HL-60 Differentiating Activity and Flavonoid Content of the Readily Extractable Fraction Prepared from *Citrus* Juices

Satoru Kawaii, Yasuhiko Tomono, Eriko Katase, Kazunori Ogawa, and Masamichi Yano*

National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, 424-0204 Japan

Citrus plants are rich sources of various bioactive flavonoids. To eliminate masking effects caused by hesperidin, naringin, and neoeriocitrin, the abundant flavonoid glycosides which make up 90% of the conventionally prepared sample, the readily extractable fraction from *Citrus* juice was prepared by adsorbing on HP-20 resin and eluting with EtOH and acetone from the resin and was subjected to HL-60 differentiation assay and quantitative analysis of major flavonoids. Screening of 34 *Citrus* juices indicated that King (*C. nobilis*) had a potent activity for inducing differentiation of HL-60, and the active principles were isolated and identified as four polymethoxylated flavonoids, namely, nobiletin, 3,3′,4′,5,6,7,8-heptamethoxyflavone, natsudaidain, and tangeretin. HPLC analysis of the readily extractable fraction also indicated that King contained high amounts of these polymethoxylated flavonoids among the *Citrus* juices examined. Principal component and cluster analyses of the readily extractable flavonoids indicated peculiarities of King and Bergamot.

Keywords: *Citrus; flavonoids; HPLC; multivariate analysis; differentiation; HL-60*

INTRODUCTION

Citrus plants are rich sources of various physiologically active substances, especially "health-promoting substances" (Benavente-García 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. In a recent literature review on *Citrus* flavonoids, a broad spectrum of biological activity including anticarcinogenic and antitumor activities was discussed (Attaway, 1994). Epidemiological studies have indicated that flavonoid consumption is associated with a reduced risk of cancer (Wattenburg, 1985, 1990; Verma et al., 1988; Wei et al., 1990). Quercetin and rutin 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 and also inhibit the development of aberrant crypt foci (Tanaka, 1997). Numerous quantitative studies (Albach and Redman, 1969; Vandercook and Stephenson, 1966; Park et al., 1983) on *Citrus* flavonoids have been conducted since the major flavonoids were identified in the late 1950s and 1960s (Horowitz, 1956, 1957; Horowitz and Gentili, 1960a,b; Gentili and Horowitz, 1964).

From a viewpoint of health promotion by dietary habits, cancer preventative activity in the *Citrus* juices is more important than that found in other inedible parts. However, few studies have focused on the biologically active substances in *Citrus* juices, possibly because the measurable activity of juice tended to be hindered by the more abundant substances, which had no activity in vitro. Sample preparation by the conventional solvent extraction using a methanol/dimethyl sulfoxide mixture [MeOH/DMSO (1:1)] is a relatively powerful and accurate way to determine flavonoid contents in *Citrus* fruits especially for chemotaxonomic study. Biological activity of such an extract, however, is hindered by the presence of high proportions of hesperidin, which comprises 50−80% of total free flavonoids in some *Citrus* juices (Kanes, 1992).

We have prepared a readily extractable flavonoid fraction from various *Citrus* juices, involving adsorption on HP-20, a porous polymer resin, and successive elution from the resin with EtOH and acetone. This method led to substantial decreases in hesperidin, naringin, and neoeriocitrin content in that fraction. We have selected 34 representative and/or economically important *Citrus* species according to Tanaka's classification. These fractions prepared from 34 *Citrus* juices were subjected to screening for cancer preventative effect, which was assayed by the differentiating activity against human promyelocytic leukemia cells (HL-60). The HL-60 cells (Collins et al., 1978), established from an acute myeloid leukemia patient, are blocked at a certain step of the cellular maturation process and display a high proliferation ability. Four polymethoxylated flavonoids were identified as active principles from King juice. HPLC analysis of the readily extractable flavonoids from *Citrus* juices was done to study the relationship between flavonoid contents and HL-60 differentiating activities.

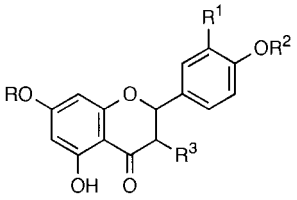
MATERIALS AND METHODS

Mass and Nuclear Magnetic Resonance Spectroscopy. Measurements of mass spectra were performed with a JEOL JMS700 mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) system. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX 400 spectrometer in $CDCl_3$. Chemical shifts were reported using residual $CHCl_3$ (δ 7.24) and $CDCl_3$ (δ 77.0) as internal standards.

Flavonoids. The flavonoids used in these experiments are listed in Figure 1. Taxifolin (TXF), eriocitrin (ERC), neoeriocitrin (NERC), narirutin (NRTN), naringin (NGIN), hesperidin (HSP), neohesperidin (NHSP), naringenin (NGEN), neoponcirin (NPNC), poncirin (PNC), rutin (RTN), isorhoifolin (IRHF), rhoifolin (RHF), diosmin (DSM), neodiosmin (NDSM), quercetin (QCT), luteolin (LTN), kaempferol (KMP), apigenin

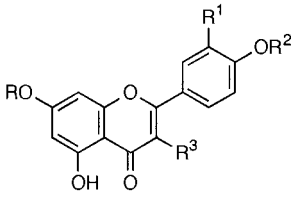
* Corresponding author (fax +81-543-69-2115; e-mail ym6082@okt.affrc.go.jp).

10.1021/jf9805101 CCC: $18.00
Published on Web 12/01/1998



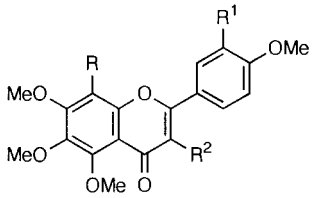
flavanone

TXF (1) : R=H, R^1=OH, R^2=H, R^3=OH
ERC (2) :R=rutinose, R^1=OH, R^2=R^3=H
NERC (3) : R=neohesperidose, R^1=OH, R^2=R^3=H
NRTN (4) : R=rutinose, R^1=R^2=R^3=H
NGIN (6) :R=neohesperidose, R^1=R^2=R^3=H
HSP (7) : R=rutinose, R^1=OH, R^2=Me, R^3=H
NHSP (9): R=neohesperidose, R^1=OH, R^2=Me, R^3=H
NGEN (14) : R=R^1=R^2=R^3=H
NPNC (15) : R=rutinose, R^1=H, R^2=Me, R^3=H
PNC (16) : R=neohesperidose, R^1=H, R^2=Me, R^3=H



flavone

RTN (5) : R=H, R^1=OH, R^2=H, R^3=O-rutinose
IRHF (8) : R=rutinose, R^1=R^2=R^3=H
RHF (10) : R=neohesperidose, R^1=R^2=R^3=H
DSM (11) : R=rutinose, R^1=OH, R^2=Me, R^3=H
NDSM (12) : R=neohesperidose, R^1=OH, R^2=Me, R^3=H
QCT (13) : R=H, R^1=OH, R^2=H, R^3=OH
LTN (17) : R=H, R^1=OH, R^2=R^3=H
KMP (18) : R=R^1=R^2=H, R^3=OH
APG (19) : R=R^1=R^2=R^3=H
ACT (21) : R=R^1=H, R^2=Me, R^3=H



polymethoxylated flavone

SNT (20) : R=H, R^1=OMe, R^2=H
NBL (22) : R=R^1=OMe, R^2=H
HPT (23) : R=R^1=R^2=OMe
NTD (24) : R=R^1=OMe, R^2=OH
TNG (25) : R=OMe, R^1=R^2=H

Figure 1. Structures of flavonoids studied. Numbers in parentheses indicate HPLC peak identification in Figure 2.

