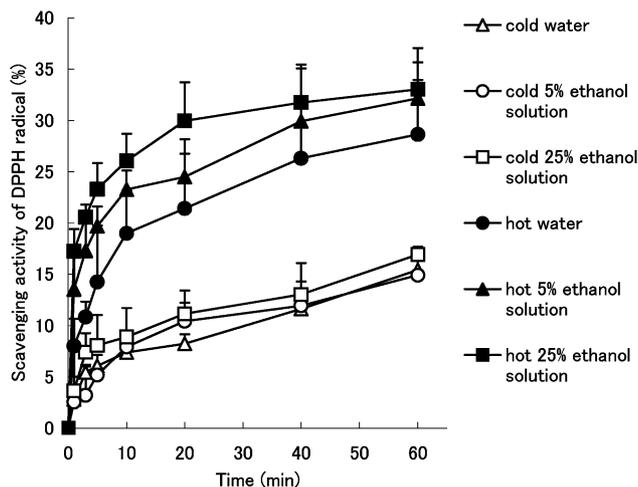


Fig. 5. DPPH radical scavenging activity of eluates of niihime fruit.

tangeretin were highest in hot 25% ethanol solution. The content of flavonoids eluted in 5 min for hot ethanol solutions was an approximate half of content eluted in 60 min. The flavonoids were eluted to some extent in hot solutions of 5% ethanol, but only to a very low content in other solutions. The elution of nobiletin and tangeretin was shown to require a hot ethanol solution with a concentration of more than 5%. Nobiletin and tangeretin are difficult to dissolve in water and ethanol solution of low concentration in comparison with eriocitrin and hesperidin because of their strong hydrophobic property. The strong hydrophobic property of nobiletin and tangeretin was suggested to affect the elution property; therefore, they are eluted from niihime fruit to some extent in hot solutions of 5% ethanol, but are eluted to the highest content in hot 25% ethanol. In food processing and cooking for niihime fruit, hot solution, preferably hot 25% ethanol was identified as a suitable solution for eluting the bioactive polymethoxyflavones nobiletin and tangeretin.

The antioxidative activity of eluates, which were eluted in hot and cold solutions of water and ethanol, were examined for DPPH radical scavenging activity (Fig. 5). The antioxidative activity of the eluates in hot solution was higher than in cold solution. The increasing change of antioxidative activity observed until 60 min (Fig. 5) was similar to that of the eluted content of eriocitrin and hesperidin (Fig. 3). Eriocitrin has been reported to have the highest antioxidative activity among citrus flavonoids (Miyake *et al.*, 1997b; 1998c). Therefore, the antioxidative activity of the eluate was suggested to be related to the elution content of eriocitrin in the solution. Hot solutions, such as water, 5% ethanol, and 25% ethanol, were suggested to be the most suitable solutions for eluting antioxidative flavonoids from niihime.

In the present study, the sour citrus fruit niihime was shown, for the first time, to contain an abundance of bioactive flavonoids, such as eriocitrin, hesperidin, nobiletin, and tangeretin. Niihime fruit exhibited high antio-

xidative activity in comparison with other sour citrus fruits. These bioactive flavonoids in niihime fruit were eluted effectively from niihime fruit in hot solution, and hot 25% ethanol was shown to be the preferable solution for the elution of nobiletin and tangeretin. These results identified the characteristics of the flavonoids in niihime and demonstrate their possible use and effective application in food processing and cooking.

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