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Flavonoids in Tropical Citrus Species

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ABSTRACT: HPLC with PDA and MS^2 detection was used to identify and quantify flavonoids in the tropical citrus species *Citrus microcarpa*, *Citrus hystrix*, *Citrus medica* var. 1 and 2, and *Citrus suhuiensis*. Most of these species contained high amounts of flavones, flavanones, and dihydrochalcone *C*- and/or *O*-glycosides, which were identified on the basis of HPLC retention times, cochromatography with available authentic standards, absorbance spectra, and mass spectral fragmentation patterns. Among the major compounds detected were apigenin-6,8-di-*C*-glucoside, apigenin-8-*C*-glucosyl-2″-*O*-rhamnoside, phloretin-3′,5′-di-*C*-glucoside, diosmetin-7-*O*-rutinoside, hesperetin-7-*O*-neohesperidoside, and hesperetin-7-*O*-rutinoside. Most of the dihydrochalcone and flavone *C*-glycosides have not previously been detected in tropical citrus. *C. microcarpa* contained a high amount of phloretin-3′,5′-di-*C*-glucoside. Most of the tropical citrus flavanones were neohesperidoside conjugates, which are responsible for imparting a bitter taste to the fruit. Only *C. suhuiensis* fruit contains rutinoside, a nonbitter conjugate.

KEYWORDS: tropical citrus, flavanones, dihydrochalcones, *C*- and *O*-glycosides, HPLC-PDA-MS^2

INTRODUCTION

The health benefits of citrus fruit have been known for centuries. In 1747 James Lind, a British naval surgeon, noted that seaman with scurvy made a full recovery after eating oranges and lemons, which we now know to be a rich source of vitamin C.[1] Several studies have shown that consumption of citrus fruits is associated with lower risk of colorectal, esophageal, and stomach cancer and stroke, improved blood lipid profiles, and improved survival of the elderly.[2]

Citrus microcarpa or musk lime is a small "orange" type fruit with a loose skin and has a sweet musky smell. It is widely used as a drink in Asia (added to either black tea or plain water), as a food (pickle), as a flavoring, and as a deodorant. *Citrus medica* var. 1 has a thin skin and excellent smell, is used in traditional medicine as a drink, or added to bath water prior to bathing, but it is not commonly cultivated in Malaysia. According to the Forest Research Institute and the Department of Agriculture, Malaysia, there are no written or scientific reports on *C. medica* var. 2, but it is being widely used as a drink in selected Chinese shops in Malaysia.

Flavonoids reported to occur in citrus include flavanone, flavone, and polymethoxyflavone aglycones, flavanone- and flavone-*O*-glycosides, and flavone-*C*-glycosides.[3] Although numerous studies have been undertaken on the analysis of polyphenolic compounds in citrus using HPLC-MS, qualitative and quantitative analysis of flavonoids in tropical citrus species is currently limited to reports by Sastry and Row[4] and Miean and Mohamed[5] as well as Kanes et al.,[6] who detected 5,6,7,8,3′,4′-hexamethoxyflavanone (citromitin), 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavanone (5-*O*-desmethylcitromitin), luteolin, isosakuranetin, hesperetin-7-*O*-rutinoside (hesperidin), and quercetin-3-*O*-rutinoside (rutin) in *C. microcarpa* (Calamansi fruit).

This paper reports the analyses of flavonoid compounds in fruits of the tropical citrus species *C. microcarpa*, *Citrus suhuiensis* (mandarin orange), *Citrus hystrix*, and *C. medica* (citron) var. 1 and 2.

MATERIALS AND METHODS

Plant Materials. Tropical citrus fruits and, in some instances, leaves were bought from Kampung Baru Market, Kuala Lumpur, Malaysia. Whenever possible, citrus fruits from the same farmer were chosen. Samples were dried using an oven at 40 °C and stored at −20 °C prior to analysis.