(APG), acacetin (ACT), and sinensetin (SNT) were purchased from Sigma (St. Louis, MO) or Funakoshi (Tokyo, Japan). Nobiletin (NBL), 3,3′,4′,5,6,7,8-heptamethoxyflavone (HPT), natsudaidain (NTD), and tangeretin (TNG) were isolated from King juice.

Isolation. Fruits were juiced by hand-squeezing, and 200 mL of juice was absorbed on 250 g of polystyrene resin (Diaion HP-20, Mitsubishi Chemical, Tokyo, Japan) which had been preconditioned by thorough washing with deionized water and eluted with EtOH and then acetone. The eluents were combined, concentrated in vacuo, and subjected to silica gel column chromatography (Wako Gel C-200, Wako Pure Chemicals, Osaka, Japan) eluted with 10% ethyl acetate in hexane, chloroform, and then 50% chloroform in methanol. Eluates with chloroform was further purified by reversed phase HPLC [mobile phase, 70% MeOH; column, Mightysil RP-18, 250 mm × 10 mm (i.d.); particle size, 5 μm; Kanto Chemicals, Tokyo, Japan], giving nobiletin (isolation yield = 12.7 mg, 6.4 × 10^{-3}%), 3,3′,4′,5,6,7,8-heptamethoxyflavone (isolation yield = 30.4 mg, 1.52 × 10^{-2}%), natsudaidain (isolation yield = 12.0 mg, 6.0 × 10^{-3}%), and tangeretin (isolation yield = 11.8 mg, 5.9 × 10^{-3}%).

Nobiletin (NBL): APCI-MS (*m*/*z*) 403 $[M + H]^+$; ^{1}H NMR data ($CDCl_3$) δ 7.54 (1H, dd, J = 2.0, 8.5 Hz, H-6′), 7.39 (1H, d, J = 2.0 Hz, H-2′), 6.97 (1H, d, J = 8.5 Hz, H-5′), 6.60 (1H, s, H-3), 4.08 (3H, s), 4.00 (3H, s), 3.95 (3H, s), 3.94 (3H, s), 3.93 (6H, s); ^{13}C NMR data ($CDCl_3$) δ 177.3 (s), 161.0 (s), 152.0 (s), 151.4 (s), 149.3 (s), 148.4 (s), 147.7 (s), 144.1 (s), 138.0 (s), 124.0 (s), 119.6 (d), 114.9 (s), 111.3 (d), 108.6 (d), 106.9 (d), 62.2 (q), 61.9 (q), 61.8 (q), 61.6 (q), 56.1 (q), 56.0 (q).

3,3′,4′,5,6,7,8-Heptamethoxyflavone (HPT) (Ito et al., 1998): APCI-MS (*m*/*z*) 433 $[M + H]^+$; ^{1}H NMR data ($CDCl_3$) δ 7.80 (1H, dd, J = 1.8, 8.5 Hz, H-6′), 7.77 (1H, d, J = 1.8 Hz, H-2′), 6.98 (1H, d, J = 8.5 Hz, H-5′), 4.06 (3H, s), 3.97 (3H, s), 3.94 (9H, s), 3.91 (3H, s), 3.85 (3H, s); ^{13}C NMR data ($CDCl_3$) δ 173.8 (s), 153.1 (s), 151.2 (s), 151.0 (s), 148.8 (s), 148.1 (s), 146.7 (s), 143.8 (s), 140.7 (s), 137.8 (s), 123.4 (s), 121.9 (d), 115.0 (s), 111.0 (d), 111.0 (d), 62.2 (q), 61.9 (q), 61.8 (q), 61.6 (q), 59.8 (q), 55.9 (q), 55.9 (q).

Natsudaidain (NTD) (Sugiyama et al., 1993): APCI-MS (*m*/*z*) 419 $[M + H]^+$; ^{1}H NMR data ($CDCl_3$) δ 7.90 (1H, dd, J = 1.9, 8.5 Hz, H-6′), 7.88 (1H, d, J = 1.9 Hz, H-2′), 7.30 (1H, br s, 3-OH), 7.01 (1H, d, J = 8.5 Hz, H-5′), 4.10 (3H, s), 4.01 (3H, s), 3.97 (6H, s), 3.95 (3H, s), 3.93 (3H, s); ^{13}C NMR data ($CDCl_3$) δ 171.9 (s), 151.7 (s), 150.6 (s), 148.9 (s), 147.6 (s), 146.9 (s), 143.5 (s), 143.0 (s), 137.9 (s), 137.4 (s), 123.9 (s), 121.0 (d), 111.7 (s), 111.1 (d), 110.3 (d), 62.3 (q), 61.9 (q), 61.8 (q), 61.7 (q), 56.0 (q), 55.9 (q).

Tangeretin (TNG) (Chen et al., 1997): APCI-MS (*m*/*z*) 373 $[M + H]^+$; ^{1}H NMR data ($CDCl_3$) δ 7.86 (2H, dd, J = 1.9, 7.0 Hz, H-2′, 6′), 7.01 (2H, dd, J = 1.9, 7.0 Hz, H-3′, 5′), 6.58 (1H, s, H-3), 4.08 (3H, s), 4.00 (3H, s), 3.93 (6H, s), 3.87 (3H, s); ^{13}C NMR data ($CDCl_3$) δ 177.3 (s), 162.3 (s), 161.2 (s), 151.4 (s), 148.4 (s), 147.7 (s), 144.1 (s), 138.1 (s), 127.7 (d), 127.7 (d), 123.9 (s), 114.9 (s), 114.5 (d), 114.5 (d), 106.7 (d), 62.2 (q), 62.0 (q), 61.8 (q), 61.6 (q), 55.5 (q).

Fruit Samples. All fruits were harvested from trees at the National Institute of Fruit Tree Science, Okitsu, Shizuoka, Japan, on December 9−10, 1996, and were juiced by hand-squeezing. Two hundred milliliters of juice was absorbed on 250 g of HP-20, which was eluted with EtOH (750 mL) and then acetone (750 mL). The combined eluates were concentrated, and each fraction was dissolved in DMSO at a concentration of 100 mg/mL as a stock solution. For HPLC analysis, the stock solution was diluted to 1 mg/mL by 50% MeOH in DMSO, and 100 μL was injected into the HPLC. HPLC analysis of flavonoids was done primarily according to the method described in the literature (Kanes et al., 1992). Analytical conditions were as follows: column, RP-18; particle size, 5 μm; 12.5 cm × 4.0 mm i. d. (Hewlett-Packard, Wilmington, DE); mobile phase; the gradient elution program, which was used for analysis of nonpolymethoxylated flavonoids, 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 polymethoxylatd flavonoid analysis, isocratic elution (mobile phase, 50% 0.01 M H_3PO_4 and 50% MeOH) was done to obtain better peak separation. Detection was done by simultaneous monitoring of UV absorption at 285 and 360 nm. Concentrations of the compounds were calculated from peak ares integration of the sample and corresponding standards by monitoring absorbance of the eluate either at 285 nm for flavanones or at 360 nm for flavones and polymethoxylated flavones.

