Quantitative Study of Flavonoids in Leaves of *Citrus* Plants

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Leaf flavonoids were quantitatively determined in 68 representative or economically important *Citrus* species, cultivars, and near-*Citrus* relatives. Contents of 23 flavonoids including 6 polymethoxylated flavones were analyzed by means of reversed phase HPLC analysis. Principal component analysis revealed that the 7 associations according to Tanaka's classification were observed, but some do overlap each other. Group VII species could be divided into two different subgroups, namely, the first-10-species class and the last-19-species class according to Tanaka's classification numbers.

Keywords: *Citrus; near-Citrus relatives; flavonoids; demethylnobiletin; HPLC; principal component analysis*

INTRODUCTION

Citrus plants are of great interest because their fruits and leaves accumulate large amounts of flavonoid glycosides, whose aglycones are early intermediates in the flavonoid biosynthetic pathway. In recent literature on *Citrus* flavonoids, a broad spectrum of biological activity including anticarcinogenic and antitumor activities has been discussed (Attaway, 1994; Sugiyama et al., 1993; Benavente-García et al., 1997). Naringenin and hesperetin widely distribute in *Citrus* species as their glycoside form; namely, naringin, neohesperidin (neohesperidosides), narirutin, and hesperidin (rutinosides). These flavanone glycosides are the four most abundant flavonoids in the edible part of many species of *Citrus* fruits (Kawaii et al., 1999a, 1999b). The *Citrus* flavonoids greatly influence the quality of both the fresh fruit and processed products. Hesperidin is a significant component of the cloud in lemon and orange juice (Nishimura et al., 1998), and naringin plays a major role in the bitterness of *Citrus* fruits (Ranganna et al., 1983; Sinclair, 1984).

On the other hand, polymethoxylated flavones are the characteristic feature of *Citrus* plants, although they are minor components that are mainly associated with the oil gland of the peel flavedo (Chen et al., 1997). Polymethoxylated flavones are reported to have many important bioactivities. For example, nobiletin has a potent inhibitory activity on cyclic adenosin monophosphate phosphodiesterase (Nikaido et al., 1982), an antifungal activity against *Deuteriophoma tracheiphila* which causes a destructive disease of *Citrus* trees (Pinka et al., 1968), and a suppressing activity of the production and gene expression of matrix metalloproteinase-9/gelatinase B (Ito et al., 1999) which plays an important role in transference of cancer cells. Tangeretin has an antiproliferative activity against HL-60 promyelocytic leukemic cells (Hirano et al., 1995; Kawaii et al., 1999c). Polymethoxylated flavones are generally more potent inhibitors of tumor cell growth than the corresponding polyhydroxylated flavones. This difference in activity may be due to greater membrane uptake of the polymethoxylated flavones (Kandaswani, 1991).

Numerous quantitative studies (Vandercook and Stevenson, 1966; Albach and Redman, 1969; Ting et al., 1979; Park et al., 1983; Gaydou et al., 1987) on *Citrus* flavonoids have been conducted since the major flavonoids of *Citrus* were identified in the late 1950s and 1960s by Horowitz and Gentili (Horowitz, 1956; 1957; Horowitz and Gentili, 1960a; 1960b; Gentili and Horowitz, 1964). Few studies, however, have been conducted with the aim of finding composition parameters that could establish similarity among *Citrus* cultivars by means of not only flavanones, flavanone glycosides, flavones, and flavone glycosides, but also by means of polymethoxylated flavones. In the previous paper, we reported quantitative analyses of 24 major flavonoids including five polymethoxylated flavones, in edible parts of various *Citrus* fruits (Kawaii et al., 1999a; 1999b). Principal component analysis revealed that the distributions of *Citrus* fruits belonging to different classes were largely in accordance with Tanaka's classification system (Tanaka, 1969).

In this study, we quantitatively estimated leaf flavonoids of various *Citrus* species, cultivars, and near-*Citrus* relatives. Contents of 24 flavonoids, including six polymethoxylated flavones(namely, sinensetin, nobiletin, natsudaidain, 3,3′,4′,5,6,7,8-heptamethoxylflavone, demethylnobiletin, and tangeretin), have been quantified by high performance liquid chromotography (HPLC) analysis. Principal component analysis (PCA) has been done in order to precisely evaluate the influence of taxa classification on flavonoid composition. The aim of the study is to carry out a comprehensive flavonoid analysis of *Citrus* plants to contribute to a better understanding of the genetic relationship in *Citrus* and related genera by flavonoid profile.

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MATERIALS AND METHODS

Flavonoids. The flavonoids used in this study are apigenin (APG), diosmin (DSM), eriocitrin (ERC), hesperidin (HSP), isorhoifolin (IRHF), kaempferol (KMP), naringenin (NGEN), naringin (NGIN), narirutin (NRTN), neodiosmin (NDSM), neoeriocitrin (NERC), neohesperidin (NHSP), neoponcirin (NPNC), poncirin (PNC), rhoifolin (RHF), and rutin (RTN), and they were purchased from Funakoshi (Tokyo, Japan); and luteolin (LTN) and quercetin (QCT) were purchased from Sigma-Aldrich (Tokyo, Japan). 3,3′,4′,5,6,7,8-Heptamethoxyflavone (HPT), natsudaidain (NTD), nobiletin (NBL), and tangeretin (TNG) were isolated from King juice (*Citrus nobilis*) (Kawaii et al., 1999a).

