Quantitation of Flavonoid Constituents in *Citrus* Fruits

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Twenty-four flavonoids have been determined in 66 *Citrus* species and near-citrus relatives, grown in the same field and year, by means of reversed phase high-performance liquid chromatography analysis. Statistical methods have been applied to find relations among the species. The *F* ratios of 21 flavonoids obtained by applying ANOVA analysis are significant, indicating that a classification of the species using these variables is reasonable to pursue. Principal component analysis revealed that the distributions of *Citrus* species belonging to different classes were largely in accordance with Tanaka's classification system.

Keywords: *Citrus; near-citrus relatives; flavonoids; HPLC; multivariate analysis*

INTRODUCTION

Flavonoids are important secondary plant metabolites and are mainly present in plant tissues in relatively high concentrations as sugar conjugates. Epidemiological studies have indicated that flavonoid consumption is associated with a reduced risk of cancer (Wattenberg, 1985, 1990; Verma et al., 1988; Wei et al., 1990). *Citrus* fruits are a rich source of flavonoids. Determination of *Citrus* fruits with high concentrations of individual flavonoids is desirable to study their biological properties. In a recent literature review on *Citrus* flavonoids, a broad spectrum of biological activity including anticarcinogenic and antitumor activities was discussed (Attaway, 1994; Benavente-García et al., 1997; Chen et al., 1997). It is commonly accepted that cancer formation can be prevented by the consumption of certain foods (Stavric, 1994), and flavonoids in *Citrus* fruits and juices are one of the most prominent cancer-preventing agents. Quercetin and rutin appear to inhibit colonic neoplasia induced by azoxymethanol (Deschner, 1992). Diosmin and hesperidin reduce the incidence and multiplicity of neoplasm in the large intestine of male F344 rats initiated with azoxymethane (Tanaka et al., 1997). These flavonoids also inhibit the development of aberrant crypt foci. Several polymethoxylated flavones show differentiation-inducing activity toward human acute promyelocytic leukemia cells (HL-60) and mouse myeloid leukemia cells (M1) (Sugiyama et al., 1993; Kawaii et al., 1999a,b). Inducers of HL-60 differentiation are recognized to have a potentially therapeutic importance (Koeffler, 1983). Luteolin and natsudaidain demonstrate strong antiproliferative activity toward several cancer cell lines, whereas they have weak antiproliferative activity against the normal human cell lines (Kawaii et al., 1999c).

Numerous quantitative studies (Vandercook and Stevenson, 1966; Albach and Redman, 1969; Ting et al., 1979; Park et al., 1983) 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,b; Gentili and Horowitz, 1964). A few studies, however, have been done with the aim of finding composition parameters that could establish similarities among *Citrus* cultivars with flavonoids by using a multivariate statistical method. Gaydou et al. (1987) has successfully applied multivariate analysis to differentiation of orange and mandarin using the composition of a few key polymethoxylated flavones in peel oils of commercially important cultivars.

In this study, high-performance liquid chromatography (HPLC) analysis of 24 flavonoids in edible parts has been done on the representative or economically important *Citrus* species, cultivars, and near-citrus relatives. We have precisely evaluated the influence of cultivar on flavonoid composition by multivariate analysis. The aim of this study is to contribute to a better understanding of genetic relationships in *Citrus* and related genera by flavonoid profile. This information will aid in horticultural breeding programs focused on health promotion by selecting varieties rich in anticancer substances.

MATERIALS AND METHODS

Flavonoids. Flavonoids used in this study are as follows: 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), rutin (RTN), and sinensetin (SNT), purchased from Funakoshi (Tokyo, Japan); and luteolin (LTN), quercetin (QCT), and taxifolin (TXF), 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).

Fruit Samples. All fruits were harvested from trees at the National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, Japan, in December 1997. The freeze-dried fruits were divided into peel and edible part. The edible part, which consisted of juice sac and segment epidermis, was ground, and 100 mg of the sample was extracted with 1 mL of MeOH/DMSO (1:1) three times. The extracts were combined and made up to 5 mL by MeOH. Twenty microliters of the combined extracts was injected into HPLC. HPLC analysis of flavonoids was done primarily according to the method described in the literature (Vandercook and Tisserat, 1989).

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Table 1. Flavonoid Contents in the Edible Part of Various Citrus Fruits