Cell Differentiation Assay. The HL-60 cell line was obtained from the Riken Gene Bank (Tsukuba, Tokyo, Japan) and was maintained in RPMI 1640 medium (Iwaki, Japan)

supplemented with 10% fetal bovine serum (FBS). The composition of RPMI 1640 medium is as follows (mg/L): L-arginine, 200.0; L-asparagine, 50.0; L-aspartic acid, 20.0; L-cystine, 50.0; L-glutamic acid, 20.0; L-glutamine, 300.0; glycine, 10.0; L-histidine, 15.0; hydroxy-L-proline, 20.0; L-isoleucine, 50.0; L-leucine, 50.0; L-lysine, 40.0; L-methionine, 15.0; L-phenylalanine, 15.0; L-proline, 20.0; L-serine, 30.0; L-threonine, 20.0; L-tryptophan, 5.0; L-tyrosine, 20.0; L-valine, 20.0; glutathione, 1.0; $Ca(NO_3)_2 \cdot 4H_2O$, 100; KCl, 400.0; $MgSO_4 \cdot 7H_2O$, 100.0; NaCl, 6000; $NaHCO_3$, 2000; $Na_2HPO_4 \cdot 7H_2O$, 1512; phenol red, 5.0; biotin, 0.2; folic acid, 1.0; nicotinamide, 1.0; calcium pantothenate, 0.25; pyridoxine·HCl, 1.0; riboflavin, 0.2; thiamin·HCl, 1.0; vitamin B_{12}, 0.005; choline chloride, 3.0; D-glucose, 2000; *i*-inositol, 35.0; *p*-aminobenzoic acid, 1.0. HL-60 cells in log phase ($\sim 10^6$ cells/mL) were diluted to 1.2×10^5 cells/mL and preincubated for 18 h in 24-well plates ($\sim 2 \times 10^5$ cells/mL). Samples dissolved in DMSO were then added, keeping the final DMSO concentrations <0.4% (v/v). In a blank experiment, the cells were treated with the same concentration of DMSO. Neither retardation of proliferation nor induction of differentiation was observed in the blank experiment. After 4 days of incubation, the cells were analyzed to determine the percentage exhibiting nitro blue tetrazolium (NBT, Kanto Chemicals, Tokyo, Japan) reducing activity. For NBT reducing assay, a 1:1 (v/v) mixture of a cell suspension (10^6 cells/500 μL) and freshly prepared 12-*O*-tetradecanoylphorbol 13-acetate (TPA, Sigma)/NBT solution (phosphate-buffered saline solution containing 1 mg/mL NBT and 1 μg/mL TPA) was incubated for 15 min at 37 °C. Cells were then smeared on slide glass and counterstained by 0.25% (w/v) safranin O in 10% ethanol. Positive cells reduce NBT to give an intracellular black-blue formazan deposit. Differentiated cells were examined by counting a minimum of 200 cells in triplicates for each experiment.

Statistical Analysis. A quantitative data set, which was composed of contents of readily extractable flavonoid, 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 and cluster analyses. For principal component analysis (PCA), minimum detectable amounts for each flavonoid were used for trace amount and values of $^1/_{10}$ of the minimum detectable amount were used for each flavonoid not detected. Each principal component was calculated by taking a linear combination of an eigenvector of the correlation matrix with a standardized original variable. Cluster analysis was carried out by using Ward's minimum variance method.

RESULTS AND DISCUSSION

Screening for HL-60 Differentiating Activity of *Citrus* Juices and Isolation of Active Compounds from King Juice. The present study was intended to survey the manifestation of HL-60 differentiating activity and the contents of major flavonoids in the readily extractable fraction prepared from 34 *Citrus* juices. The readily extractable fraction was prepared from *Citrus* juice by adsorbing on HP-20 resin and eluting from the resin with organic solvents. This procedure could efficiently reduce the total proportion of HSP, NGIN, and NERC from 90% of the total flavonoid in the MeOH/DMSO extract from the freeze-dried endocarp to 57% in the readily extractable fraction prepared from King fruits (Table 2). These results demonstrate the feasibility of the physiological screening on the readily extractable fraction to identify *Citrus* species and cultivars rich in anticancer substances.

The readily extractable fractions from 34 *Citrus* juices were examined for their HL-60 differentiating activity by monitoring the NBT reducing cell (Table 1). Total activity was obtained from a division of the amount of fraction yield by the minimum effective dose, that is, 200 μg/mL. *C. nobilis* (common name King), *C. bergamia* (Bergamot), and *C. limettioides* (sweet lime) exhibited strong activity, and *C. depressa* (Shiikuwasha) showed moderate activity.

Table 1. Induction of NBT Reducing Activity by Readily Extractable Fraction Prepared from Various *Citrus* Juices

conventional name[a]	scientific name[a]	yield[b] (mg)	NBT reducing cell[c] (%)	total activity[d]
Tahiti lime	*C. latifolia*	1203	14	
sweet lime	*C. limettioides*	908.9	41	4545
Bergamot	*C. bergamia*	1024	59	5120
Eureka lemon	*C. limon*	1178.2	8	
Hirado	*C. grandis*	525.1	11	
Marsh	*C. paradisi*	977.2	22	
Red Blush	*C. paradisi*	1829.1	17	
Kinukawa	*C. glaberrima*	240.9	18	
Hassaku	*C. hassaku*	627.5	10	
Natsudaidai	*C. natsudaidai*	1198.6	22	
Sanbokan	*C. sulcata*	620.2	15	
sour orange	*C. aurantium*	898.3	9	
Valencia	*C. sinensis*	610.9	17	
Morita navel	*C. sinensis*	565.1	14	
Oto Mikan	*C. sinograndis*	549.6	12	
Iyo	*C. iyo*	606.7	8	
Hyuganatsu	*C. tamurana*	1073.8	15	
Yuzu	*C. junos*	1023.3	11	
Kunenbo	*C. nobilis*	415	13	
King	*C. nobilis*	1172	62	5860
Sugiyama Unshiu	*C. unshu*	520.3	23	
Okitsu Wase	*C. unshu*	171.8	18	
Yatsushiro	*C. yatsushiro*	541.2	15	
Kabuchi	*C. keraji*	375.3	8	
Ota Ponkan	*C. reticulata*	510.1	12	
Dancy tangerine	*C. tangerina*	180.3	11	
Clementine	*C. clementina*	586.6	24	
Jimikan	*C. succosa*	1473	11	
Shikaikan	*C. suhuiensis*	581.7	12	
Kobeni Mikan	*C.erythrosa*	231.4	48	1157
Hirakishu	*C. kinokuni*	637.4	12	
Shiikuwasha	*C. depressa*	458.6	44	2293
Koji	*C. leiocarpa*	369.4	20	
Shikikitsu	*C. madurensis*	1012.2	12	

[a] The classification and the nomenclature of *Citrus* plants were based on Tanaka's classification. [b] Yield from 100 mL of juice. [c] HL-60 differentiation assay was done at a concentration of 200 μg/mL. [d] Total activity of the readily extractable fraction was calculated from a division of the fraction yield by the concentration examined for HL-60 differentiation activity (200 μg/mL), and when >40% of HL-60 cells were induced to have NBT reducing activity, a sample was judged as active.