Leaf samples. Leaves of 68 taxonomically representative *Citrus* and related genera were collected at random from adult trees at the National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, Japan in December 1997. The sample of leaves was divided into three replicates consisting of five leaves. The freeze-dried leaves were ground, and 100 mg of the sample was extracted with 1 mL of MeOH−DMSO (1:1) 3 times. The extracts were combined and filled up to 5 mL by MeOH. A 5-μL portion of the combined extracts was injected to HPLC. HPLC analysis of flavonoids was done primarily according to the method described in the literature (Vandercook and Tisserat, 1989). Analytical conditions included the following column: TSK gel Super-ODS, particle size 2 μm, 10 cm × 4.6 mm (i.d.) (Tosoh, Tokyo, Japan). Mobile phase; the gradient elution program consisted of an initial 2 min of 80% 0.01 M H_3PO_4 and 20% MeOH, followed by a linear gradient to 55% 0.01 M H_3PO_4 and 45% MeOH in 43 min, and was used for analysis of ERC, NERC, RTN, NRTN, NGIN, HSP, IRHF, NHSP, RHF, DSM, NDSM, KMP, and APG. For analysis of polymethoxylated flavones (namely, SNT, NBL, NTD, HPT, DNBL, and TNG), isocratic elution (mobile phase; 50% 0.01 M H_3PO_4 and 50% MeOH) was done to obtain better peak separation. Another isocratic elution (mobile phase; 70% 0.01M H_3PO_4 and 30% MeOH) was used for QCT, NGEN, LTN, NPNC, and PNC analysis. Under these conditions, the peaks were reasonably separated to integrate peak area. Assignment of flavonoids was basically done on the co-chromatographed peak by examination of changes in the chromatogram and UV spectrum detected by photodiode array detector. Concentrations of the compounds were calculated from integration peak areas of the sample and the corresponding authentic standards. The UV diode array detector was set to measure spectra from 200 to 400 nm and the eluent was monitored at 285 nm for flavanones and 360 nm for flavones and polymethoxylated flavones.

Statistical Analysis. The quantitative data set, which was composed of values taken from HPLC analysis of the leaves of 68 *Citrus* species, cultivars, and near-*Citrus* relatives, was used for principal component analysis. The statistical analysis program JMP (SAS Institute, Inc., Cary, NC) was used to calculate the data, and the graph software Kaleida Graph (Synergy Software, Reading, PA) was used to plot the results from principal component analysis. Each principal component was calculated by taking a linear combination of an eigenvector of the correlation matrix with a standardized original variable.

RESULTS AND DISCUSSION

Isolation and Identification of Demethylnobiletin. We reported the isolation of four polymethoxylated flavones from King (*Citrus nobilis*) juice as differentiation-inducing compounds toward HL-60 promyelocytic leukemic cells (Kawaii et al., 1999a). These compounds shared the most part of flavonoids in the region of polymethoxylated flavones of *Citrus* juices as indicated by HPLC analysis. However, a considerable amount of another polymethoxylated flavone, which was hardly observed in *Citrus* juices, was detected in various *Citrus* leaves. In the following experiment to obtain the unknown polymethoxylated flavone, Shikikitsu (*C. madurensis*) leaves were used.

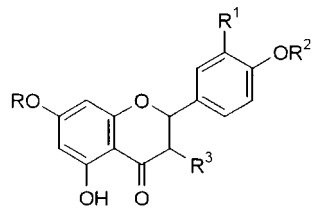
The fresh leaves (450 g) of Shikikitsu grown in the orchard of the National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, Japan, was extracted with ethanol at room temperature (× 3), and the combined extracts were evaporated under reduced pressure. The residue was dissolved in ethanol. The soluble portion was submitted in silica gel column chromatography eluted by benzene, benzene−isopropyl ether (3:1, 1:3), acetone, and methanol. The acetone elution was chromatographed over silica gel with isopropyl ether−acetone (10:1, 5:1, 4:1, 2:1, 1:1) to give five fractions. Further treatment by preparative thin-layer chromatography gave the unknown peak compound from isopropyl ether−acetone (10:1) fraction.

This compound had the molecular formula $C_{20}H_{20}O_8$ as determined by HREIMS (observed *m*/*z* 388.1166, calcd. for $C_{20}H_{20}O_8$, Δ + 0.8 mmu). The UV absorptions at λ_{max} 252, 284, and 337 nm were consistent with a flavonoid having a hydroxyl group (Voirin, 1983). The ^{1}H NMR spectrum revealed resonances for five aromatic methoxyl groups, one phenolic hydroxyl proton (which was strongly hydrogen bonded), one olefinic proton, and three aromatic protons. Coupling constants of these three aromatic protons were characteristic of a 1,3,4-trisubstituted benzene ring. ^{1}H- and ^{13}C NMR data were reminiscent of nobiletin. The positions of substituents at ring B were deduced by NOE correlations between H-3/H-2′, H-6′, 3′-OCH_3/H-2′, and 4′-OCH_3/H-5′. One hydroxyl group could be placed at C-5 because of its deshielded position. From these data, this unknown peak compound was identified as demethylnobiletin (Figure 1). The chemical shifts of ^{1}H- and ^{13}C NMR were unambiguously assigned from the HMQC and HMBC spectra (Table 1). To the best of our knowledge, this is the first report on the complete NMR signal assignment of demethylnobiletin (DNBL).