Tanaka's no.	scientific name	conventional name	TXF	ERC	NERC	NRTN	RTN	NGIN	HSP	IRHF	NHSP	RHF	DSM	NDSM
II-4-14	*C. latifolia*	Tahiti lime	0	186	0	0	18.1	0	572	55.1	0	0	83.6	0
II-5-17	*C. bergamia*	Bergamot	0	22.6	670	20.9	5.4	598	42.0	22.0	508	107	0	44.5
III-8-36	*C. limon*	Eureka lemon	0	245	0	0	22.7	0	358	0	0	0	73.2	0
III-8-37	*C. limonia*	Rangpur lime	0	33.1	0	2.2	35.9	0	472	0	0	0	51.1	0
III-8-40	*C. meyerii*	Sweet lemon	0	2.7	7.8	14.1	18.7	0	1099	12.0	0	0	57.9	0
III-8-40	*C. meyerii*	Meyer lemon	0	2.0	0	2.4	3.1	0	855	0	0	0	39.6	0
III-9-48	*C. lumia*	Lumie	0	143	0	0	9.1	0	985	0	0	0	38.2	0
IV-10-56	*C. grandis*	Hirado buntan	8.1	6.7	8.1	0	0	397	8.5	0	0	24.7	0	0
IV-10-61	*C. panuban*	Shaten yu	16.9	3.6	3.7	2.5	0	170	42.6	0	0	13.6	0	0
IV-11-62	*C. paradisi*	Red blush	1.7	1.8	9.9	285	0	1143	19.0	0	76.2	9.1	0	0
IV-11-62	*C. paradisi*	Marsh grapefruit	3.8	1.2	3.5	500	0	1459	5.0	0	88.3	12.8	0	0
IV-11-63	*C. glaberrima*	Kinukawa	0	9.6	17.5	42.6	0	85.2	0	0	35.5	7.0	0	0
IV-12-74	*C. hassaku*	Hassaku	0	7.1	4.2	98.2	0	138	33.7	0	67.6	9.9	0	0
IV-12-76	*C. tengu*	Tengu	0	0	0	634	0	0	29.1	9.8	0	3.3	0	0
V-13-78	*C. natsudaidai*	Natsudaidai	8.1	6.1	6.4	61.2	0	636	9.7	0	141	34.1	0	0
V-13-78	*C. natsudaidai*	Kawano Natsudaidai	0	1.3	4.9	45.8	0	440	11.9	0	97.8	19.5	0	0
V-13-84	*C. sulcata*	Sanbokan	0	73.4	1.4	502	0	4.7	189	0	0	0	0	0
V-14-93	*C. aurantium*	sour orange	0	13.2	331	11.8	0	377	10.6	0	324	26.5	0	5.7
V-16-100	*C. sinensis*	Valencia	0	6.9	0	75.7	3.5	0	698	0	0	0	0	0
V-16-101	*C. sinograndis*	Oto mikan	0.9	3.7	0	433	0	0	583	106	0	0	28.7	0
V-16-100	*C. sinensis*	Morita navel	0	11.9	4.1	444	13.7	0	1080	0	0	6.6	9.0	0
V-16-105	*C. iyo*	Iyo	0	7.9	0	251	0	0	406	0	0	0	0	0
V-16-105	*C. iyo*	Miyauchi iyo	0	3.4	0	108	0	0	264	0	0	0	0	0
V-17-107	*C. tamurana*	Hyuganatsu	0	3.4	0	167	0	0	165	0	0	0	0	0
V-19-111	*C. shunkoukan*	Shunkoukan	0	23.2	3.5	1022	20.7	0	387	107	0	4.6	10.2	0
VI-21-113	*C. junos*	Yuzu	0.9	2.8	3.4	309	8.1	162	94.8	0	64.4	0	0	0
VI-21-113	*C. junos*	Mukaku yuzu	0	1.6	5.1	303	0	134	156	0	84.0	0	0	0
VI-21-114	*C. hanayu*	Hanayu	0	3.3	2.7	35.6	0	32.5	2001	0	209	0	4.3	0
VI-21-115	*C. sudachi*	Sudachi	0.8	149	80.6	270	0	98.4	163	0	220	0	7.9	0
VI-21-116	*C. inflata*	Mochiyu	0.6	2.5	8.2	203	0	103	96.1	0	69.5	0	0	0
VI-21-121	*C. sphaerocarp*	Kabosu	1.0	4.6	5.2	90.1	0	30.2	65.2	0	23.4	0	0	0
VI-21-120	*C. wilsonii*	Ichang lemon	1.4	0	4.8	0	9.4	482	60.7	0	5.1	10.6	0	0
VI-22-122	*C. nippokorean*	Kourai tachibana	0	2.8	19.7	689	0	0	177	0	0	0	0	0
VII-23-123	*C. nobilis*	Kunenbo	0.8	20.8	5.0	634	12.0	0	588	5.1	0	9.5	13.0	0
VII-23-123	*C. nobilis*	King	0	1.6	0	319	0	0	1172	0	0	3.3	0	4.7
VII-23-124	*C. unshu*	Unshu	0	5.9	11.5	61.1	0	0	1596	0	0	0	4.1	0
VII-23-124	*C. unshu*	Sugiyama unshiu	0	8.4	3.1	625	23.0	0	664	0	0	10.4	12.9	0
VII-23-124	*C. unshu*	Okitsu wase	0	0	11.1	157	4.2	0	456	0	0	9.3	11.0	0
VII-23-125	*C. yatsusiro*	Yatsusiro	0	0	1.4	4.0	3.0	0	549	0	0	0	0	0
VII-24-126	*C. keraji*	Keraji	0	19.8	1.8	99.2	39.7	0	843	0	0	0	0	0
VII-24-126	*C. keraji*	Kabuchi	0	3.0	4.3	11.4	0	0	379	0	0	0	0	0
VII-24-127	*C. oto*	Oto	0	0	0	22.6	0	0	1030	0	0	0	0	0
VII-25-130	*C. reticulata*	Ponkan	0	1.3	1.7	148	0	0	1199	0	0	0	0	0
VII-25-130	*C. reticulata*	Ota ponkan	0	5.7	3.2	73.4	0	0	676	0	0	0	0	0
VII-25-131	*C. deliciosa*	Mediterranean mandarin	0	0	1.6	174	0	0	1464	18.5	0	0	12.5	0
VII-25-133	*C. tangerina*	Dancy tangerin	0	6.7	1.9	192	0	0	1513	37.6	0	0	0	0
VII-25-133	*C. tangerina*	Obenimikan	0	8.0	1.5	121	0	0	1734	0	0	0	0	0
VII-25-134	*C. clementina*	Clementine	0	3.1	4.5	51.1	0	0	852	0	0	0	3.3	0
VII-25-136	*C. succosa*	Jimikan	0	2.2	6.3	276	0	0	1326	0	0	3.1	4.6	0
VII-25-140	*C. suhuiensis*	Shikaikan	0	5.2	2.0	193	0	0	1197	0	0	0	0	0
VII-26-143	*C. tachibana*	Tachibana	0	13.1	5.5	14.2	0	24.4	626	0	3.4	0	11.5	0
VII-26-144	*C. erythrosa*	Kobeni mikan	0	1.7	14.1	94.0	0	0	869	0	0	0	14.1	0
VII-26-145	*C. kinokuni*	Hirakishu	0	3.3	8.9	27.1	0	0	621	0	0	0	12.4	0
VII-26-145	*C. kinokuni*	Sokitsu	0.5	1.7	1.5	167	13.4	0	409	0	0	13.8	8.0	0
VII-26-145	*C. kinokuni*	Mukaku kishu	0	4.6	7.3	129	0	0	1319	9.9	0	0	25.9	0
VII-26-148	*C. sunki*	Sunki	0	9.1	9.7	68.5	tr	5.9	1520	0	0	0	8.4	0
VII-26-149	*C. reshni*	Cleopatra	0	3.9	0	95.9	0	0	1905	30.5	0	3.3	4.0	0
VII-26-150	*C. tardiva*	Giri-mikan	0	64.6	0	349	3.7	0	119	0	0	0	0	0
VII-27-153	*C. depressa*	Shiikuwasha	0	0	5.1	18.4	4.2	0	992	0	0	0	12.9	0
VII-27-154	*C. leiocarpa*	Koji	0	142	1.9	189	0	0	180	0	0	0	3.3	0
VII-27-155	*C. tumida*	Fukure mikan	0	10.1	8.9	24.1	0	0	980	0	0	0	13.1	0
VIII-28-159	*C. madurensis*	Shikikitsu	0	0	1.4	142	25.5	0	50.3	0	26.6	0	0	0
102-302	*F. crassifolia*	Neiha kumquat	0	1.5	2.5	320	0	6.2	2.4	0	1.4	74.7	0	0
102-303	*F. japonica*	round kumquat	0.9	1.5	0	460	33.9	0	9.3	0	0	18.9	0	0
102-304	*F. margarita*	Naga kumquat	0	1.4	0	289	0	0	5.4	0	0	25.5	0	0
103-401	*P. trifoliate*	Trifoliate orange	0	0	23.2	20.7	0	123	0	20.1	0	3.5	8.9	0