The readily extractable fraction prepared from 200 mL of King juice was subjected to purification of an active principle under a guide of HL-60 differentiating activity. The fraction was partitioned between diethyl ether (Et_2O) and water, and the bioactive Et_2O fraction was chromatographed on a silica gel column eluted with $CHCl_3$. The active fraction was finally purified by HPLC to give four active compounds. These compounds were NBL (isolation yield = 12.7 mg), HPT (30.4 mg), NTD (12.0 mg), and TNG (11.8 mg). NBL was identified by direct comparison of NMR and MS data with those of the authentic compound, and HPT, NTD, and TNG were identified by comparison of ^{1}H and ^{13}C NMR spectral data with those reported in the literature (Ito et al., 1998; Chen et al., 1997). These four polymethoxylated flavonoids as active principles have already been isolated and identified from the fruit peels of *C. nobilis* as HL-60 differentiation inducer (Sugiyama et al., 1993).

Table 3 summarizes induction of NBT reducing cells upon treatment by 25 citrus flavonoids. LTN, APG, QCT, HPT, NTD, and TNG induced NBT reducing activity at a concentration of 2.5 μM. No flavonoids

Table 2. Flavonoid Contents of the Readily Extractable Fraction and the MeOH/DMSO Extract[a]

conventional name	TXF	ERC	NERC	NRTN	RTN	NGIN	HSP	IRHF	NHSP	RHF	DSM	NDSM	QCT	NGEN	NPNC	PNC	LTN	KMP	APG	SNT	ACT	NBL	HPT	NTD	TNG
Readily Extractable Fraction (mg/100 mL of Juice)																									
Tahiti lime	nd[b]	0.29	nd	nd	nd	nd	2.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.61	nd	nd	nd	nd	nd	nd	nd	nd
sweet lime	0.04	nd	0.01	nd	nd	nd	1.52	nd	nd	nd	0.08	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.52	0.12	0.04	0.18
Bergamot	nd	nd	1.38	nd	nd	2.23	nd	nd	1.60	0.37	0.39	0.30	nd	nd	nd	6.41	nd	nd	nd	nd	nd	nd	nd	nd	nd
Eureka lemon	nd	1.67	nd	nd	nd	nd	3.84	nd	nd	nd	0.51	nd	nd	nd	nd	nd	0.08	nd	nd	nd	nd	nd	nd	nd	nd
Hirado	0.08	nd	0.08	nd	nd	1.45	nd	nd	0.31	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.12	nd	nd	nd
Marsh	nd	nd	0.33	2.49	nd	16.38	0.25	nd	1.28	0.28	nd	nd	0.17	nd	nd	1.51	nd	nd	nd	nd	nd	0.17	0.11	nd	0.12
Red Blush	0.16	nd	0.30	2.98	nd	13.16	0.44	nd	1.58	nd	nd	nd	0.21	nd	nd	1.58	nd	nd	nd	nd	nd	0.12	0.01	nd	nd
Kinukawa	0.03	nd	0.17	0.13	0.07	0.95	nd	nd	0.41	0.09	nd	nd	nd	nd	nd	0.06	nd	nd	nd	nd	nd	nd	nd	nd	nd
Hassaku	0.09	nd	nd	0.63	nd	1.59	0.18	nd	1.41	nd	nd	nd	nd	nd	nd	0.09	nd	nd	nd	nd	nd	nd	nd	nd	nd
Natsudaidai	0.21	nd	0.01	nd	nd	5.93	nd	nd	1.58	nd	nd	nd	nd	nd	nd	0.26	nd	nd	nd	nd	nd	tr[c]	tr	nd	0.01
Sanbokan	nd	0.33	1.89	nd	nd	nd	0.99	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
sour orange	nd	nd	0.77	nd	nd	1.97	nd	nd	0.87	nd	0.15	nd	nd	nd	nd	0.73	nd	0.14	nd	nd	nd	0.20	nd	nd	0.08
Valencia	nd	nd	nd	0.55	nd	nd	3.51	nd	nd	nd	0.09	0.08	nd	nd	nd	0.49	nd	nd	nd	0.37	nd	0.46	0.12	nd	0.07
Morita navel	0.03	nd	0.59	2.54	nd	nd	8.02	0.07	nd	0.05	nd	nd	nd	nd	nd	1.59	nd	nd	nd	nd	0.03	0.19	0.04	nd	0.01
Oto Mikan	nd	nd	0.01	2.55	nd	nd	3.00	0.21	nd	nd	nd	nd	nd	nd	0.27	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Iyo	nd	nd	0.02	1.71	nd	nd	3.51	nd	nd	nd	nd	nd	nd	nd	0.12	nd	nd	nd	nd	nd	nd	0.01	0.01	nd	0.04
Hyuganatsu	nd	nd	0.06	0.49	nd	nd	0.91	nd	nd	nd	nd	nd	nd	nd	0.34	0.01	nd	nd	nd	nd	nd	0.01	tr	nd	tr
Yuzu	nd	nd	nd	1.27	nd	0.73	0.52	nd	0.15	nd	nd	nd	nd	nd	0.11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
King	nd	nd	nd	0.63	nd	nd	6.12	nd	nd	nd	nd	nd	nd	nd	0.79	nd	nd	nd	nd	nd	nd	0.97	1.92	0.82	0.71
Kunenbo	nd	0.11	0.09	1.02	nd	nd	0.97	0.12	nd	nd	nd	nd	0.04	nd	nd	0.02	nd	nd	nd	nd	nd	0.18	0.41	nd	0.14
Sugiyama Unshiu	nd	nd	0.08	0.76	nd	nd	1.93	nd	nd	nd	nd	nd	nd	nd	0.17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Okitsu Wase	nd	nd	0.06	0.55	0.08	0.04	1.44	nd	nd	0.05	0.05	nd	nd	nd	0.13	tr	nd	nd	nd	nd	nd	nd	nd	nd	nd
Yatsushiro	nd	nd	0.05	nd	nd	nd	2.54	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.15	0.15	0.08	0.14
Kabuchi	nd	nd	0.01	nd	nd	nd	0.92	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.26	0.02	nd	0.23
Ota Ponkan	nd	nd	nd	0.52	nd	nd	5.10	nd	nd	nd	nd	nd	nd	nd	0.35	nd	nd	nd	nd	nd	nd	0.21	nd	nd	0.19
Dancy tangerine	tr	0.09	0.04	0.23	nd	nd	0.81	nd	nd	nd	nd	nd	nd	nd	0.06	nd	nd	nd	nd	nd	0.02	0.35	nd	nd	0.19
Clementine	nd	nd	0.06	0.12	nd	nd	2.41	nd	nd	nd	nd	nd	nd	nd	0.05	nd	nd	nd	nd	nd	nd	0.20	0.07	nd	0.07
Jimikan	nd	nd	0.03	nd	nd	nd	3.18	nd	nd	nd	nd	nd	nd	nd	0.17	nd	nd	nd	nd	nd	nd	0.54	nd	nd	0.33
Shikaikan	nd	nd	0.15	0.63	nd	nd	6.57	nd	nd	nd	nd	nd	nd	nd	0.30	nd	nd	nd	nd	nd	nd	0.68	nd	nd	0.27
Kobenimikan	nd	nd	0.27	0.44	nd	nd	8.01	nd	nd	nd	nd	nd	nd	nd	0.33	nd	nd	nd	nd	nd	nd	0.61	nd	nd	0.30
Hirakishu	nd	nd	0.01	nd	nd	nd	3.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.33	nd	nd	0.14
Shiikuwasha	nd	nd	0.05	nd	nd	nd	1.88	nd	nd	nd	nd	nd	nd	nd	0.04	nd	nd	nd	nd	nd	nd	0.14	nd	nd	0.08
Koji	tr	1.03	0.03	1.16	nd	nd	0.63	nd	nd	nd	0.08	nd	nd	nd	0.10	nd	nd	nd	nd	nd	nd	0.09	nd	0.08	0.04
Shikikitsu	nd	nd	nd	nd	nd	nd	0.35	0.36	0.15	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DMSO/MeOH Extract (mg/100 g of Dry Wt)																									
King	nd	5.7	231	7.9	10.5	308	239	nd	nd	56.6	5.4	7.4	nd	nd	110	153	nd	nd	nd	nd	nd	4.7	8.3	6.2	1.7