Quantitation of Flavonoid Contents in *Citrus* leaves. All of the *Citrus* leaves studied in this paper were collected in the same field and year and, therefore, were produced under the same conditions of climate to reduce this additional source of variance. Table 2 summarizes the quantitative determination of the major flavonoids in 68 representative and/or economically important *Citrus* leaves according to Tanaka's classification (Tanaka, 1969).

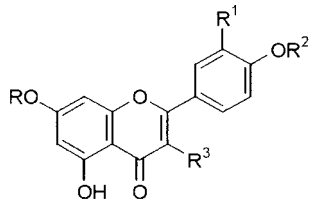
HSP was the most abundant flavonoid in the *Citrus* samples studied, followed by RTN and DSM; whereas NBL is the most widely distributed flavonoid (detected in 57 samples). No HSP, or only a small amount, was found in the samples from group IV, several species belonging to group V (namely, Natsudaidain (*C. natsudaidai*), Kawano Natsudaidai (*C. natsudaidai*), Sanbokan (*C. sulcata*), and sour orange (*C. aurantium*)), group VIII, kumquats (*Fortunella crassifolia*, *F. japonica*, *F. margarita*), and trifoliate orange (*Poncirus trifoliate*).

Most *Citrus* fruits and leaves can be classified by their glycosilation pattern as either predominately neohesperidosyl flavonoids, such as RHF, NGIN, and NHSP, or predominately rutinosyl flavonoids, such as HSP, DSM, and NRTN (Albach and Redman, 1969; Kanes et al., 1992; Tsuchida et al., 1997). To study the distribution of the rutinosyl-flavonoid predominant species and the neohesperidosyl-flavonoid predominant species, the scores for HSP (as a representative of rutinosyl flavonoids) and NGIN (as a representative of neohesperi-



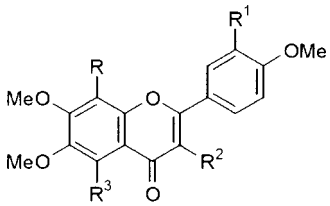
flavanones

Eriocitrin (ERC):R=rutinosyl, R^1=OH, R^2=R^3=H
Neoeriocitrin (NERC): R=neohesperidosyl, R^1=OH, R^2=R^3=H
Narirutin (NRTN): R=rutinosyl, R^1=R^2=R^3=H
Naringin (NGIN):R=neohesperidosyl, R^1=R^2=R^3=H
Hesperidin (HSP) : R=rutinosyl, R^1=OH, R^2=Me, R^3=H
Neohesperidin (NHSP): R=neohesperidosyl, R^1=OH, R^2=Me, R^3=H
Naringenin (NGEN) : R=R^1=R^2=R^3=H
Neoponcirin (NPNC) : R=rutinosyl, R^1=H, R^2=Me, R^3=H
Poncirin (PNC) : R=neohesperidosyl, R^1=H, R^2=Me, R^3=H



flavones

Rutin (RTN): R=H, R^1=OH, R^2=H, R^3=O-rutinose
Isorhoifolin (IRHF): R=rutinosyl, R^1=R^2=R^3=H
Rhoifolin (RHF): R=neohesperidosyl, R^1=R^2=R^3=H
Diosmin (DSM) : R=rutinose, R^1=OH, R^2=Me, R^3=H
Neodiosmin (NDSM): R=neohesperidosyl, R^1=OH, R^2=Me, R^3=H
Quercetin (QCT): R=H, R^1=OH, R^2=H, R^3=OH
Luteolin (LTN): R=H, R^1=OH, R^2=R^3=H
Apigenin (APG): R=R^1=R^2=R^3=H



polymethoxylated flavones

Sinensetin (SNT): R=H, R^1=OMe, R^2=H, R^3=OMe
Nobiletin (NBL): R=R^1=OMe, R^2=H, R^3=OMe
3,3',4',5,6,7,8-Heptamethoxyflavone (HPT)
:R=R^1=R^2=R^3=OMe
Natsudaidain (NTD): R=R^1=OMe, R^2=OH, R^3=OMe
5-Demethylnobiletin (DNBL): R=R^1=OMe, R^2=H, R^3=OH
Tangeretin (TNG): R=OMe, R^1=R^2=H, R^3=OMe

Figure 1. Structures of the flavonoids.

dosyl flavonoids) are plotted as a scatter diagram in Figure 2. Figure 2 clearly demonstrates the mutually exclusive relationship between HSP and NGIN content. The highest amount of HSP was found in Bilolo (*C. montana*, group II), and the high-HSP-containing species are largely in group VII. On the other hand, the highest amounts of NGIN were found in group IV, such as Hirado buntan (*C. grandis*), Shaten yu (*C. pauban*), and Marsh grapefruit (*C. paradisi*).There were some exceptions that contained a considerable amount of both HSP and NGIN. These species, which had mixed glycosilation patterns (indicated as R/N in Table 2) were mostly species of the IV and VI groups, and several species belonging to the group V. The mixed-glycosilation species in group IV are likely hybrids between pummelo and mandarin (Swingle, 1943); the results of comprehensive study on the inheritance of rutinosyl-flavonoid and neohesperidosyl-flavonoid alleles has been reported already (Albach and Redman, 1969; Kanes et al., 1993).