Chemicals. Diosmetin, apigenin, phloretin, hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, diosmetin-7-*O*-rutinoside, and quercetin-3-*O*-rutinoside were obtained from AASC Ltd. (Southampton, U.K.). Apigenin-8-*C*-glucosyl-2″-*O*-rhamnoside (vitexin-2″-*O*-rhamnoside), quercetin, and diosmetin-7-*O*-neohesperidoside were purchased from Extrasynthase (Genay, France). Apin Chemical Ltd. (Abingdon, Oxon, U.K.) supplied luteolin. Hesperetin-7-*O*-neohesperidoside, isosakuranetin-7-*O*-rutinoside, eriodictyol-7-*O*-rutinoside, eriodictyol-7-*O*-neohesperidoside, and formic acid were acquired from Sigma-Aldrich (Poole, Dorset, U.K.). HPLC solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland, U.K.). Hydrochloric acid was purchased from Fisher Scientific (Loughborough, Leicestershire, U.K.). Methanol was supplied from Rathburn Chemicals (Walkerburn, Scotland, U.K.). All other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated.

Extraction of Citrus Tissues. Five gram aliquots of dried citrus tissues were soaked in 10 mL of acidified methanol (0.1% HCl) for 60 min and were centrifuged at 4000*g* for 20 min at 4 °C. The pellet was extracted twice more with the same solvent and the combined methanolic extract reduced to dryness in vacuo using a rotary evaporator and redissolved in 10 mL of acidified methanol. All samples were subdivided into 2 mL aliquots and stored at −20 °C before analysis.

Acid Hydrolysis. Conjugated flavonoids in a *C. microcarpa* flesh extract were subjected to acid treatment using a method adapted from Maatta et al.[7] Six hundred microliters of 5 M HCl was added to 1400 μL of flesh extract in a 3 mL glass V-vial. A Teflon-coated magnetic stirrer

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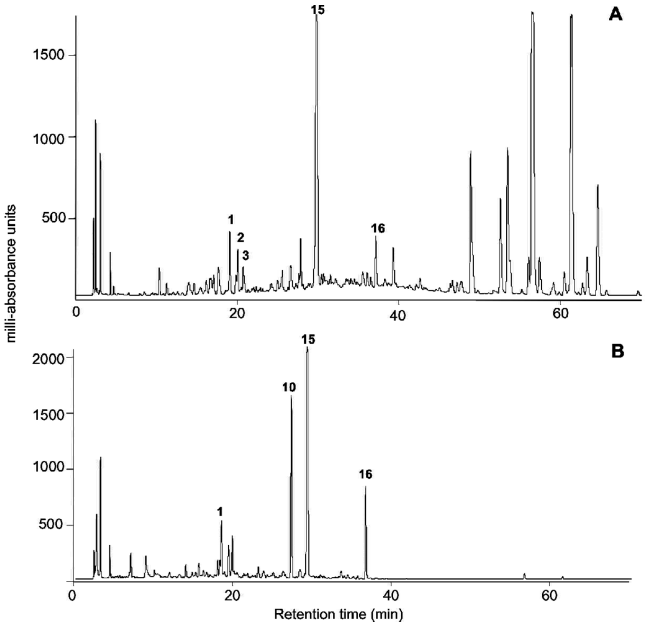

Figure 1. Gradient reversed phase HPLC analysis with detection at 280 nm of extracts of (A) *Citrus suhuiensis* fruit peel and (B) *C. suhuiensis* fruit flesh. Samples were analyzed using a 70 min gradient of 5−30% acetonitrile in 0.1% aqueous formic acid. For identification of numbered peaks, see Table 1.

was placed in the vial and sealed tightly with a PTFE-faced septum before heating in a Reacti-Therm heating/stirring module (Pierce, Rockford, IL). After 2 h at 90 °C, the sample was cooled, filtered, diluted with an equal volume of methanolic HCl (0.1%), and analyzed using HPLC-PDA-MS^2.