Analytical conditions were as follows: column, Hypersil RP-18; particle size, 5 μm; 10 cm × 4.6 mm (i.d.) (Hewlett-Packard, Wilmington, DE); 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 100% MeOH in 55 min. For analysis of polymethoxylated flavones, 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% 0.01 M H_3PO_4 and 30% MeOH) was used for analysis of QCT, NGEN, LTN, NPNC, and PNC. Assignments of flavonoids were basically done by cochromatography with the authentic flavonoid and then by careful comparison

Table 1 (Continued)

Tanaka's no.	scientific name	conventional name	QCT	NGEN	LTN	NPNC	PNC	KMP	APG	SNT	NBL	HPT	NTD	TNG
II-4-14	*C. latifolia*	Tahiti lime	0	0	0	0.2	0	0	0	0	0	2.5	1.1	1.4
II-5-17	*C. bergamia*	Bergamot	0	0	0	222	0	0	0	0	0.1	0.2	0.3	0.2
III-8-36	*C. limon*	Eureka lemon	0	0	0	0	0	0	0	0	0.1	0.1	0.1	0.0
III-8-37	*C. limonia*	Rangpur lime	1.0	0	0.2	0.9	0	0	0	0	0.6	0.1	0.2	1.4
III-8-40	*C. meyerii*	Sweet lemon	0.8	0	0	2.5	0	0	0	0	0.1	0	0.2	0
III-8-40	*C. meyerii*	Meyer lemon	0.3	0	0	1.4	0	0	0	0	0.4	0.2	0	0.1
III-9-48	*C. lumia*	Lumie	0.7	0	0	0.4	0	0	0	0	0.1	0.1	0	0.0
IV-10-56	*C. grandis*	Hirado buntan	0.3	0	0	9.3	0	0	0	0	0.1	0	0	0.7
IV-10-61	*C. panuban*	Shaten yu	0	0	0	178	0	0	0	0	0	0	0	0
IV-11-62	*C. paradisi*	Red blush	0	0	0	15.1	84.1	0	0	0	0.4	0.2	0.1	0.1
IV-11-62	*C. paradisi*	Marsh grapefruit	0	0	0	12.2	67.8	0	0	0	0.2	0.2	0	0.2
IV-11-63	*C. glaberrima*	Kinukawa	0	0	0	2.7	3.2	0	0	0	0.1	0.2	0	0.2
IV-12-74	*C. hassaku*	Hassaku	0	0	0	3.0	4.1	0	0	0	0.2	0.5	0	0.3
IV-12-76	*C. tengu*	Tengu	0	0	0	8.2	0	0	0	0	0.4	0.1	0	0.2
V-13-78	*C. natsudaidai*	Natsudaidai	0	0	0	7.4	7.1	0	0	0	0.4	0.5	0.5	1.0
V-13-78	*C. natsudaidai*	Kawano Natsudaidai	0.3	0	0	9.7	2.3	0	0	0	0.6	0.2	0.9	0.5
V-13-84	*C. sulcata*	Sanbokan	0	0	0	1.1	0	0	0	0	0.9	1.6	0.5	1.1
V-14-93	*C. aurantium*	sour orange	0	0	0	21.6	0	0	0	0	0.7	0.5	0.5	0.5
V-16-100	*C. sinensis*	Valencia	3.1	0	0	9.8	0.4	0	0	4.0	1.3	0.4	0.2	0.3
V-16-101	*C. sinograndis*	Oto mikan	0.4	0	0	19.6	0	0	0	0	0.4	0.2	0	0.3
V-16-100	*C. sinensis*	Morita navel	1.0	0	0.2	39.9	0	0	0	0	2.2	0.5	0	0.3
V-16-105	*C. iyo*	Iyo	1.3	0	0	7.7	0	0	0	0	2.0	0.8	0	1.1
V-16-105	*C. iyo*	Miyauchi iyo	0.8	0	0.1	3.8	0	0	0	0	1.2	0.4	0	0.6
V-17-107	*C. tamurana*	Hyuganatsu	0	0	0	23.0	0	0	0	0	1.5	0.9	0	1.3
V-19-111	*C. shunkoukan*	Shunkoukan	0.6	0	0	6.9	0	0	0	0	3.7	0.7	0	1.6
VI-21-113	*C. junos*	Yuzu	0	0	0	3.6	3.0	0	0	0	0.1	0.0	0.1	0.1
VI-21-113	*C. junos*	Mukaku yuzu	1.4	0.3	0.6	3.5	2.2	0	0	0	0	0.4	0.3	0.1
VI-21-114	*C. hanayu*	Hanayu	0	0	0	0.8	1.7	0	0	0	4.6	0	0	2.4
VI-21-115	*C. sudachi*	Sudachi	0	0	0	0.9	1.1	0	0	0	0.2	0.3	0	0