Table 1. Chemical Shifts, HMBC, and NOE Correlations of Demethylnobiletin in $CDCl_3$

position	δ ^{13}C (multiplicity[a])	δ ^{1}H (multiplicity, J_{H-H})	HMBC	NOE
2	164.0 (s)			
3	104.0 (d)	6.62 (s)	C-2,4,10,1′	H-2′,6′
4	183.0 (s)			
5	149.6 (s)			
6	136.6 (s)			
7	153.0 (s)			
8	133.0 (s)			
9	145.8 (s)			
10	107.0 (s)			
1′	123.8 (s)			
2′	108.8 (d)	7.43 (d, 2)	C-2,4′,6′	
3′	149.4 (s)			
4′	152.5 (s)			
5′	111.3 (d)	7.01 (d, 8)	C-1′,3′	
6′	120.2 (d)	7.60 (dd, 2, 8)	C-2,2′,4′	
5-OH		12.54 (s)	C-5,6,10	
6-OCH_3	61.2 (q)	3.96 (s)	C-6	
7-OCH_3	61.8 (q)	4.11 (s)	C-7	
8-OCH_3	62.1 (q)	3.984 (s)	C-8	7-OCH_3
3′-OCH_3	56.0 (q)	3.99 (s)	C-3′	
4′-OCH_3	56.2 (q)	3.979 (s)	C-4′	H-5′

[a] Multiplicities were established by an HMQC experiment.

Evaluation of the coefficients of correlation between HSP and other flavonoids and between NGIN and other flavonoids (see Table 3 in Supporting Information) indicates that HSP content positively correlates with the flavonoids possessing rutinose as a sugar moiety, namely RTN (0.22), IRHF (0.22), and DSM (0.69), whereas NGIN has a positive correlation with the flavonoids possessing neohesperidose as a sugar part, namely NERC (0.15), RHF (0.45), and NDSM (0.23).

NBL was the most abundant polymethoxylated flavone, and the highest concentrations of it were observed in Obeni mikan (*C. tangerina*, group VII), Cleopatra (*C. reshni*), and Dancy tangerine (*C. tangerina*, group VII). NTD content is of great interest because NTD has the most potent antitumor (Kawaii et al., 1999d) and differentiation-inducing activities (Kawaii et al., 1999c).

Previously we have reported that *Citrus* species can be classified by their pattern of polymethoxylated flavones in the edible part of fruits, i.e., the high-NBL-TNG species and the high-HPT-NTD species (Kawaii et al., 1999b). The significance of correlation has been shown between NBL and TNG, and between NTD and HPT in leaves (see Table 3 in the Supporting Information). Similarly strong correlation was observed among NBL, DNBL, and TNG contents in *Citrus* leaves (NBL−DNBL, r = 0.85; NBL−TNG, r = 0.90; DNBL−TNG, r = 0.81). Content of NTD and HPT in leaves also positively correlated, but the correlation coefficient was

Table 2. Flavonoid Constituents in Leaves of the Various Citrus Species[a]