HPLC-PDA-MS^2 Analysis. Methanolic extracts of citrus were analyzed in triplicate on a Surveyor HPLC comprising an HPLC pump, an AS 3000 autosampler at 6 °C, and a UV6000 photodiode array detector (PDA) scanning from 250 to 700 nm (Thermo Finnigan, San Jose, CA). Separation of flavonoids was carried out using a Synergi RP-Max, Phenomenex (Torrance, CA) 4 μm, 250 × 4.6 mm i.d. C_{18} reverse-phase column at 40 °C, eluted at 1 mL/min with a gradient of acetonitrile in water containing 0.1% formic acid. Flavanones were detected at 280 and 365 nm, whereas dihydrochalcone was monitored at 280 nm. After the extract passed through the flow cell of the PDA monitor, the column eluate was split, and 20% was directed to a Finnigan LCQ Decca mass spectrometer with an electrospray interface (ESI), operating in full-scan data-dependent MS mode from 100 to 2000 amu. Samples were analyzed using the negative ion mode. ESI-MS parameters were as follows: potential of ESI source, 4 kV; capillary temperature, 400 °C. Eriodictyol-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, isosakuranetin-7-*O*-rutinoside, diosmetin-7-*O*-rutinoside, hesperetin-7-*O*-rutinoside, and hesperetin-7-*O*-neohesperidoside were quantified by reference to standard calibration curves obtained with the PDA at λ_{max} values. Quantifications of phloretin-3′,5′-diC-glucoside, apigenin-6,8-di-*C*-glucoside, diosmetin-6,8-di-*C*-glucoside, quercetin-3-*O*-rutinoside-7-*O*-glucoside, luteolin-7-*O*-rutinoside, and diosmetin-6-di-*C*-glucoside were based on their aglycones.

RESULTS AND DISCUSSION

Methanolic extracts of citrus fruits were analyzed by gradient reversed phase HPLC and ESI-MS^2 detection. Typical HPLC traces with numbered peaks are illustrated in Figures 1−3, and the identifications discussed below are based on the negative ion mass spectra summarized in Tables 1−3.

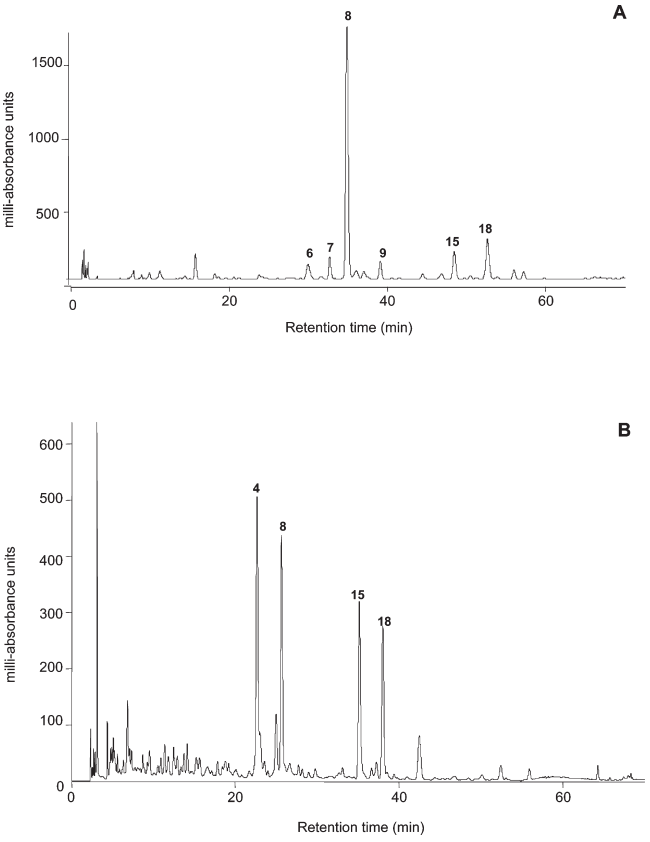

Figure 2. Gradient reversed phase HPLC analysis with detection at 280 nm of *Citrus microcarpa* fruit peel (A), analyzed using a 70 min gradient of 5−30% acetonitrile in 0.1% aqueous formic acid. Gradient reversed phase HPLC analysis with detection at 280 nm of *Citrus medica* var. 1 fruit flesh (B), analyzed using a 70 min gradient of 10−60% acetonitrile in 0.1% aqueous formic acid. For identification of numbered peaks, see Tables 1 and 2.