VI-21-116	*C. inflata*	Mochiyu	0	0	0	10.0	1.5	0	0	0	0.3	0.3	0	0.3
VI-21-121	*C. sphaerocarp*	Kabosu	0	0	0	1.0	0	0	0	0	0.5	0.5	0.4	0.5
VI-21-120	*C. wilsonii*	Ichang lemon	0	0	0	1.1	0	0	0	0	8.9	1.5	1.4	6.5
VI-22-122	*C. nippokorean*	Kourai tachibana	0	0	0	14.2	0	0	0	10.7	3.2	0.3	1.7	1.2
VII-23-123	*C. nobilis*	Kunenbo	0.3	0	0	22.5	0	0	0	0	1.3	2.0	0	0.9
VII-23-123	*C. nobilis*	King	0.7	0	0	45.9	0	0	0	0	1.4	4.0	2.5	2.0
VII-23-124	*C. unshu*	Unshu	2.0	0	0	35.2	0	0	0	0	8.6	0	0	4.7
VII-23-124	*C. unshu*	Sugiyama unshiu	1.1	0	0	34.7	0	0	0	0	0.9	1.2	0	0.5
VII-23-124	*C. unshu*	Okitsu wase	0	0	0	10.6	0.8	0	0	0	0.9	0.9	0	0.4
VII-23-125	*C. yatsusiro*	Yatsusiro	0.4	0	0	2.2	0	0	0	0	0.8	1.1	0	0.8
VII-24-126	*C. keraji*	Keraji	0	0	0	2.1	0	0	0	0	2.1	1.2	0.2	1.7
VII-24-126	*C. keraji*	Kabuchi	0	0	0	3.5	0	0	0	0	5.2	1.3	0	7.4
VII-24-127	*C. oto*	Oto	1.3	0	0.1	5.3	0	0	0	0	1.7	1.8	1.9	1.1
VII-25-130	*C. reticulata*	Ponkan	0	0	0.2	32.8	0	0	0	0	12.8	0	0	9.1
VII-25-130	*C. reticulata*	Ota ponkan	0	0	0	13.5	0	0	0	0	5.3	0	0	5.2
VII-25-131	*C. deliciosa*	Mediterranean mandarin	0	0	0	25.7	0	0	0	0	5.7	0	0	3.5
VII-25-133	*C. tangerina*	Dancy tangerin	1.5	0.2	0.4	21.2	0	0	0	0	4.5	0.1	0	1.5
VII-25-133	*C. tangerina*	Obenimikan	0	0	0	11.3	0	0	0	0	6.1	0	0	2.4
VII-25-134	*C. clementina*	Clementine	0.9	0	0	8.4	0	0	0	0	0.8	0.8	0	0.3
VII-25-136	*C. succosa*	Jimikan	0.8	0	0	27.5	0	0	0	0	5.5	0	0	3.2
VII-25-140	*C. suhuiensis*	Shikaikan	0.9	0	0	24.5	0	0	0	0	3.5	0.3	0	1.4
VII-26-143	*C. tachibana*	Tachibana	0	0	0	3.9	2.1	0	0	66.6	8.2	0.1	0.1	4.1
VII-26-144	*C. erythrosa*	Kobeni mikan	0	0	0.2	22.4	0	0	0	0	5.2	0.1	0	3.5
VII-26-145	*C. kinokuni*	Hirakishu	0.8	0	0	3.2	0	0	0	0	6.7	0	0	3.9
VII-26-145	*C. kinokuni*	Sokitsu	1.3	0	0	14.4	0	0	0	0	0.4	0.3	0.2	0.1
VII-26-145	*C. kinokuni*	Mukaku kishu	1.5	0	0	7.7	0	0	0	0	12.5	0.3	0	6.0
VII-26-148	*C. sunki*	Sunki	2.6	0	0	18.6	0	0	0	0	3.4	0.1	0	0.9
VII-26-149	*C. reshni*	Cleopatra	0	0	0	8.8	0	0	0	0	4.0	1.0	0	4.4
VII-26-150	*C. tardiva*	Giri-mikan	0	0	0	27.1	0	0	0	0	1.8	0	0	1.9
VII-27-153	*C. depressa*	Shiikuwasha	0.4	0	0.3	5.3	0.6	3.3	0	6.0	21.0	0	0	8.8
VII-27-154	*C. leiocarpa*	Koji	0	0	0	15.3	0	0	0	0	2.7	0	3.1	2.4
VII-27-155	*C. tumida*	Fukure mikan	0	0	0	5.4	1.3	0	0	0	10.0	0	0	6.4
VIII-28-159	*C. madurensis*	Shikikitsu	0	0	0	0.7	0	0	0	0	2.0	0.1	0	1.0
102-302	*F. crassifolia*	Neiha kumquat	3.4	0	13.8	1.4	9.7	32.1	0	0	0.1	0.1	0	0.1
102-303	*F. japonica*	round kumquat	8.3	0	8.7	3.1	4.0	20.4	0	0	0.1	0	0	0.1
102-304	*F. margarita*	Naga kumquat	0	0	6.8	0.8	2.9	16.1	0	0	0	0	0	0
103-401	*P. trifoliate*	Trifoliate orange	4.6	0	0	41.3	289	8.8	0	0	0.7	0	0	0