Tanaka's no.	scientific name	conventional name	glycosilation pattern[b]	ERC (R)	NERC (N)	RTN (R)	NRTN (R)	NGIN (N)	HSP (R)	IRHF (R)	NHSP (N)	UF1	RHF (N)	DSM (R)	NDSM (N)	QCT	NGEN	NPNC	PNC	APG	SNT	NBL	HPT	NTD	DNBL	TNG
II-4−14	C. latifolia	Tahiti lime	R	250.0	0	254.0	0	0	53.4	97.7	0	0	0	124.7	0	0	0	0	0	0	0	0	0	2.1	4.2	0
II-4−15	C. limettioides	Sweet lime	R	0	0	153.7	0	0	109.0	410.8	0	0	0	57.2	0	0	0	0	0	0	0	0	3.3	1.6	0.9	1.6
II-5−17	C. bergamia	Bergamot	N	0	216.5	0	0	0	0	0	0	0	309.0	0	0	0	0	0	1.7	0	10.0	0	0	1.6	0	0
II-6−29	C. montana	Bilolo	R	0	0	1587	0	0	2774	0	0	193.9	0	1331	0	0	0.7	0	0	0	14.3	115.9	9.6	0	65.1	13.7
III-8−36	C. limon	Eureka lemon	R	60.0	0	591.3	0	0	0	142.7	0	0	0	204.0	0	0	0	0.7	0	0	0	0.5	0	1.3	2.8	0
III-8−37	C. limonia	Rangpur lime	R	161.1	0	741.1	0	0	190.2	121.5	0	0	0	327.0	0	0	0	2.5	0	0	9.3	58.8	0	0	52.4	131.1
III-8−40	C. meyerii	Meyer lemon	R	0	0	269.3	0	0	405.5	134.3	0	0	0	220.2	0	0	0	1.2	0	0	1.2	9.2	1.4	0.8	2.2	4.4
III-9−48	C. lumia	Lumie	R	1132.9	0	1821	0	0	0	151.3	0	289.6	0	556.8	0	0	0	2.4	0	0	0	3.4	0	0	2.2	1.0
IV-10−56	C. grandis	Hirado buntan	N	0	73.7	0	0	1076	0	0	0	0	562.1	0	50.3	0	0	0	0	0	0	0.7	0.7	0.7	0	0
IV-10−61	C. panuban	Shaten yu	N	0	0	0	0	455.8	0	0	0	0	414.7	0	0	0	0	0	0	0	0	0	0	2.0	1.1	0
IV-11−62	C. paradisi	Marsh GF	R/N	0	0	0	80.5	402.2	0	91.8	0	0	352.9	0	0	0	0	3.2	3.0	0	0	3.6	0.6	2.2	2.2	1.8
IV-11−62	C. paradisi	Red blush	R/N	0	0	105.1	100.5	378.8	0	113.8	0	0	0	0	0	0	0	0	1.4	0	0.8	5.0	0	1.6	2.5	1.0
IV-11−63	C. glaberrima	Kinukawa	R/N	0	113.9	0	0	0	0	0	539.6	129.5	0	99.1	63.2	0	0	0	0	0	2.8	18.2	1.8	1.7	4.9	7.0
IV-12−74	C. hassaku	Hassaku	R/N	0	18.3	0	0	0	44.9	0	340.5	0	0	0	32.9	0	0	0.7	0	0	0	2.0	0.8	1.6	2.0	1.7
IV-12−76	C. tengu	Tengu	R	0	0	75.0	829.3	0	0	590.4	0	0	0	43.1	0	0	0	2.5	0	0	4.3	43.7	5.8	2.0	19.1	25.4
V-13−78	C. natsudaidai	Natsudaidai	R/N	0	27.4	0	0	347.2	0	55.1	1123	0	166.6	0	77.9	0	0	0.6	0	0	7.8	15.3	0	0	5.2	7.7
V-13−78	C. natsudaidai	Kawano natsudaidai	R/N	0	25.1	0	0	93.7	0	0	529.8	0	110.2	47.4	61.6	0	0	0.8	0	0	1.5	10.4	0.7	1.2	3.9	4.5
V-13−84	C. sulcata	Sanbokan	R	191.4	0	297.4	173.2	0	79.3	78.5	0	342.8	0	196.7	0	0	0	0	0	0	0	24.2	3.5	0	10.0	18.1
V-14−93	C. aurantium	Sour orange	N	0	217.6	0	0	159.6	0	0	0	234.0	0	0	36.8	0	0	0	0	0	0	20.6	0	1.8	4.5	6.9
V-16−100	C. sinensis	Valencia	R	0	0	215.1	0	0	498.6	233.6	0	204.8	0	163.2	0	0	0	0	0	0	19.8	30.8	0	2.0	19.9	7.2
V-16−100	C. sinensis	Morita navel	R	0	0	212.5	0	0	741.2	283.6	0	260.2	0	206.9	0	0	0	0.8	1.5	0	24.3	38.7	0	0	23.5	8.9
V-16−101	C. sinograndis	Oto	R	0	0	95.6	0	0	550.1	147.4	0	169.1	0	213.3	0	0	0	1.1	0	0	3	21.4	0	0	9.9	23.7
V-16−105	C. iyo	Iyo	R	0	0	81.5	15.0	0	356.3	0	0	203.0	0	90.9	0	0	0	0.8	0	0	0	52.6	7.1	0	16.0	18.9
V-16−105	C. iyo	Miyauchi iyo	R	0	0	155.3	84.8	0	601.5	0	0	290.2	0	108.9	0	0	0.4	0	0	0	8.8	68.4	6.8	0	9.0	23.6
V-17−107	C. tamurana	Hyuganatsu	R/N	0	0	0	0	305.0	348.9	418.1	0	131.7	0	127.8	0	0	0	0	0	0	0	16.9	4.0	1.7	7.0	16.6
V-19−111	C. shunkoukan	Shunkoukan	R/N	0	0	177.9	126.2	126.7	612.5	182.4	0	0	0	126.8	0	0	0.3	0	0	0	0	37.2	0	1.5	22.2	6.8