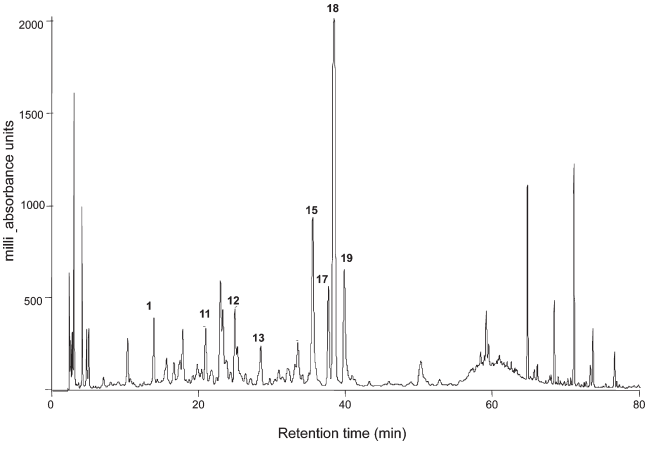

Figure 3. Gradient reversed phase HPLC analysis with detection at 280 nm of *Citrus hystrix* leaf. Samples were analyzed using 10−90% acetonitrile in 0.1% formic acid for 80 min. For identification of numbered peaks, see Table 3.

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Table 1. Summary of Flavonoids Detected in *Citrus suhuiensis* and *Citrus microcarpa* Fruit Using HPLC with Diode Array and MS^2 Detection[a]

			C. suhuiensis			
	t_R (min)					
peak	peel[b]	flesh[b]	compound	λ_{max}(nm)	$[M-H]^-$ (m/z)	MS^2 fragment ions (m/z)
1	19.1	18.4	apigenin-6,8-di-*C*-glucoside	335, 270	593	575 ($[M-H]^- - H_2O$), 533 ($[^{0,4}X_0]^-$; $[M-H]^- - 60$), 503 ($[^{0,3}X_0]^-$; $[M-H]^- - 90$) and 473 ($[^{0,2}X_0]^-$; $[M-H]^- - 120$)
2	20.1	nd	diosmetin-6,8-di-*C*-glucoside	345, 270	623	605 ($[M-H]^- - H_2O$), 533 ($[^{0,3}X_0]^-$ $[M-H]^- - 90$), 503 ($[^{0,2}X_0]$)$^-$; $[M-H]^- - 120$) and 383 ($[^{0,2}X_0{}^{0,2}X_1]^-$; $[M-H]^- - 120 - 120$)
3	20.8	nd	chysoeriol-6,8-di-*C*-glucoside	335, 270	623	605 ($[M-H]^- - H_2O$), 533 ($[^{0,3}X_0]^-$ $[M-H]^- - 90$), 503 ($[^{0,2}X_0]$)$^-$; $[M-H]^- - 120$), and 383 ($[^{0,2}X_0{}^{0,2}X_1]^-$ $[M-H]^- - 120 - 120$)
10	nd	27.2	naringenin-7-*O*-rutinoside	330, 275	579	271[narin] ($[M-H]^-$ − rut)
15	29.8	29.2	hesperetin-7-*O*-rutinoside	285, 230	609	301 [hesp] ($[M-H]^-$ − rut)
16	37.2	36.6	isosakuranetin-7-*O*-rutinoside	280, 230	593	285 ($[M-H]^-$ − rut)
			C. microcarpa			
	t_R (min)					
peak	peel[c]	flesh[d]	compounds	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 fragment ions (m/z)
1	nd	28.5	apigenin-6,8-di-*C*-glucoside	335, 270	593	575 ($[M-H]^- - H_2O$), 533 ($[^{0,4}X_0]^-$; $[M-H]^- - 60$), 503 ($[^{0,3}X_0]^-$; $[M-H]^-$−90) and 473 ($[^{0,2}X_0]^-$; $[M-H]^- - 120$)
6	30.3	49.0	apigenin-8-*C*-glucosyl-2″-*O*-rhamnoside	340, 265	577	457 $[^{0,2}X_1]^-$, 413 $[Z_1]^-$
7	33.1	52.4	unknown apigenin conjugate	340, 270	577	457 $[^{0,2}X_1]^-$, 413 $[Z_1]^-$
8	35.2	56.0	phloretin-3′,5′-di-*C*-glucoside	285, 235	597	477 ($[^{0,2}X_1]^-$; $[M-H]^- - 120$), 417 ($[^{0,3}X_1{}^{0,3}X_0]^-$; $[M-H]^- - 90 - 90$), 387 ($[^{0,2}X_1{}^{0,3}X_0]^-$; $[M-H]^- - 120 - 90$) and 357 ($[^{0,2}X_1{}^{0,2}X_0]^-$; $[M-H]^- - 120 - 120$)
9	39.4	61.2	unknown diosmetin conjugate	350, 230	607	443, 323
15	48.7	61.2	hesperetin-7-*O*-rutinoside	285, 230	609	325, 323, 301 [hesp] ($[M-H]^-$ − rut)
18	52.8	74.2	hesperetin-7-*O*-neohesperidoside	285, 235	609	301 [hesp] ($[M-H]^-$ − neohesp)

