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High-performance liquid chromatographic determination of naturally occurring flavonoids in *Citrus* with a photodiode-array detector

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Abstract

High-performance liquid chromatography (HPLC) coupled with ultraviolet–visible spectrophotometry using a photodiode-array detector was used as a routine method for the simultaneous separation and determination of 25 naturally occurring *Citrus* flavonoids. The separation system consisted of a C_{18} reversed-phase column, a gradient system of 0.01 *M* phosphoric acid (A) and methanol (B), and a photodiode-array detector. Each of the 25 flavonoids was eluted from the column with a gradient system composed of three periods: (1) 0–55 min, 70–55% (v/v) A in B, (2) 55–95 min, 55–0% A in B, and (3) 95–100 min, isocratic, 100% B, and quantified by spectrophotometric detection at 285 nm. Identifications of specific flavonoids were made by comparing their retention times (t_R) and UV spectra with those of standards. The relative standard deviations of t_R values were 0.029–0.321%. The recoveries of pure eriocitrin, naringin, hesperidin and tangeretin added to tissues prepared from Unshiu (*Citrus unshiu* Marc.) and Hirado-buntan (*Citrus grandis* Osbeck f. Hirado) and subsequent extraction were 97.47–103.13% from the mesocarp and 96.87–104.93% from the juice with standard deviations of 2.32–5.72% and 2.18–5.96%, respectively.

1. Introduction

The flavonoid constituents of *Citrus* continue to claim attention not only for their remarkable taste properties [1–3] but also their therapeutic and pharmacological activities. For example, they have been shown to possess biological activities such as anti-carcinogenic effects [4–9], anti-inflammatory properties [10,11], and inhibitory activities against histamine release [12,13]. Further, in early chemotaxonomic studies, several flavanone glycosides unique to *Citrus* and even to specific cultivars were examined in relation to taxonomic classification [14–16]. For these kinds of studies, precise quantitative data on the occurrence of flavonoids in *Citrus* are needed.

There have been many reports on the HPLC of flavonoids [17–20]. Wulf and Nagel [21] elaborated the theory and practicality of separating a dozen flavone compounds. They used a solvent system consisting of methanol–acetic acid–water (30:5:65) on a C_{18} column. Daigle and Conker-

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ton [22] reported the HPLC of 34 selected flavonoids using a water–acetic acid (495:5)–methanol isocratic system on a μBondapak C_{18} column. Van de Casteele *et al.* [23] reported retention times (t_R) of 141 flavonoids ranging from triglycosides to aglycones using a C_{18} column and a gradient elution system of formic acid–water (5:95)–methanol. However, reports on the application of HPLC to the determination of flavonoids are limited both in number and scope. In the determination of flavonoids in *Citrus*, attention has been paid only to particular flavones such as polymethoxylated flavones [24,25] and certain major flavonoids [26,27].

The objective of this work was to develop a routine, dependable and accurate method for the determination of flavonoids in extracts prepared from various *Citrus* tissues using reversed-phase HPLC with photodiode-array detection [28].

2. Experimental

2.1. Apparatus for HPLC

The system consisted of Model L-6210 and L-6010 pumps, a Model AS-2000 autosampler, a Model L-3000 photodiode-array detector, a Model D-6100 interface (Hitachi, Tokyo, Japan), a C_{18} reversed-phase analytical HPLC column (LiChrospher 100 RP-18, 250 mm × 4.0 mm I.D., 5 μm particle size; Merck, Darmstadt, Germany), a Model 545B degassing unit (GL Sciences, Tokyo, Japan), a Model CTO-6A column oven (Shimadzu, Kyoto, Japan) and a Model 3852-2 colour printer (IBM, Armonk, NY, USA). The system was controlled by a Hitachi D-6100 Data Station HPLC Manager.

2.2. Chemicals and standards of flavonoids

Neodiosmin was isolated and characterized by NMR at the Fruit and Vegetable Chemistry Laboratory, US Department of Agriculture, Agricultural Research Service, Pasadena, CA, USA, and other HPLC-grade flavonoid standards were purchased from Extrasynthese (Genay, France). Sep-Pak C_{18} cartridges, used for sample clean-up, were obtained from Waters (Milford, MA, USA). All other chemicals were of analytical-reagent grade (Wako, Osaka, Japan).