VI-21−113	C. junos	Yuzu	R/N	0	0	145.5	0	54.2	577.6	0	909.7	0	0	37.8	38.0	0	0.6	0	0	0	0	0	0	0.9	12.5	0
VI-21−113	C. junos	Mukaku yuzu	R/N	0	0	163.2	0	0	182.8	0	368.9	0	0	0	31.4	0	0	0	0	0	0	0	0	2.0	0	0
VI-21−114	C. hanayu	Hanayu	R/N	253.4	154.6	50.3	0	34.5	111.9	0	593.6	0	0	0	0	0	0	0.9	0	0	19.9	132.7	0	5.9	85.4	83.4
VI-21−115	C. sudachi	Sudachi	R/N	33.8	42.5	75.6	0	0	0	0	60.1	0	0	0	38.6	0	0	0	0	0	0	1.2	0	1.5	0	1.3
VI-21−116	C. inflata	Mochiyu	R/N	0	0	255.0	0	0	131.3	0	344.7	0	0	0	54.5	0	0	0	0	0	0	12.2	2	1.4	4.6	4.5
VI-21−120	C. wilsonii	Ichang lemon	N	0	0	0	0	382.3	0	0	0	55.7	0	0	0	0	0	1.5	0	0	0	0	0	1.7	0	0
VI-21−121	C. sphaerocarp	Kabosu	R/N	0	0	43.8	0	0	105.6	0	198.8	0	0	0	62.8	0	0	2.1	0	0	0.8	7.0	2.4	1.6	5.3	5.3
VI-22−122	C. nippokorean	Kourai tachibana	R/N	521.7	0	0	581.8	320.3	109.5	0	0	0	0	0	0	0.7	1.8	0	0	45.8	73.5	0	0	44.5	36.9
VII-23−123	C. nobilis	King	R	0	0	146.2	0	0	1543	123.0	0	100.0	0	239.2	0	0	0	0	0	0	19.3	205.1	17.5	0	39.2	101.0
VII-23−123	C. nobilis	Kunenbo	R	0	0	0	0	0	491.6	252.9	0	494.7	0	131.0	0	0	0	0	0	0	3.5	31.0	25.9	1.9	3.1	18.4
VII-23−124	C. unshu	Unshu	R	0	0	0	0	0	2336	231.9	0	455.7	0	324.3	0	0	0	0	0	0	17.6	43.5	10.8	0	8.0	16.4
VII-23−124	C. unshu	Sugiyama unshiu	R	0	0	0	0	0	1873	217.8	0	358.0	0	293.6	0	0	0	0.8	0	0	5.3	21.3	5.3	1.8	8.6	8.8
VII-23−124	C. unshu	Wase unshiu	R	0	0	0	0	0	2467	285.2	0	317.6	0	322.4	0	0	0	0.6	0	0	6.7	26.5	4.7	0	8.1	9.7
VII-23−124	C. unshu	Okitsu wase	R	0	0	267.3	0	0	920.4	199.9	0	293.3	0	235.0	0	0	0	0	0	0	4.6	20.6	3.4	2.9	10.1	9.2
VII-23−125	C. yatsusiro	Yatsusiro	R	0	0	0	0	0	558.6	60.1	0	87.2	0	45.9	0	0	0	0	0	0	6.1	58.1	10.9	18.2	17.1	49.5
VII-24−126	C. keraji	Keraji	R	0	0	0	0	0	614.5	71.1	0	170.1	0	72.9	0	0	0	0	0	0	3.3	63.8	10.2	1.4	8.4	48.1
VII-24−126	C. keraji	Kabuchi	R	0	0	179.8	0	0	600.5	99.3	0	256.4	0	119.4	0	0	0	0	0	0	9.5	134.4	12.1	0	30.6	138.8
VII-24−127	C. oto	Oto	R	0	0	0	0	0	963.8	0	0	84.0	0	49.6	0	0	0	0	0	0	6.9	67.3	14.2	0	5.4	39.8
VII-25−130	C. reticulata	Ponkan	R	0	0	0	0	0	1481	64.7	0	150.9	0	229.7	0	0	0.4	0.7	0	0	23.1	354.8	0	0	192.4	233.0
VII-25−130	C. reticulata	Ota ponkan	R	0	0	0	0	0	1239	129.6	0	223.1	0	308.5	0	0	0	0.7	0	0	30.2	371.3	0	0	151.0	217.9
VII-25−131	C. deliciosa	Mediterranean mandarin	R	0	0	459.9	0	0	1263	112.2	0	368.0	0	458.0	0	0	0.4	2.6	0	0	39.1	708.6	0	0	398.6	349.9
VII-25−133	C. tangerina	Obenimikan	R	0	0	307.7	0	0	1538	161.7	0	413.2	0	359.6	0	0	0.5	1.0	0	0	79.9	913.5	0	0	285.1	322.7
VII-25−133	C. tangerina	Dancy tangerine	R	0	0	298.3	0	0	2185	130.0	0	0	0	339.2	0	0	0	1.1	0	0	74.0	876.4	0	0	270.0	318.3
VII-25−134	C. clementina	Clementine	R	0	0	885.1	0	0	763	420.8	0	428.6	0	253.0	0	0	0	1.4	0	0	46.4	137.6	0	0	67.4	67.0
VII-25−136	C. succosa	Jimikan	R	0	0	342.3	0	0	1986	143.3	0	268.4	0	420.6	0	0	0.4	1.9	0	0	76.7	726.8	0	0	372.9	753.6
VII-25−140	C. suhuiensis	Shikaikan	R	0	0	0	0	0	639.5	0	0	43.5	0	65.9	0	0	0	0.8	0	0	77.8	615.8	0	0	96.6	474.6
VII-26−143	C. tachibana	Tachibana	R	0	0	0	0	0	72.9	89.7	0	101.9	0	90.7	0	0	0.4	0	0	0	71.7	586.4	0	0	243.3	371.8
VII-26−144	C. erythrosa	Kobeni mikan	R	0	0	0	0	0	813.6	0	0	181.7	0	342.5	0	0	0	0	0	0	32.7	328.4	0	0	114.5	181.7