[a] All values are given in μg/100 mg of dried sample for $n = 3$. Listed in increasing retention order on an RP-18 HPLC column. 0, not detected. tr, detected but too small to quantify.

of test samples with the authentic flavonoid mixture. 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 at 360 nm for flavones. Under these conditions the authentic flavonoids were separated from each other.

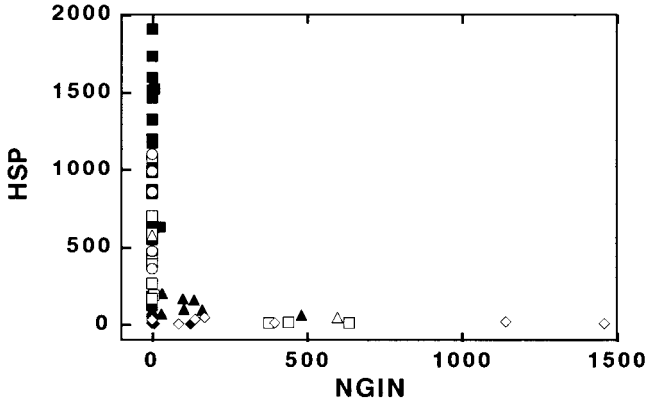

Figure 1. Exclusive relationship between HSP and NGIN contents: (△) group II; (○) group III; (◇) group IV; (□) group V; (▲) group VI; (■) group VII; (●) group VIII; (◆) kumquats and Trifoliate orange according to Tanaka's classification. Open symbols indicate *Archicitrus* and closed symbols *Metacitrus* subgenera.

Statistical Analysis. A quantitative data set, which was composed of values taken from HPLC analysis of the edible parts of 66 citrus fruits, was used for multivariate analysis. The statistical analysis program JMP (SAS Institute Inc., Cary, NC) was used to calculate and plot results from principal component analysis (PCA). One-way ANOVA, according to the Tukey−Kramer honestly significant difference (HSD) test, was applied to the contents of flavonoids that could contribute to find differences among the *Citrus* cultivars. The statistical significance at $p = 0.05$ was estimated by the actual absolute difference in the means minus the least significant difference (LSD), which is the difference that would be significant. PCA was done to find associations among the citrus samples by taking into account the 21 flavonoids determined. PCA was applied to data that had been previously autoscaled, using the eigenvalues >1. Each principal component is calculated by taking a linear combination of an eigenvector of the correlation matrix with a standardized original variable.

RESULTS AND DISCUSSION

All of the *Citrus* samples studied in this paper were harvested in the same field and year and, therefore, were produced under the same conditions of climate to reduce additional sources of variance. Table 1 summarizes the quantitative determination of flavonoids in various samples classified according to Tanaka's system (Tanaka, 1969). HSP [548.2 ± 523.7 μg/100 mg of dry weight (mean ± standard deviation, $n = 66$), detected in 64 of 66 samples] is the most abundant flavonoid and widely distributed in the citrus samples studied, followed by NRTN (185.5 ± 208.2 μg/100 mg, detected in 62 samples), NGIN (100.7 ± 257.9 μg/100 mg, detected in 31 samples), NHSP (31.0 ± 83.3 μg/100 mg, detected in 27 samples), NERC (20.5 ± 90.4 μg/100 mg, detected in 50 samples), and ERC (20.2 ± 46.8 μg/100 mg, detected in 57 samples). HSP was not found in Trifoliate orange (*Poncirus trifoliate*), and only a small amount was detected in group IV and several species belonging to group V, namely, Natsudaidai, Kawano Natsudaidai, and sour orange. In contrast, these species contain higher amounts of NGIN than those of the high-HSP-containing species. HSP and NGIN seem to be mutually exclusive; thus, they cannot exist together at high concentrations. A relationship of this kind has been already reported in several kinds of *Citrus* fruits (Albach and Redman, 1969; Kanes et al., 1992; Tsuchida et al., 1997). Figure 1 also shows the exclusive relationship between HSP and NGIN contents. The high-NGIN-containing species are largely group VII species. Exceptions are the group VI species. These species contain perceptible amounts of both HSP and NGIN and seem to form an independent group on the basis of the pattern of HSP and NGIN contents. LTN and KMP were mainly found in *Fortunella* species.