2.3. Materials

Unshiu (*Citrus unshiu* Marc.) and Hirado-buntan (*Citrus grandis* Osbeck f. Hirado) were grown at the Okitsu Branch, National Fruit Tree Research Station, Shimizu, Japan. From the same tree, 5–20 leaves (young but well expanded) were harvested in June and 5–15 fruits were harvested in November 1992. Four different tissue samples were dissected from the detached fruits and stored at −20°C until processed for analysis: the epicarp, the mesocarp, the endocarp and the juice. Leaves were frozen directly in liquid nitrogen and stored at −20°C until processed.

2.4. Sample preparation

After lyophilization, the epicarp, mesocarp, endocarp and leaf tissue samples were ground using an Ultra Centrifugal Mill (Mitamura Riken Kogyo, Tokyo, Japan) with a 0.5-mm filter. Portions (100 mg) of these powdered tissues were extracted for 12 h with 5 ml of extraction solvent [methanol–dimethyl sulphoxide (DMSO) (1:1, v/v)] in glass-stopped vessels on a wrist-action shaker at ambient temperature. After centrifugation at 3000 *g* for 10 min, the extract of each sample was decanted and the remaining solid residue was extracted twice more with 1 ml of the same solution. To remove polar compounds, the combined extract for each sample was diluted tenfold with water and passed through a Sep-Pak cartridge that had been preconditioned with 5 ml of methanol followed by 10 ml of 10% methanol. The eluate was discarded and the cartridge was washed with 15 ml of 10% methanol. The retained flavonoids were eluted with 4.8 ml of elution solvent [methanol–DMSO (1:1, v/v)]. The final volume of the eluate for each sample was adjusted to 5 ml with the same solvent.

Juice samples were prepared by homogenizing

the sarcocarp tissue after another three fruit tissues had been removed from each sample fruit with a mixer. The juice was clarified by centrifugation at 15 000 *g* for 20 min, and 3 ml of each juice sample were passed through a Sep-Pak cartridge as described above. Solutions to be analysed by HPLC were filtered through membrane filters (0.5-μm pore size; Advantec, Tokyo, Japan) prior to injection.

2.5. HPLC conditions

The detector monitored the eluent at 285 nm and measured spectra from 200 to 360 nm. A two-solvent gradient system was used. The gradient programme consisted of three periods: (1) 0–55 min, 70–55% (v/v) A (0.01 *M* phosphoric acid) in B (methanol), (2) 55–95 min, 55–0% A in B, and (3) 95–100 min, isocratic, 100% B. The resulting chromatographic data on the absorbing peaks was integrated up to 90 min. The flow-rate was 0.6 ml/min with a column head pressure of 1000–1550 p.s.i. (1 p.s.i. = 6894.76 Pa). The column was operated at 40°C. The sample injection volume was 10 μl. Identifications of compounds were made by comparing their t_R values and UV spectra with those of standards stored in a data bank. Concentrations of the compounds were calculated from integrated peak areas of the sample and corresponding standards.

2.6. Recovery studies

The recovery efficiency was determined by adding measured amounts of pure flavonoid standards (eriocitrin, naringin, hesperigin and tangeretin) to either the extraction solvent for mesocarp tissues described above or directly to juice samples to a final concentration of 100 ppm. The samples were prepared as described above and 10 μl of the filtrate were injected on to the HPLC column. Controls were prepared from the same tissue samples. The recoveries were determined by subtracting the values obtained for the control tissue preparation from those of the samples prepared with the added standards. The recovery experiment was performed with five replicates and mean values with standard deviations are reported.

3. Results and discussion

3.1. Separation and identification

Fig. 1 illustrates the separation of 25 flavonoids on LiChrospher 100 RP-18 using the 0.01 *M* phosphoric acid–methanol gradient elution system. As a means of qualitative identification, retention data including mean values of t_R with standard deviation (S.D.) of five replicates, the relative retention value (α) and UV absorption maxima of each standard are given in Table 1. Because the S.D. values were within 0.02–0.10 min and not dependent on t_R, the relative standard deviations of t_R grew smaller with the progression of retention time, 0.284% for the early-eluting flavonoid standard eriocitrin and 0.047% for the final-eluting standard tangeretin.