[a] Listed in increasing retention order on an RP-18 HPLC column. [b] nd, not detected. [c] tr, trace; detected but too small to quantify.

Table 3. Induction of NBT Reducing Activity by 25 *Citrus* Flavonoids[a]

flavonoid	NBT reducing cell (%)	flavonoid	NBT reducing cell (%)
TXF	12.0 ± 3.5	NGEN	12.5 ± 3.2
ERC	8.8 ± 3.3	NPNC	10.3 ± 3.8
NERC	7.3 ± 2.5	PNC	10.5 ± 1.7
NRTN	11.0 ± 2.1	LTN	33.5 ± 5.7[b]
RTN	13.5 ± 3.6	KMP	10.5 ± 2.1
NGIN	10.8 ± 2.8	APG	25.3 ± 2.6[b]
HSP	8.3 ± 3.3	SNT	10.3 ± 2.3
IRHF	10.8 ± 3.3	ACT	14.5 ± 3.0
NHSP	10.5 ± 0.9	NBL	28.3 ± 2.7[b]
RHF	11.8 ± 1.1	HPT	24.0 ± 2.9[b]
DSM	6.8 ± 3.6	NTD	29.8 ± 1.9[b]
NDSM	9.5 ± 3.0	TNG	27.3 ± 6.2[b]
QCT	25.3 ± 2.7[b]	control	10.0 ± 2.9

[a] Percent of NBT reducing cells following treatment with 2.5 μM flavonoids. Each result represents the mean of triplicates of experiments with standard deviation (SD). No flavonoid induced HL-60 differentiation at concentrations <2.5 μM. [b] Significant at $p < 0.05$ (Student's t test).

examined in this study induced HL-60 differentiation at concentrations <2.5 μM.

Flavonoid Contents in the Readily Extractable Fraction. Isolation of polymethoxylated flavonoids as biologically active principles suggested the correlation between the flavonoid content and the differentiating activity in the readily extractable fraction. To study this, the polymethoxylated flavonoid contents along with other *Citrus* flavonoids were compared within 34 *Citrus* juices by HPLC analysis. Figure 2 shows HPLC profiles of a standard flavonoid mixture (1) and the readily extractable fraction of King juice (2). Quantitative determination of the flavonoids for various samples is given in Table 2. HSP was distributed most widely among the juices examined (detected in 29 samples of 34 samples), followed by NBL (22 samples), TNG (21 samples), and NRTN (20 samples). NGIN presented the highest variability among the samples [1.3 ± 3.6 mg/100 mL of juice (mean ± SD)].

King juice was indicated to be rich in the polymethoxylated flavonoids. HPLC analysis indicated that the four polymethoxylated flavonoids made up ~2% of the conventionally prepared sample from the freeze-dried King fruit segments on the basis of UV absorbance at 360 nm. It was assumed that HL-60 differentiating activity of the conventionally prepared sample was masked by inactive flavonoids, such as HSP and NGIN.

Test medium of the readily extractable fraction of King contained totally 1.8 μM polymethoxylated flavonoids (NBL, 0.41 μM; HPT, 0.76 μM; NTD, 0.33 μM; TNG, 0.33 μM). Assuming that these polymethoxylated flavonoids had the same potency of inducing activity of NBT reduction, the total concentration of polymethoxylated flavonoid was very close to the minimum effective concentration examined for these flavonoids (2.5 μM). We therefore considered that these polymethoxylated flavonoids mainly contributed the differentiation inducing activity of the readily extractable fraction of King juice.

Extracts from Bergamot, sweet lime, and Shiikuwasha induced HL-60 differentiation. These species, which contained no or small amounts of polymethoxylated flavonoids, seemed to have active principles other than polymethoxylated flavonoids.

Multivariate Analysis. NGEN and APG were excluded from PCA, because no juice contained these flavonoids (Table 2). The PCA was used to represent the 23-dimensional data structure in a smaller number of dimensions. The principal component (PC) with eigenvalue >1 reflects more of the original data structure than each of the original variables (23 flavonoid content), because the average of the eigenvalues is 1. Eight PCs with eigenvalues >1 account for 79.0% of the total variance (Table 4).