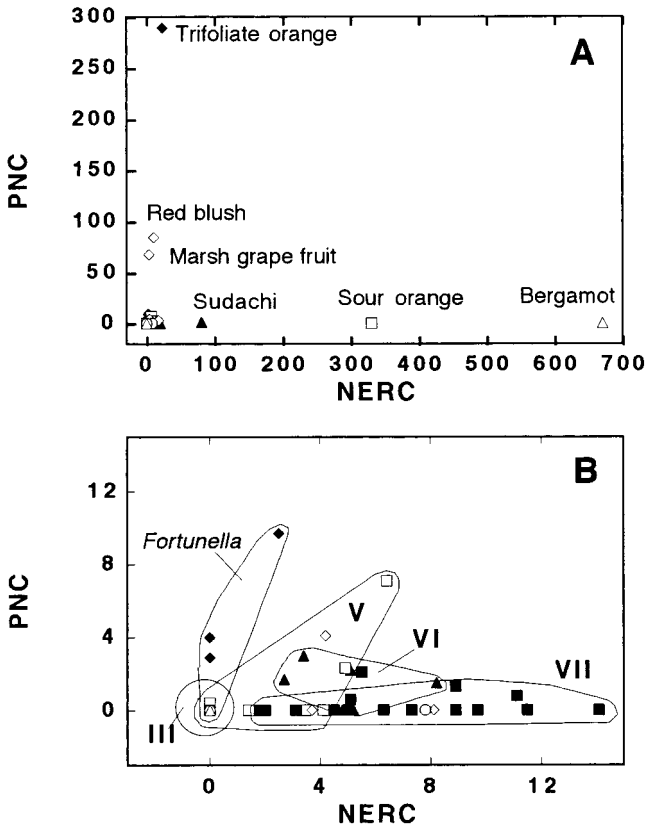

Figure 2. Projection of scatter diagram of (A) NERC vs PNC and (B) zoom of the plot of NERC vs PNC: (△) group II; (○) group III; (◇) group IV; (□) group V; (▲) group VI; (■) group VII; (●) group VIII; (◆) kumquats and Trifoliate orange.

Table 2. *F*-Ratio Values When the Species Are Compared in Pairs for All of the Flavonoids Considered

flavonoid	*F* ratio	flavonoid	*F* ratio
TXF	1.06	QCT	3.94
ERC	34.80	NGEN	0.97
NERC	182.61	LTN	15.05
NRTN	6.96	NPNC	9.34
RTN	1.18	PNC	417.20
NGIN	45.16	KMP	4.75
HSP	7.52	APG	ND
IRHF	7.14	SNT	0.99
NHSP	15.73	NBL	7.95
RHF	8.93	HPT	14.63
DSM	5.66	NTD	3.83
NDSM	19.38	TNG	8.40

Precise information is provided by one-way ANOVA using the Tukey−Kramer HSD test, which allows all pairwise comparisons of means. The *F*-ratio values for each variable (Table 2) are >1 in 21 of 24 flavonoids studied, indicating that these flavonoids show differences among the citrus cultivars examined. It is likely that flavonoids with high *F*-ratio values, such as PNC (*F* ratio = 417.20) and NERC (*F* ratio = 182.61) could differentiate among cultivars. Figure 2 shows the scatter diagram for NERC and PNC. These graphics show the peculiarities of Trifoliate orange, red blush (IV), Marsh grapefruit (IV), Bergamot (II), sour orange (V), and Sudachi (IV). Magnification of the complicated part in

Table 3. Correlation Coefficient Matrix for the Flavonoids and Principal Components

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
eigenvalue	4.41	3.06	2.36	1.98	1.51	1.35	1.13	1.07
%	20.99	14.55	11.25	9.44	7.20	6.44	5.37	5.12
cum %	20.99	35.54	46.80	56.24	63.44	69.87	75.25	80.36
eigenvectors								
TXF	0.13	−0.01	−0.02	−0.37	−0.17	−0.01	−0.28	0.66
ERC	−0.03	−0.05	0.43	0.18	−0.19	−0.28	0.05	−0.00
NERC	0.41	−0.17	−0.01	0.24	−0.00	0.02	0.06	−0.13
NRTN	−0.01	0.19	0.13	−0.06	0.39	0.50	−0.14	−0.13
RTN	−0.04	0.15	0.30	0.26	−0.13	0.06	−0.11	0.10
NGIN	0.25	−0.05	−0.01	−0.35	−0.07	0.01	0.21	−0.03
HSP	−0.23	−0.19	−0.16	0.31	0.00	0.15	0.04	0.18
IRHF	−0.01	−0.00	0.20	0.19	0.06	0.62	0.09	0.12
NHSP	0.40	−0.16	0.02	0.06	−0.04	−0.04	0.03	−0.29
RHF	0.41	0.12	−0.09	0.17	0.06	−0.08	−0.09	0.07
DSM	−0.12	−0.04	0.38	0.30	−0.37	−0.06	0.08	0.14
NDSM	0.40	−0.17	−0.01	0.28	0.06	0.04	0.05	−0.01
QCT	0.00	0.40	−0.12	0.18	−0.04	0.05	0.29	0.17
LTN	0.08	0.48	−0.15	0.19	0.09	−0.17	−0.15	0.04
NPNC	0.34	−0.16	−0.07	0.08	−0.02	0.15	−0.05	0.46
PNC	0.06	0.15	−0.05	−0.19	−0.21	0.15	0.77	0.06
KMP	0.08	0.49	−0.17	0.18	0.04	−0.15	0.01	0.06
NBL	−0.18	−0.22	−0.41	0.23	−0.01	0.01	0.09	0.06
HPT	−0.05	−0.08	0.25	0.04	0.55	−0.10	0.16	0.24
NTD	0.00	−0.10	0.19	−0.01	0.50	−0.37	0.27	0.19
TNG	−0.18	−0.25	−0.37	0.21	0.06	−0.07	0.09	0.10