The widths of the peaks of compounds eluted after 60 min were narrower than those eluted before 60 min. For compounds that eluted before 60 min, such as hesperidin and rutin, α values of more than 1.05 were needed to establish a baseline separation, whereas the separation of acacetin and tangeretin, which eluted after 60 min, was successfully achieved with even smaller α values of 1.03 or less.

The elution order of the standards in this system was the same as for corresponding com-

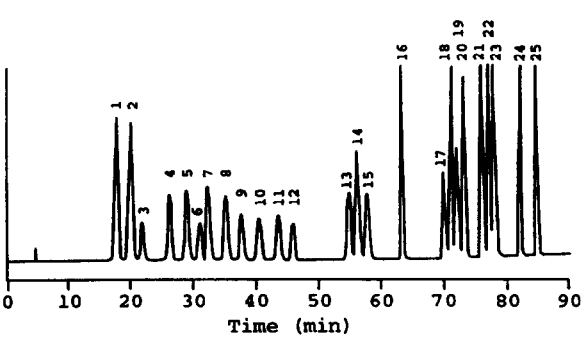

Fig. 1. Separation of flavonoid standards. For the elution system, see Experimental. For the identity of peaks, see Table 1.

Table 1
Retention times (t_R), capacity factors (k'), relative retentions (α) and UV absorbance maxima (λ_{max}) of the flavonoids investigated

No.	Common name	Systematic name	$t_R \pm$ S.D.[a] (min)	α	λ_{max}[b] (nm)
1	Eriocitrin	Eriodictyol-7-β-rutinoside	17.59 ± 0.05		285
2	Neoeriocitrin	Eriodictyol-7-β-neohesperidoside	19.85 ± 0.05	1.17	285
3	Robinetin	3,7,3',4',5'-Pentahydroxyflavone	21.81 ± 0.07	1.23	251, 318
4	Narirutin	Naringenin-7-β-rutinoside	25.97 ± 0.07	1.24	282
5	Naringin	Naringenin-7-β-neohesperidoside	28.81 ± 0.07	1.13	284
6	Rutin	Quercetin-3-β-rutinoside	30.81 ± 0.07	1.08	258, 360
7	Hesperidin	Hesperetin-7-β-rutinoside	32.09 ± 0.08	1.05	285
8	Neohesperidin	Hesperetin-7-β-neohesperidoside	34.95 ± 0.08	1.10	284
9	Isorhoifolin	Apigenin-7-β-rutinoside	37.33 ± 0.08	1.08	267, 336
10	Rhoifolin	Apigenin-7-β-neohesperidoside	40.27 ± 0.08	1.09	268, 336
11	Diosmin	Diosmetin-7-β-rutinoside	43.17 ± 0.07	1.08	253, 268, 345
12	Neodiosmin	Diosmetin-7-β-neohesperidoside	46.11 ± 0.06	1.08	255, 268, 345
13	Neoponcirin	Isosakuranetin-7-β-rutinoside	54.64 ± 0.06	1.20	284
14	Quercetin	3,3',4',5,7-Pentahydroxyflavone	56.32 ± 0.10	1.03	256
15	Poncirin	Isosakuranetin-7-β-neohesperidoside	57.65 ± 0.06	1.03	284
16	Luteolin	3',4',5,7-Tetrahydroxyflavone	62.93 ± 0.06	1.10	242, 256, 351
17	Kaempferol	3,4',5,7-Tetrahydroxyflavone	70.02 ± 0.02	1.12	253, 266
18	Apigenin	4',5,7-Trihydroxyflavone	71.55 ± 0.06	1.02	269, 335
19	Isorhamnetin	3,4',5,7-Tetrahydroxy-3'-methoxyflavone	72.01 ± 0.08	1.01	253
20	Diosmetin	3',5,7-Trihydroxy-4'-methoxyflavone	73.25 ± 0.04	1.02	252, 268, 347
21	Rhamnetin	3,5,3',4'-Tetrahydroxy-7-methoxyflavone	76.51 ± 0.03	1.05	256
22	Isosakuranetin	5,7-Dihydroxy-4'-methoxyflavanone	77.28 ± 0.05	1.01	282
23	Sinensetin	3',4',5,6,7-Pentamethoxyflavone	77.84 ± 0.06	1.01	240, 265, 326
24	Acacetin	5,7-Dihydroxy-4'-methoxyflavone	82.19 ± 0.04	1.06	269, 301, 329
25	Tangeretin	4',5,6,7,8-Pentamethoxyflavone	84.88 ± 0.04	1.03	271, 322