Factor loadings indicate the relative extent to which each original variable contributes to the variance contained in each PC. As listed in Table 5, the four flavonoids that contributed most to PC1 were all polymethoxylated flavonoids. Factor loadings for PC2 show that first four constituents contain neohesperidose as a sugar part (NDSM, PNC, NERC, RHF). For PC3, loading values in Table 5 show that three of the first four constituents were flavanones (NGIN, TXF, and

Table 4. Correlation Coefficient Matrix for the Flavonoids and Principal Components

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
eigenvalue	5.21	3.38	2.87	1.70	1.48	1.25	1.20	1.06
%	22.66	14.70	12.47	7.40	6.44	5.44	5.23	4.61
cum %	22.66	37.36	49.83	57.23	63.68	69.12	74.35	78.97
eigenvectors								
TXF	0.16	0.05	−0.33	−0.22	0.01	0.18	−0.06	−0.07
ERC	0.02	−0.13	0.19	−0.16	0.67	−0.04	0.07	−0.02
NERC	0.19	0.16	0.23	0.11	−0.12	0.08	0.35	−0.10
NRTN	0.08	0.16	−0.33	0.40	0.24	−0.11	0.03	0.04
RTN	0.03	−0.09	0.01	−0.10	−0.26	−0.45	−0.16	−0.48
NGIN	0.24	0.26	−0.35	−0.07	0.14	0.04	−0.03	−0.03
HSP	−0.27	0.10	0.08	0.37	0.19	0.16	−0.03	−0.18
IRHF	0.00	−0.11	−0.06	0.27	−0.06	−0.34	0.14	0.70
NHSP	0.32	0.25	−0.14	−0.21	−0.06	0.09	0.05	0.03
RHF	0.26	0.31	0.18	0.11	−0.06	−0.24	−0.09	−0.10
DSM	0.16	0.07	0.40	−0.14	0.37	0.03	0.02	0.06
NDSM	0.21	0.25	0.37	0.07	−0.12	−0.03	−0.21	0.11
QCT	0.19	0.23	−0.37	0.01	0.22	0.01	−0.04	0.01
NPNC	−0.32	0.24	−0.01	−0.02	0.01	−0.21	0.04	0.01
PNC	0.28	0.32	0.24	0.15	−0.03	−0.03	−0.02	0.01
LTN	0.00	−0.13	0.06	−0.12	0.30	−0.07	−0.08	−0.11
KMP	0.05	0.00	0.07	−0.20	−0.17	0.36	0.57	0.10
SNT	−0.02	0.01	0.09	0.08	−0.05	0.41	−0.60	0.25
ACT	−0.01	0.01	−0.01	0.54	0.07	0.15	0.23	−0.30
NBL	−0.30	0.27	0.02	0.01	−0.05	0.31	−0.09	−0.05
HPT	−0.27	0.34	−0.01	−0.16	0.05	−0.18	0.04	0.12
NTD	−0.27	0.33	0.01	−0.19	0.07	−0.19	0.08	0.07
TNG	−0.33	0.30	0.01	−0.09	−0.04	0.08	0.03	−0.04

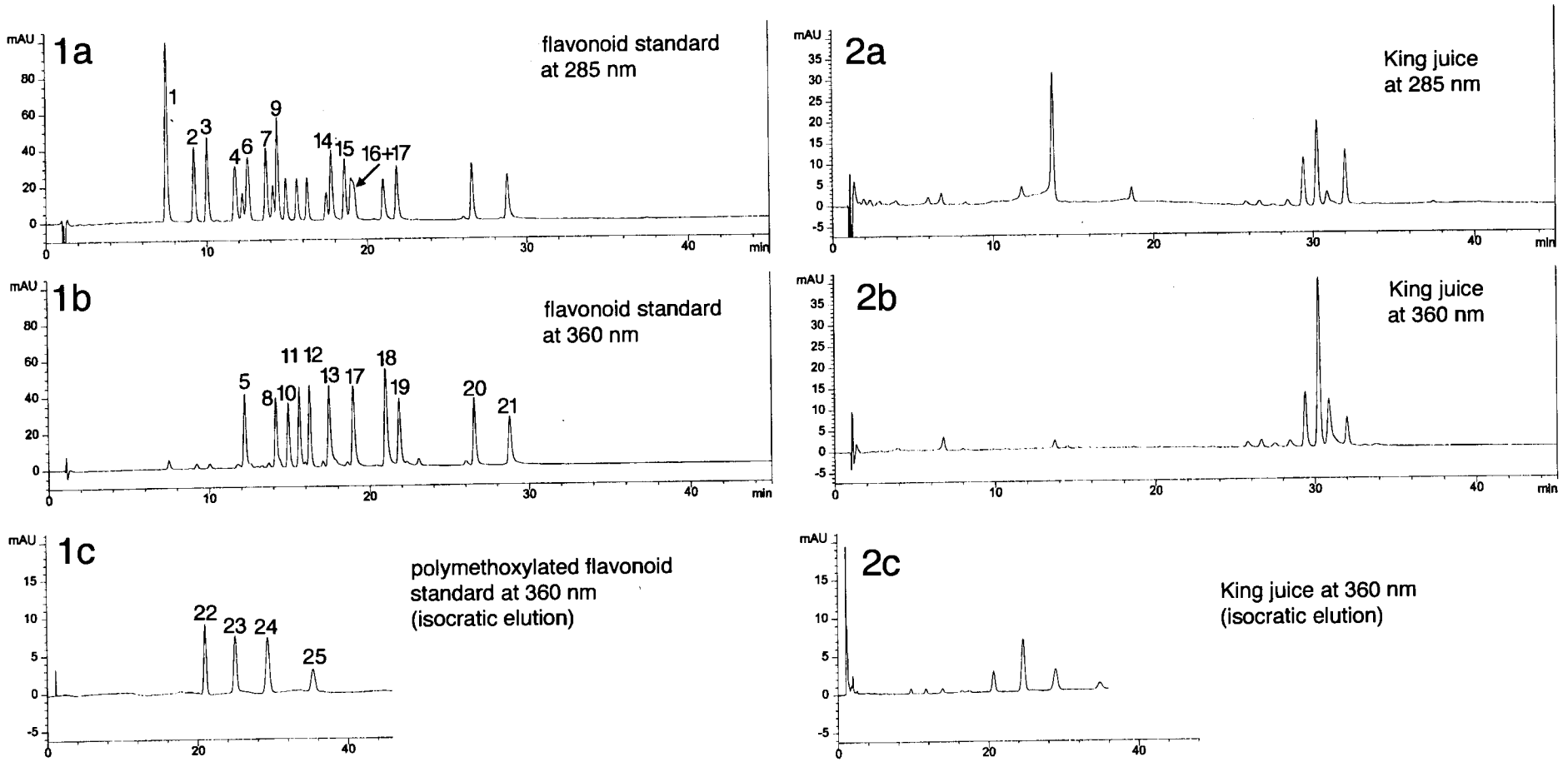


Figure 2. HPLC profiles of (1) a standard flavonoid mixture and (2) the readily extractable fraction of King juice. Chromatograms with gradient elution monitored by UV absorption at (a) 285 and (b) 360 nm and with (c) isocratic elution at 360 nm. Peaks: 1, TXF; 2, ERC; 3, NERC; 4, NRTN; 5, RTN; 6, NGIN; 7, HSP; 8, IRHF; 9, NHSP; 10, RHF; 11, DSM; 12, NDSM; 13, QCT; 14, NGEN; 15, NPNC; 16, PNC; 17, LTN; 18, KMP; 19, APG; 20, SNT; 21, ACT; 22, NBL; 23, HPT; 24, NTD; 25, TNG. Flavanones, peaks 1−4, 6, 7, 9, and 14−16; flavones, peaks 5, 8, 10−13, and 17−21; polymethoxylated flavones, peaks 22−25. PNC and LTN can be distinguished by their UV absorption spectra.