Table 2. (Continued)

Tanaka's no.	scientific name	conventional name	glycosilation pattern[b]	ERC (R)	NERC (N)	RTN (R)	NRTN (R)	NGIN (N)	HSP (R)	IRHF (R)	NHSP (N)	UF1	RHF (N)	DSM (R)	NDSM (N)	QCT	NGEN	NPNC	PNC	APG	SNT	NBL	HPT	NTD	DNBL	TNG
VII–26–145	C. kinokuni	Hirakishu	R	0	0	332.4	0	0	1213	94.9	0	0	0	466.6	0	0	0	0.7	0	0	80.2	619.9	0	0	217.2	378.4
VII–26–145	C. kinokuni	Mukaku kishu	R	0	0	206.2	0	0	1010	51.9	0	0	0	271.2	0	0	0.4	0	0	0	78.5	645.7	0	3.6	196.4	368.8
VII–26–145	C. kinokuni	Sokitsu	R	0	0	198.4	0	0	1141	82.7	0	0	0	300.7	0	0	0	0.5	0	0	67.1	425.5	0	1.3	110.6	358.0
VII–26–148	C. sunki	Sunki	R	0	0	368.7	0	0	1039	192.0	0	0	0	405.5	0	0	0	1.3	0	0	457.9	634.5	0	2.3	164.4	119.8
VII–26–149	C. reshni	Cleopatra	R	0	0	301.4	0	0	1074	168.7	0	219.6	0	530.3	0	0	0	2.2	0	0	88.3	906.0	0	0	64.7	478.9
VII–26–150	C. tardiva	Giri mikan	R	55.1	0	41.2	0	0	341.4	0	0	0	0	102.9	0	0	0	1.9	0	0	10.4	125.1	0	2.3	10.2	212.6
VII–27–153	C. depressa	Shiikuwasha	R	0	0	106.8	0	0	1113	117.2	0	47.7	0	154.7	0	0	0	0	0	0	30.3	360.5	0	2.7	43.7	263.8
VII–27–154	C. leiocarpa	Koji	R/N	750.0	0	514.8	0	84.4	145.0	215.5	0	258.4	0	444.5	0	0	0	1.9	0	0	31.4	279.9	0	1.3	286.2	195.9
VII–27–155	C. tumida	Fukure mikan	R/N	207.2	0	167.8	0	213.8	504.7	73.5	0	179.7	0	340.6	0	0	0	0.6	0	0	49.7	478.6	0	1.7	173.1	226.1
VIII–28–159	C. madurensis	Shikikitsu	N	0	0	0	0	0	0	0	0	0	174.3	0	71.6	0	0	0	0	0	17.5	147.1	0	1.0	15.7	71.6
102–302	F. crassifolia	Neiha kumquat	N	0	0	0	0	0	0	0	0	0	628.2	0	0	2.9	0	0	0	330.5	0	0	0	1.4	0	0
102–303	F. japonica	Round kumquat	N	0	0	0	0	0	0	0	0	0	473.3	0	0	4.2	0	0	0	175.8	0	0	0	1.0	0	0
102–304	F. margarita	Naga kumquat	N	0	0	0	0	0	0	0	0	0		0	0	0.7	0	0	0	252.9	0	0	0	0	1.4	0	0
103–401	P. trifoliate	Trifoliate orange	R/N	0	0	38.7	0	36.3	19.2	0	0	0	73.0	0	0	0	0	0	0	0	0	0	0	0	0.8	0	0

[a] All values are given in μg/100 mg of dried sample. Listed in increasing retention order on an RP-18 HPLC column. 0: not detected. UF1: unidentified flavonoids, possibly apigenin glycoside. [b] Glycosilation pattern: R, predominantly rutinosyl flavonoid; N, predominantly neohesperidosyl flavonoid; R/N, mixture type (R, rutinosyl flavonoid; N, neohesperidosyl flavonoids).

Figure 2. Relationship between HSP and NGIN contents. Symbols: Δ, group II; ○, group III; ◊, group IV; □, group V; ▲, group VI; ■, group VII; ●, group VIII; ♦, kumquats and Trifoliate orange, according to Tanaka's classification. Open symbols indicate Archicitrus and closed symbols indicate Metacitrus.

Figure 3. Relationship between NBL and NTD contents. Symbols: Δ, group II; ○, group III; ◊, group IV; □, group V; ▲, group VI; ■, group VII; ●, group VIII; ♦, kumquats and Trifoliate orange.

much lower ($r = 0.13$) than that found in the edible part of *Citrus* fruits ($r = 0.48$) (Kawaii et al., 1999b).

To study distribution of the high-NBL-TNG species and the high-HPT-NTD species, the scatter diagram for NBL and NTD content in *Citrus* leaves was plotted (Figure 3). This graphic shows that high-polymethoxylated-flavone-containing species belong largely to group VII according to Tanaka's classification. Figure 3 shows the peculiarity of Yatsusiro (*C. yatsusiro*), which contained the highest amount of NTD. However, we could not demonstrate differentiation between the high-NBL-TNG species and the high-HPT-NTD species in leaves.

Principal Component Analysis. Principal component analysis has been done in order to differentiate *Citrus* plants based on their flavonoid profile. KMP and LTN were excluded from PCA, because no leaf sample contained these flavonoids. The data for the 23 flavonoids, including an unidentified flavonoid (UF1), were used to perform PCA, which can reduce the dimensionality of a set of data. The eigenvalues are 5.20, 2.50, 2.27, 2.04, 1.67, 1.27, 1.22, and 1.01 (Table 4 in the Supporting Information). Choosing only eigenvalues >1