[a] Peak numbers and HPLC retention times refer to HPLC trace in Figures 1 and 2. ($[M-H]^-$, negatively charged molecular ion; rut, rutinoside; hesp, hesperetin; t_R, retention time; nd, not detected. [b] Samples were analyzed using 5−30% acetonitrile in 0.1% formic acid for 70 min. [c] Samples were analyzed using 10−30% acetonitrile in 0.1% formic acid for 70 min. [d] Samples were analyzed using 5−20% acetonitrile in 0.1% formic acid for 110 min.

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Table 2. Summary of Flavonoids Detected in *Citrus medica* var. 1 and 2 Fruit Using HPLC with Diode Array and MS^2 Detection[a]

C. medica var. 1						
	t_R (min)					
peak	peel[b]	flesh[c]	compound	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)
4	23.7	22.7	eriodictyol-7-*O*-rutinoside	285, 230	595	287 [erid] ($[M-H]^- -$ rut)
8	24.9	25.7	phloretin-3′,5′-di-*C*-glucoside	285, 235	597	477 ($[^{0,2}X_1]^-$; $[M-H]^- - 120$), 417 ($[^{0,3}X_1{}^{0,3}X_0]^-$; $[M-H]^- - 90$; $[M-H]^- - 90$), 387 ($[^{0,2}X_1{}^{0,3}X_0]^-$; $[M-H]^- - 120$; $[M-H]^- - 120$), 357 ($[^{0,2}X_1{}^{0,2}X_0]^-$; $[M-H]^- - 120$; $[M-H]^- - 120$)
15	29.2	35.2	hesperetin-7-*O*-rutinoside	285, 230	609	301 [hesp] ($[M-H]^- -$ rut)
17	29.6	nd	diosmetin-7-*O*-rutinoside	345, 230	607	299 ($[M-H]^- -$ rut)
18	30.2	38.0	hesperetin-7-*O*-neohesperidoside	285, 235	609	301 ($[M-H]^- -$ neohesp)
C. medica var. 2						
	t_R (min)					
peak	peel[d]	flesh[e]	compound	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 fragment ions (m/z)
2	16.9	20.4	diosmetin-6,8-di-*C*-glucoside	345, 270	623	605 ($[M-H]^- - H_2O$), 533 ($[^{0,3}X_0]^-$ $[M-H]^- - 90$), 503 ($[^{0,2}X_0]$)$^-$; $[M-H]^- - 120$) and 383 ($[^{0,2}X_0{}^{0,2}X_1]^-$ $[M-H]^- - 120 - 120$)
4	22.9	24.1	eriodictyol-7-*O*-rutinoside	285, 230	595	287 [erid] ($[M-H]^- -$ rut)
14	nd	27.4	diosmetin-6-*C*-glucoside	330, 245	461	383 ($[M-H]^- - 60 - H_2O$), 371 $[^{0,3}X_0]^-$; $[M-H]^- - 90$) and 341 ($[^{0,2}X_0]^-$; $[M-H]^- - 120$)
15	28.9	29.5	hesperetin-7-*O*-rutinoside	285, 230	609	325, 323, and 301 [hesp] ($[M-H]^- -$ rut)
17	29.4	29.8	diosmetin-7-*O*-rutinoside	345, 250	607	299 ($[M-H]^- -$ rut)
18	30.0	30.7	hesperetin-7-*O*-neohesperidoside		609	301 [hesp] ($[M-H]^--$ neohesp)