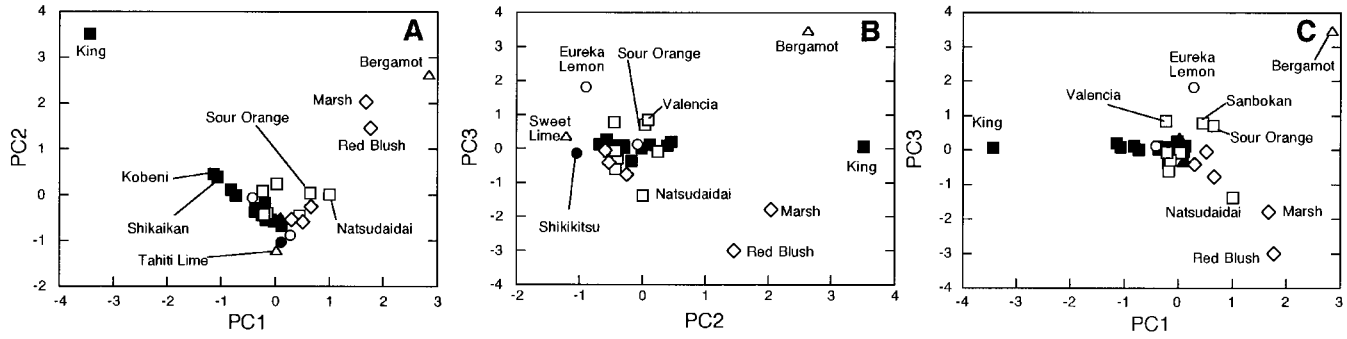


Figure 3. Projections of scatter diagram from a PCA: (A) PC1 vs PC2; (B) PC1 vs PC3; (C) PC2 vs PC3. Symbols: △, group II; ○, group III; ◇, group IV; □, group V; ▲, group VI; ■, group VII; ●, group VIII, according to Tanaka's classification. Open symbols indicate Archicitrus and solid symbols Metacitrus.

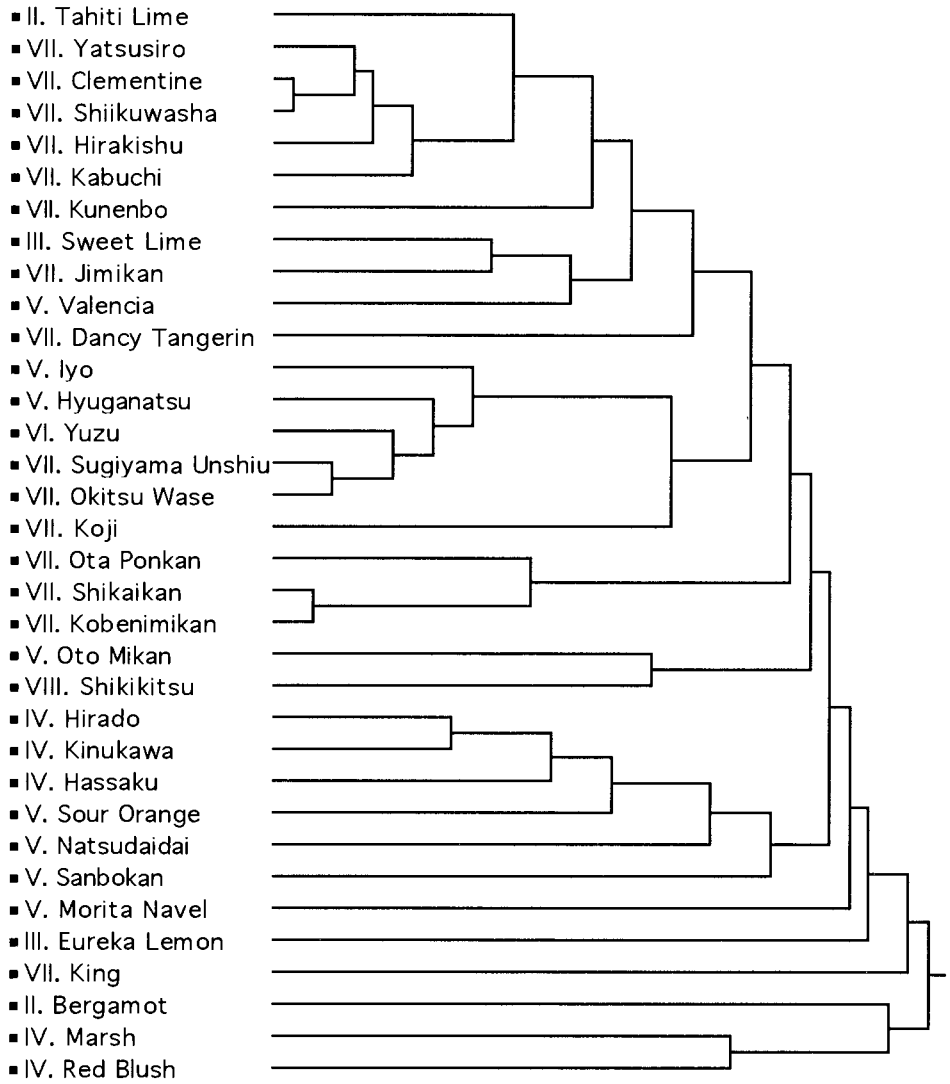


Figure 4. Dendrogram from cluster analysis based on the readily extractable flavonoids from *Citrus* fruits. Roman numerals before each common name indicate Tanaka's categorical number.

NHSP). The scores for the first two PCs (A), for the second and third PCs (B), and for the first and the third PCs (C) are plotted as scatter diagrams in Figure 3. These graphics show the peculiarities of King, Bergamot, Red Blush, and Marsh grapefruit.

According to Tanaka's classification (Tanaka, 1969), *Citrus* species can be divided into Archicitrus (open symbols in Figure 3) and Metacitrus (solid symbols in Figure 3). Figure 3 shows that Metacitrus samples, except for King, were clustered in a relatively small area, whereas Archicitrus samples seemed to be more widely dispersed. We considered that the result of PCA based on flavonoid content will help identification of varieties rich in anticancer substances, because both King and Bergamot, which had peculiar flavonoid profiles, exerted potent anticarcinogenic activity.