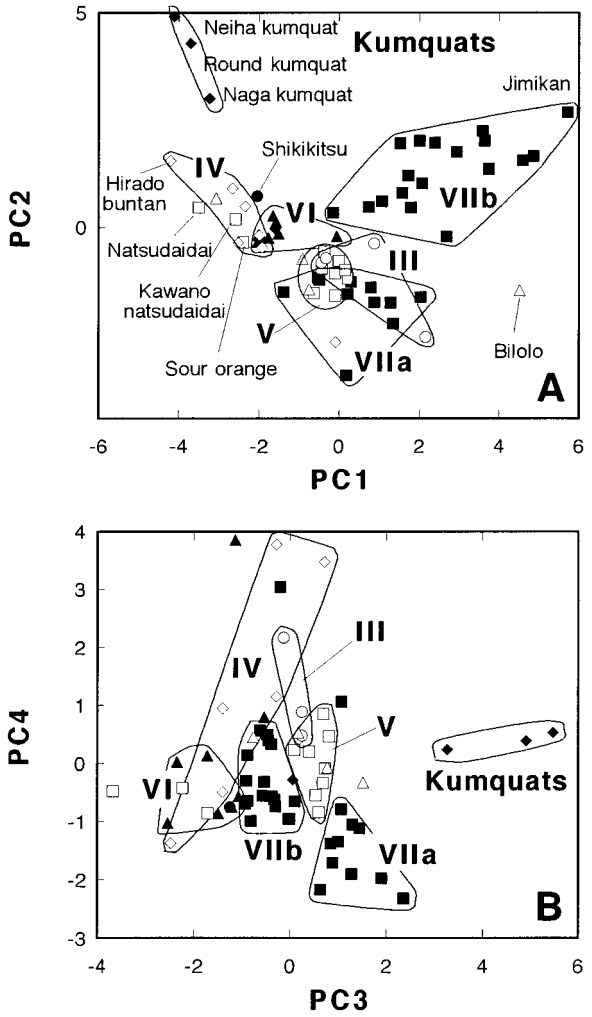


Figure 4. Projection of scatter diagram from a principal component analysis. (A) PC1 vs PC2; (B) zoom of the plot of PC1 vs PC2. Symbols: Δ, group II; ○, group III; ◊, group IV; □, group V; ▲, group VI; ■, group VII; ●, group VIII; ♦, kumquats and Trifoliate orange.

led to the reduction of 23 variables to 8 principal components (PC), according 74.7% of the total variability. The percentages of variance for the four principal components are 22.6% for the first one, 10.9% for the second one, 9.9% for the third one, and 8.9% for the last one.

Factor loadings (Table 5 in the Supporting Information) indicate the relative extent to which each original variable contributes to the variance contained in each principal component. With first principal component (PC1), four polymethoxylated flavones (i.e., SNT (0.22), NBL (0.28), DNBL (0.24), and TNG (0.27)) are positively correlated. RHF (0.26) and two free flavones (i.e., QCT (0.38) and APG (0.39)) are positively correlated with the second principal component (PC2). The third principal component (PC3) is positively correlated with PNC (0.23) and HPT (0.20), and negatively correlated with two neohesperidosyl flavonoids (i.e., NHSP (−0.38) and NDSM (−0.38)). The fourth principal component (PC4) is strongly correlated with three rutinosyl flavonoids (i.e., NRTN (0.62), IRHF (0.23), and NPNC (0.25)).

The scores for the first two PCs are plotted as a scatter diagram in Figure 4A. This graphic shows that the distribution of *Citrus* species belonging to different classes can be observed. This graphic shows that seven associations according to Tanaka's classification appeared to be separate, but that some do overlap each other. Interestingly, group VII species could be divided into 2 different subgroups, named VIIa and VIIb on the basis of flavonoid profiles. Subgroup VIIa consists of the first 10 species of the VII group (i.e., King (VII-23-123), Kunenbo (VII-23-123), Unshu (VII-23-124), Sugiyama unshu (VII-23-124), Wase unshu (VII-23-124), Okitsu wase (VII-23-124), Yatsusiro (VII-23-125), Keraji (VII-24-126), Kabuchi (VII-24-126), and Oto (VII-24-127)), whereas subgroup VIIb contained the remaining last 19 species of group VII (i.e., Ponkan (VII-25-130), Ota ponkan (VII-25-130), Mediterranean mandarin (VII-25-131), Obenimikan (VII-25-133), Dancy tangerine (VII-25-133), Clementin (VII-25-134), Jimikan (VII-25-136), Shikaikan (VII-25-140), Tachibana (VII-26-143), Kobeni mikan (VII-26-144), Hirakishu (VII-26-145), Mukaku Kishu (VII-26-145), Sokitsu (VII-26-145), Sunki (VII-26-148), Cleopatra (VII-26-149), Girimikan (VII-26-150), Shiikuwasha (VII-27-153), Koji (VII-27-154), and Fukuremikan (VII-27-155)), according to the Tanaka's classification number. Separation of the VIIa and VIIb subgroups is also observed in a scatter diagram plotting the scores for the third (PC3) and fourth (PC4) principal components (Figure 4B). Peculiarities of Natsudaidai and Kawano Natsudaidai, which is a variation of Natsudaidai, could be explained by hybridity with sour orange (*C. aurantium*) (Swingle, 1943).

Citrus taxonomy is one of the most complex taxonomies, because of hybridization, apomixis, and many centuries of cultivation. These compositional data may contribute to information toward *Citrus* genetics and inheritance. Yamamoto et al. (1998) studied variety difference of limonoid (bitter-taste component) contents in 54 *Citrus* cultivars, and reported that the pummelos and the pummelo-like miscellaneous contained high amounts of limonoids in their fruits. The flavonoid profiles presented herein seemed to be independent of the limonoid contents; therefore, principal component analysis based on the contents of not only flavonoids but also limonoids would give us more comprehensive results on *Citrus* chemotaxonomy.

Supporting Information Available: Tables containing the correlation coefficient matrix, principal components and eigenvectors, and factor loadings and communality values. This material is available free of charge via the Internet at http://pubs.acs.org.

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