Table 4. Factor Loadings and Communality Values

	factor loading								
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	communality
TXF	0.09	0.01	−0.02	0.02	−0.00	−0.04	0.07	0.74	0.95
ERC	−0.02	−0.03	−0.08	0.36	−0.07	−0.18	0.00	−0.05	0.68
NERC	−0.27	−0.02	0.03	0.03	0.02	0.01	−0.01	−0.13	0.96
NRTN	0.04	−0.01	−0.13	−0.20	−0.03	0.50	0.14	−0.11	0.77
RTN	−0.01	0.08	−0.02	0.29	0.04	0.12	0.09	0.06	0.49
NGIN	−0.03	−0.11	−0.15	−0.12	0.00	−0.07	−0.26	0.04	0.58
HSP	−0.02	0.01	0.32	0.07	−0.01	0.13	−0.01	0.07	0.66
IRHF	−0.05	−0.05	0.08	0.08	0.04	0.55	−0.12	0.05	0.70
NHSP	−0.22	−0.09	−0.10	−0.03	0.08	−0.08	0.01	−0.22	0.87
RHF	−0.19	0.16	−0.01	−0.01	−0.02	−0.03	0.10	0.07	0.90
DSM	−0.01	−0.01	0.05	0.46	0.06	−0.03	−0.09	0.06	0.84
NDSM	−0.27	0.01	0.08	0.04	−0.04	0.06	0.00	−0.05	0.95
QCT	0.01	0.28	0.11	0.07	−0.04	0.06	−0.28	0.04	0.73
LTN	−0.00	0.35	−0.01	−0.01	−0.01	−0.07	0.16	0.00	0.93
NPNC	−0.17	0.03	0.15	0.04	−0.06	0.13	−0.03	0.43	0.86
PNC	0.02	0.01	0.02	0.01	−0.04	0.02	−0.77	−0.06	0.92
KMP	−0.00	0.35	0.01	0.00	−0.02	−0.07	0.00	−0.01	0.94
NBL	−0.04	0.03	0.35	−0.07	0.03	−0.04	−0.04	−0.02	0.82
HPT	0.01	0.02	0.03	−0.02	−0.54	0.11	−0.01	0.05	0.74
NTD	−0.01	0.04	0.01	−0.02	−0.60	−0.13	−0.07	0.00	0.79
TNG	−0.03	0.02	0.34	−0.07	−0.06	−0.09	−0.02	0.00	0.79

Figure 2A shows some associations of groups III and V−VII and *Fortunella* species, whereas group IV species are relatively dispersed.

In this study, *F*-ratio values of NGEN, APG, and SNT are <1, and therefore these flavonoids are excluded from consideration in the next statistical analysis. The data for the 21 flavonoids were used for PCA, which can reduce the dimensionality of a set of data, and thus plotted in conventional two-dimensional graphics. The eigenvalues obtained from the correlation matrix are 4.41, 3.06, 2.36, 1.98, 1.51, 1.35, 1.13, and 1.07 (Table 3). Choosing only eigenvalues >1 led to the reduction of 21 flavonoids to 8 principal components (PC), according 80.4% of the total variability. The percentages of variance for the four PCs are 20.99% for the first one, 14.55% for the second one, 11.25% for the third one, and 9.44% for the last one.

The correlation between each PC and each original variable is given in Table 4. The four flavonoids that contributed most to PC1 possess neohesperidose as a sugar part (NERC, NHSP, RHF, and NDSM). The factor loadings for NERC, NHSP, RHF, and NDSM were very similar, suggesting the existence of some common biosynthetic pathway in which they take part. This suggestion is supported by correlation analysis on these flavonoids, by which positive correlations between NERC and NHSP of 0.8621, between NERC and RHF of 0.7163, and between NERC and NDSM of 0.9330 are found. QCT, LTN, and KMP are positively correlated with the second principal component (PC2). With the third principal component (PC3), HSP and two polymethoxylated flavones, namely, NBL and TNG, have strong correlation. The forth principal component (PC4) is positively correlated with ERC, RTN, and DSM and negatively correlated with NRTN and NGIN.

The score for the first two PCs is plotted as a scatter diagram in Figure 3A. This graphic shows the peculiarities of Trifoliate orange, *Fortunella* species, Bergamot, and sour orange. The rest of the *Citrus* species form a jungle. After a zoom of the thicket, the distribution of