The communality value of a variable is the percentage of variance of the variable that is explained by the retained principal components. These values can be used to remove those variables that do not contribute to the retained PCs. In this study, communality values of RTN, LTN, and SNT are <0.10, as shown in Table 5,

Table 5. Factor Loadings and Communality Values

	factor loading				
	PC1	PC2	PC3	PC4	communality
TXF	0.00	−0.09	0.23	−0.10	0.53
ERC	−0.03	0.01	−0.08	−0.16	0.21
NERC	−0.01	0.19	−0.05	0.04	0.44
NRTN	−0.02	0.00	0.12	0.36	0.71
RTN	−0.03	−0.03	0.00	−0.08	0.05
NGIN	0.03	0.00	0.27	0.02	0.88
HSP	0.08	0.04	−0.14	0.27	0.67
IRHF	−0.07	−0.02	−0.05	0.19	0.17
NHSP	0.02	0.07	0.22	−0.12	0.86
RHF	0.03	0.23	0.02	0.06	0.79
DSM	0.00	0.18	−0.10	−0.17	0.64
NDSM	0.03	0.27	−0.08	−0.01	0.85
QCT	0.03	−0.01	0.25	0.09	0.76
NPNC	0.19	−0.01	−0.01	0.02	0.71
PNC	0.02	0.27	0.00	0.07	0.95
LTN	−0.03	−0.03	−0.03	−0.11	0.09
KMP	0.01	0.01	0.02	−0.16	0.10
SNT	0.00	0.04	−0.06	0.04	0.04
ACT	−0.06	0.07	−0.11	0.39	0.50
NBL	0.20	0.02	−0.01	0.03	0.73
HPT	0.23	0.02	0.06	−0.08	0.79
NTD	0.23	0.01	0.05	−0.11	0.81
TNG	0.23	0.00	0.01	−0.03	0.89

and therefore these three flavonoids as well as NGEN and APG are excluded from consideration in the following cluster analysis. As the result of the application of cluster analysis, the obtained dendrogram indicates that King, Bergamot, Eureka lemon, and Morita navel are totally separated from the rest of the cultivars along with the association of Marsh grapefruit and Red Blush (Figure 4).

The results of PCA and cluster analysis, however, were not entirely in agreement with Tanaka's classification system, presumably because the flavonoid contents of the readily extractable fraction prepared from *Citrus* juices were influenced not only by subgenera classification but also by a multitude of factors, such as flavonoid localization, pulp contents, and water contents, which are not fully understood.

In conclusion, our results suggested that readily extractable polymethoxylated flavonoids are important candidates for cancer-protective action. The active principle survey on the readily extractable fraction of both commercial cultivars and important hybrids will aid in horticultural breeding programs focused on health promotion by selecting varieties rich in anticancer substances. However, further study on their bioavailability and metabolic fate is necessary to state their action in vivo.

ACKNOWLEDGMENT

We thank Prof. Y. Sashida (Tokyo University of Pharmacy and Life Science) for his gift of nobiletin. We also thank Dr. M. Koizumi (National Institute of Fruit Tree Science) for his gift of natsudaidain.

LITERATURE CITED

Albach, R. F.; Redman, G. H. Composition and inheritance of flavanones in citrus fruit. *Phytochemistry* **1969**, *8*, 127−143.

Attaway, J. A. Citrus juice flavonoids with anticarcinogenic and antitumor properties. In *Food Phytochemicals for Cancer Prevention I*; Maple Press: York, PA, 1994; pp 240−248.

Benavente-García, O.; Castillo, J.; Marin, F. R.; Ortuño, A.; Del Río, J. A. Use and properties of *Citrus* flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4506−4515.

Chen, J.; Montanari, A. M.; Widmer, W. W. Two new polymethoxylated flavones, a class of compounds with potential anticancer activity, isolated from cold pressed Dancy Tangerine peel oil solids. *J. Agric. Food Chem.* **1997**, *45*, 364−368.

Collins, S. J.; Ruscetti, F. W.; Gallagher, R. E.; Gallo, R. C. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2458−2462.

Deschner, E. E.; Ruperto, J.; Wong, G.; Newmark, H. L. Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenesis* **1991**, *12*, 1193−1196.

Gentili, B.; Horowitz, R. M. Flavonoids of citrus−VII. Limocitrol and isolimocitrol. *Tetrahedron* **1964**, *20*, 2313−2318.

Horowitz, R. M. Flavonoids of citrus. I. Isolation of diosmin from lemons (*Citrus limon*). *J. Org. Chem.* **1956**, *21*, 1184−1185.

Horowitz, R. M. Flavonoids of citrus. II. Isolation of a new flavonol from lemons. *J. Am. Chem. Soc.* **1957**, *79*, 6561−6562.

Horowitz, R. M.; Gentili, B. Flavonoid compounds of Citrus. III. Isolation and structure of eriodictyol glycoside. *J. Am. Chem. Soc.* **1960a**, *82*, 2803−2806.

Horowitz, R. M.; Gentili, B. Flavonoids of citrus. IV. Isolation of some aglycones from the lemon (*Citrus lemon*). *J. Org. Chem.* **1960b**, *25*, 2183−2187.

Ito, C.; Fujiwara, K.; Koizumi, M.; Furukawa, H. Isolation and characterization of an antibacterial substance from citrus plant. *J. Chinese Chem. Soc.* **1998**, *45*, 89−91.

Kanes, K.; Tisserat, B.; Berhow, M.; Vandercook, C. Phenolic composition of various tissues of Rutaceae species. *Phytochemistry* **1992**, *31*, 967−974.

Park, G. L.; Avery, S. M.; Byers, J. L.; Nelson, D. B. Identification of bioflavonoids from citrus. *Food Technol.* **1983**, *37*, 98−105.

Stavric, B. Antimutagens and anticarcinogens in foods. *Food Chem. Toxicol.* **1994**, *32*, 79−90.

Sugiyama, S.; Umehara, K.; Kuroyanagi, M.; Ueno, A.; Taki, T. Studies on the differentiation inducers of myeloid leukemic cells from *Citrus* species. *Chem. Pharm. Bull.* **1993**, *41*, 714−719.

Tanaka, T. Misunderstanding with regards citrus classification and nomenclature. *Bull. Univ. Osaka Prefect., Ser. B* **1969**, *21*, 139.

Tanaka, T.; Makita, H.; Kawabata, K.; Mori, H.; Kakumoto, M.; Satoh, K.; Hara, A.; Sumida, T.; Tanaka, T.; Ogawa, H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis* **1997**, *18*, 957−965.

Vandercook, C. E.; Stevenson, R. G. Lemon juice composition. Identification of the major phenolic compounds and estimation by paper chromatography. *J. Agric. Food Chem.* **1966**, *14*, 450−454.

Verma, A. K.; Johnson, J. A.; Gould, M. N.; Tanner, M. A. Inhibition of 7,12-dimethylbenz[*a*]anthracene and *N*-nitromethylurea induced rat mammary cancer by dietary flavonol quercetin. *Cancer Res.* **1988**, *48*, 5754−5758.

Wattenbrug, L. W. Chemoprevention of cancer. *Cancer Res.* **1985**, *45*, 1−8.

Wattenburg, L. Inhibition of carcinogenesis by minor anutrient constituents of the diet. *Proc. Nutr. Soc.* **1990**, *49*, 173−183.

Wei, H.; Tye, L.; Bresnick, E.; Birt, D. F. Inhibitory effect of apigenin, a plant flavonoid, on epidermal ornithine decarboxylase and skin tumour promotion in mice. *Cancer Res.* **1990**, *50*, 499−506.

Received for review May 15, 1998. Revised manuscript received September 14, 1998. Accepted October 15, 1998. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences from the Bio-oriented Technology Research Advancement Institution.

JF9805101