[a] Mean values and standard deviations of retention times for five replicate determinations.
[b] Measured in the eluate, the composition of which varies with t_R.

pounds reported by Daigle and Conkerton [22], except that rutin eluted immediately prior to hesperidin (Fig. 1). In the separation system developed by Van de Casteele *et al.* [23], naringin, rutin, hesperidin and neohesperidin were not separated well with t_R values of 15.71, 15.76, 16.3 and 16.62 min, respectively. These compounds were separated sufficiently for quantification in our system (Fig. 1 and Table 1).

The column was found to perform satisfactorily when the precolumn was changed periodically and the column was cleaned occasionally with methanol. However, individual columns may require slightly different conditions for optimum performance.

The UV maxima in absolute methanol for the flavonoid compounds studied in this work have been reported [29,30]. To determine the authenticity of our standards, each was dissolved in absolute methanol and their UV maxima were determined. There was excellent agreement with the reported values. The reported maxima also correlated well with those obtained by the photodiode-array detector during the experimental runs (Table 1). The values obtained differed not more than 3 nm from the reported values. Therefore, the identification of neighbouring peaks was ensured by comparing t_R values and their spectra.

3.2. *Quantitative analysis*

In order to check the linearity of the relationship between flavonoid concentration and peak area, solutions of 200 ppm of the each standard

were prepared and suitably diluted with methanol. Aliquots of five different concentrations (10, 25, 50, 100 and 200 ppm) of each of these standard solutions were injected on to the HPLC column and the peak areas were determined at 285 nm. The relationship between the concentration and the peak area is shown by the a, b and r values in Table 2, where a and b are the coefficients of the regression equation $y = ax + b$, x being the concentration of flavonoid (ppm) and y the peak area, and r is the correlation coefficient of the equation. All the flavonoids exhibited good linearity ($r = 0.988–1.000$) and obeyed Beer's law in the investigated concentration range of 10–200 ppm.

The detection limits, which were arbitrarily determined as the amounts exhibiting the area of the compound equivalent to that of the largest noise peak, ranged from 0.5 ppm for apigenin and acacetin to 2.5 ppm for rutin and robinetin. These results suggest that the proposed HPLC method is sufficiently sensitive for the determination of flavonoids.

3.3. Recovery of flavonoids from Citrus mesocarp and juice segment

Known amounts of four flavonoid standards were added to the extraction solvents or dissolved in juice samples. The samples were then extracted and prepared as described under Experimental. To elute the retained flavonoids from the Sep-Pak cartridge, we used methanol–DMSO (1:1) because of the insolubility of hesperidin and certain flavone compounds in methanol. Hesperidin does not dissolve completely in methanol at 200 ppm at ambient temperature and diosmin and diosmetin are almost insoluble in methanol. Methanol–DMSO (1:1) dissolved hesperidin up to 3000 ppm and diosmetin up to 400 ppm. This solvent was used for all Sep-Pak elutions in the sample preparations.

The recoveries of the added flavonoids are given in Table 3. In this study, eriocitrin is the most and tangeretin the least polar compound among the selected flavonoids. It is obvious that these two compounds are retained on the Sep-Pak cartridge and efficiently recovered; the recoveries were from 101.93–102.82% and 99.68–103.13% with S.D.s 2.18–2.52% and 2.85–3.34% for added eriocitrin and tangeretin, respectively. There were slight differences in the recoveries of these two flavonoids from mesocarp extraction and juice samples between the two *Citrus* species. Hesperidin is known to be the major flavonoid constituent in Unshiu tissues, whereas naringin is the major flavonoid constituent in Hirado-buntan tissues [15]. The S.D.s of the recovery of hesperidin from Unshiu and of naringin from Hirado-buntan vary slightly more than those of the other standards from each segment.