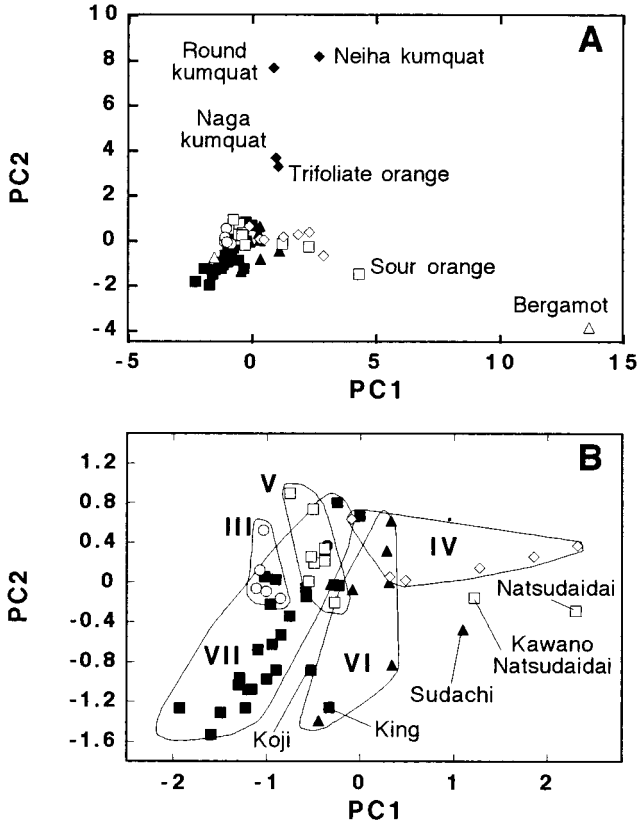

Figure 3. (A) Projection of scatter diagram from a PCA, PC1 vs PC2, and (B) zoom of the plot of PC1 vs PC2: (△) group II; (○) group III; (◇) group IV; (□) group V; (▲) group VI; (■) group VII; (●) group VIII; (◆) kumquats and Trifoliate orange.

Citrus species belonging to different classes can then be observed (Figure 3B). This graphic shows that five groups according to Tanaka's classification appeared to be separated apart from some overlap of groups III, V, and VII. Exceptions are Natsudaidai (*C. natsudaidai*), Kawano Natsudaidai, which is a variation of Natsudaidai, and Sudachi. The hybridity of Natsudaidai according to Swingle's classification (Swingle, 1943) could explain their peculiarities in group V. Numerical taxonomic study also reveals that morphology of Natsudaidai is different from that of sweet oranges according to Swingle's classification (Handa and Oogaki, 1985).

Tanaka's classification system is an excellent descriptive morphology of *Citrus* biotype and equates morphology diversity with speciation. Tanaka classified the forms of *Citrus* into two subgenera, namely Archicitrus and Metacitrus subgenera, based on their inflorescence pattern, 8 sections, 13 subsections, 8 groups, and 145 species (Tanaka, 1969). Quantitation of flavonoids in the edible part of various *Citrus* fruits suggested that flavonoid content followed the variation of morphological citrus biotypes because some agreement with Tanaka's classification system is observed.

Factor loadings (Table 4) indicate the relative extent to which each original variable contributes to the variance contained in each PC. PC3 and PC5 strongly correlated with NBL and TNG with and HPT and NTD, respectively. Similar factor loadings of NBL and TNG for PC3 (0.35 and 0.34, respectively) and the coefficient of correlation ($r = 0.9075$) between these polymethoxylated flavones suggest possible relationship between them. A similar relationship was also suggested for HPT and NTD (factor loadings are −0.54 and −0.60 for PC5,

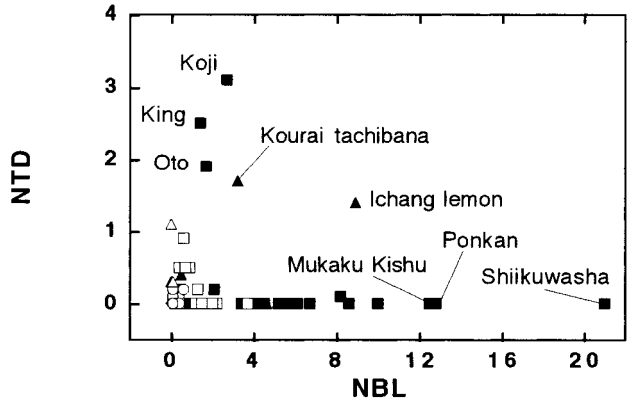

Figure 4. Relationship between NBL and NTD contents: (△) group II; (○) group III; (◇) group IV; (□) group V; (▲) group VI; (■) group VII; (●) group VIII; (◆) kumquats and Trifoliate orange.

respectively; $r = 0.4810$). This significance of correlation between NBL and TNG and between HPT and NTD suggests the existence of a common biosynthetic pathway. TNG has a structure similar to that of NBL, but it lacks the C-3′ methoxyl group of NBL. HPT is a 3-OH methylated derivative of NTD. These structural similarities also support the above-mentioned suggestion.

Previously we have reported the potent differentiation-inducing activity toward HL-60 promyelocytic leukemia cells in the readily extractable fraction prepared from King juice (Kawaii et al., 1999a). The polymethoxylated flavones, namely, NBL, HPT, NTD, and TNG, seemed to be responsible for the biological activity. Among these polymethoxylated flavones, NTD demonstrated most potent activity.

The scatter diagram for NBL and NTD (Figure 4) demonstrates peculiarities of King, Koji, Oto Mikan, Ichang lemon, Kourai Tachibana, Shiikuwasha, and Ponkan. Especially, King, Koji, and Oto Mikan demonstrate characteristic patterns, because these species contain higher concentrations of HPT and NTD than of NBL and TNG, ,whereas other species show reversed profiles of polymethoxylated flavone contents (Table 1). Characterization of polymethoxylated flavones in hybrids of the high-NBL−TNG species and the high-HPT−NTD species will give us important information on the inheritance of polymethoxylated flavones.

Daily consumption of health-promoting food seems to link disease prevention and, therefore, flavonoids in the edible part of *Citrus* are more beneficial than those in the other inedible parts. However, only a few studies have been done on flavonoids in the edible part of *Citrus* fruits. The data presented in this paper provide a qualitative and quantitative survey of the major flavonoids in the edible part of *Citrus* species, cultivars, and near-citrus relatives. This flavonoid survey on both the commercial cultivars and important hybrids will aid not only in taxonomic evaluation by genetics and inheritance patterns but also in horticultural breeding programs geared toward health promotion.

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