3.4. Application

The flavonoid content in the two *Citrus* species were determined to demonstrate the validity of this method. Table 4 gives the results of the HPLC analysis expressed as milligrams of flavonoid per gram (fresh mass). The smaller peaks, which were insufficient for the measurement of their UV spectra, were not quantified even if one had a t_R value that corresponded to that of the standard. Nishiura *et al.* [14,15] investigated the occurrence and distribution of ten flavanone glycosides in five tissues from several varieties of *Citrus* using thin-layer chromatography. The main flavonoids that they found in Unshiu tissues were narirutin and hesperidin. On the basis of their t_R values and UV spectra, these compounds are readily identified. Among the five different tissues examined, the narirutin content was lowest in the leaves, which agreed with the results reported elsewhere [15]. The less polar flavonoids occurred more commonly in the epicarp than in the other four tissues. The highest concentrations of flavonoids occurred in the mesocarp, which had concentrations about 100 times greater than those found in the juice. Leaves had considerably higher concentrations of flavones and flavon-3-ols, whereas juice and endocarp had little of these compounds.

In Hirado-buntan tissues, naringin and rhoifolin predominate among the flavonoids, as reported previously [14]. In addition to these compounds, we found that neohesperidin occurs

Table 2
Relationships between flavonoid levels and peak areas of flavonoids investigated

Flavanones					Flavones					Flavon-3-ols				
No.	Compound	$a(\times 10^6)^a$	$b(\times 10^3)^a$	r^b	No.	Compound	$a(\times 10^6)^a$	$b(\times 10^3)^a$	r^b	No.	Compound	$a(\times 10^6)^a$	$b(\times 10^3)^a$	r^b
1	Eriocitrin	2.89	2.50	0.999	9	Isorhoifolin	1.27	−0.73	0.999	3	Robinetin	0.74	−3.22	1.000
2	Neoeriocitrin	2.41	−3.56	0.999	10	Rhoifolin	1.01	−0.36	0.999	6	Rutin	0.65	−13.63	0.988
4	Narirutin	2.37	2.36	0.999	11	Diosmin	1.23	−1.82	0.997	14	Quercetin	1.11	−8.32	1.000
5	Naringin	2.20	−2.30	0.999	12	Neodiosmin	1.04	−3.69	1.000	17	Kaempferol	1.47	−1.64	1.000
7	Hesperidin	2.21	−9.31	1.000	16	Luteolin	2.00	10.82	0.999	19	Isorhamnetin	1.63	−15.79	1.000
8	Neohesperidin	2.16	1.33	0.999	18	Apigenin	3.22	0.72	1.000	21	Rhamnetin	1.41	−8.00	0.999
13	Neoponcirin	1.54	5.13	0.999	20	Diosmetin	2.23	−1.82	0.997					
15	Poncirin	1.46	5.75	0.999	23	Sinensetin	2.40	9.27	0.999					
22	Isosakuranetin	3.17	8.29	1.000	24	Acacetin	3.39	−4.63	1.000					
					25	Tangeretin	3.12	−0.73	0.999					

[a] Coefficients of the regression equation $y = ax + b$, where x is flavonoid concentration (ppm) and y is peak area, for concentrations ranging from 10 to 100 ppm.
[b] Correlation coefficients of the regression equation.

Table 3
Recoveries of eriocitrin, naringin, hesperidin and tangeretin from mesocarp and juice samples of Unshiu (*Citrus unshiu* Marc.) and Hirado-buntan (*Citrus grandis* Osbeck F. Hirado)

Compound	Mean recovery ± S.D.[a] (%)			
	Unshiu		Hirado-buntan	
	Mesocarp	Juice	Mesocarp	Juice
Eriocitrin	102.46 ± 2.38	102.82 ± 2.52	101.93 ± 2.32	102.68 ± 2.18
Naringin	97.47 ± 3.71	96.87 ± 3.77	102.58 ± 5.13	101.14 ± 5.03
Hesperidin	101.55 ± 5.72	104.93 ± 5.96	98.46 ± 4.09	98.22 ± 3.90
Tangeretin	103.13 ± 3.12	102.96 ± 3.34	99.68 ± 2.85	102.34 ± 3.05

For operating conditions, see text.
[a] Mean values and standard deviations for five replicates.

in all five tissues examined. The flavones and flavon-3-ols are also abundant in leaves as found with Unshiu. The flavonoid content in the epicarp is considerably lower than that found in the Unshiu epicarp, whereas Hirado-buntan juice contained relatively larger concentrations of flavonoids than did Unshiu juice. The occurrence and distribution of flavonoids in each tissue of

Table 4
Contents of flavonoids in epicarp, mesocarp, endocarp, juice and leaf samples of Unshiu and Hirado-buntan

No.	Compound	Content[a] (mg per g fresh mass)									
		Unshiu					Hirado-buntan				
		Epicarp	Mesocarp	Endocarp	Juice	Leaf	Epicarp	Mesocarp	Endocarp	Juice	Leaf
1	Eriocitrin	0.020	0.028	0.018	0.002	0.123	–[b]	–	–	–	–
2	Neoeriocitrin	–	–	–	–	–	–	–	–	0.020	0.130
4	Narirutin	0.745	4.938	2.896	0.154	0.084	0.026	0.015	0.014	–	0.141
5	Naringin	–	–	–	–	–	1.363	13.952	11.187	0.785	4.013
6	Rutin	0.126	tr[c]	tr	0.029	1.170	0.053	–	–	–	0.975
7	Hesperidin	9.452	21.030	4.225	0.087	9.155	–	0.011	0.013	0.017	–
8	Neohesperidin	–	–	–	–	–	0.074	0.120	0.060	0.015	0.671
9	Isorhoifolin	0.048	0.029	–	–	0.681	–	–	–	–	–
10	Rhoifolin	0.101	0.026	–	–	0.441	0.105	0.161	0.061	0.056	2.323
11	Diosmin	0.044	0.020	–	0.014	0.775	0.036	–	–	0.006	0.119
12	Neodiosmin	0.019	0.023	–	–	0.282	–	0.028	0.023	0.011	–
13	Neoponcirin	0.195	0.871	0.343	tr	tr	0.015	0.064	0.052	tr	–
15	Poncirin	–	–	–	–	–	–	–	–	–	tr
16	Luteolin	–	–	–	–	–	0.019	0.011	–	tr	0.058
17	Kaempferol	0.017	–	–	–	tr	–	–	–	–	–
18	Apigenin	0.008	–	–	–	–	–	–	–	–	–
20	Diosmetin	0.057	–	–	–	–	0.017	–	–	–	–
23	Sinensetin	0.022	–	–	–	0.031	0.021	–	0.014	tr	–
24	Acacetin	0.025	tr	tr	–	0.076	–	–	–	–	–
25	Tangeretin	0.076	0.019	–	–	0.117	0.078	–	–	–	–
	Total	10.955	26.984	7.482	0.286	12.935	1.807	14.362	11.424	0.910	8.430

[a] Mean values for four replicates.
[b] Dashes indicate not detected.
[c] tr = trace, not measurable in UV spectra.

Hirado-buntan was as characteristic as that found in Unshiu. Robinetin, quercetin, isorhamnetin, rhamnetin and isosakuranetin either were not present or were present in insufficient concentrations to be detected in these two *Citrus* species.

4. Conclusions

A routine and simultaneous HPLC method for the determination of 25 flavonoids that naturally occur in *Citrus* tissues was established. The flavonoids in epicarp, mesocarp and endocarp tissues of fruits and in leaf tissues were lyophilized, milled and extracted with methanol–DMSO (1:1). The resulting supernatant from these extractions was diluted ten-fold with water and the flavonoids were retained on a Sep-Pak C_{18} cartridge. After elution with methanol–DMSO (1:1), the extracts were filtered and injected on to an HPLC column. Clarified juice samples were examined in a similar fashion. The separated compounds were identified by comparing both their t_R values and UV spectra.

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