[a] Peak numbers and HPLC retention times. ($[M-H]^-$, negatively charged molecular ion; erid, eriodictyol; rut, rutinoside; neohesp, neohesperidoside; hesp, hesperetin; t_R, retention time; nd, not detected. [b] Samples were analyzed using 10−40% acetonitrile in 0.1% formic acid for 80 min. [c] Samples were analyzed using 10−60% acetonitrile in 0.1% formic acid for 70 min. [d] Samples were analyzed using 10−60% acetonitrile in 0.1% formic acid for 55 min. [e] Samples were analyzed using 5−30% acetonitrile in 0.1% formic acid for 70 min.

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Table 3. Summary of Flavonoids Detected in *Citrus hystrix* Using HPLC with Diode Array and MS^2 Detection[a]

peak	t_R (min) leaf[b]	peel[c]	flesh[d]	compound	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 fragment ions (m/z)
1	13.8	nd	nd	apigenin-6,8-di-*C*-glucoside	335, 270	593	575 ($[M-H]^- - H_2O$), 533 ($[^{0,4}X_0]^-$; $[M-H]^- - 60$), 503 ($[^{0,3}X_0]^-$; $[M-H]^- - 90$) and 473 ($[^{0,2}X_1]^-$; $[M-H]^- - 120$)
4	nd	nd	22.8	eriodictyol-7-*O*-rutinoside	285, 230	595	287 [erid] ($[M-H]^- - rut$)
5	nd	nd	25.2	eriodictyol-7-*O*-neohesperidoside	285, 230	595	459 ($[^{1,3}A]^-$; $[M-H]^- - 136$), 286 ($[Y_0]^-$ $[M-H]^- - 308$), and 235 ($[^{1,3}X_0]$; $[M-H]^- - 360$)
8	nd	nd	26.2	phloretin-3′,5′-di-*C*-glucoside	285, 235	597	477 ($[^{0,2}X_1]^-$; $[M-H]^- - 120$), 417 ($[^{0,3}X_1\ ^{0,3}X_0]^-$; $[M-H]^- - 90 - 90$), 387 ($[^{0,2}X_1\ ^{0,3}X_0]^-$; $[M-H]^- - 120 - 90$) and 357 ($[^{0,2}X_1\ ^{0,2}X_0]^-$; $[M-H]^- - 120 - 120$)
11	20.8	nd	nd	quercetin-3-*O*-rutinosyl-7-*O*-glucoside	280, 230	771	609 ($[M-H]^-$ − glu), 463 ($[M-H]^- - rut$), 301
12	24.8	nd	nd	quercetin-3-*O*-rutinoside	350, 255	609	343, 325, and 301 [quer] ($[M-H]^- - rut$)
13	28.3	nd	nd	luteolin-7-*O*-rutinoside	340, 230	593	285 ($[M-H]^- - rut$)
15	35.4	31.6	35.6	hesperetin-7-*O*-rutinoside	285, 230	609	323, 325, and 301 [hesp] ($[M-H]^- - rut$)
17	37.5	32.1	nd	diosmetin-7-*O*-rutinoside	345, 250	607	299 ($[M-H]^- - rut$)
18	38.3	32.8	38.6	hesperetin-7-*O*-neohesperidoside	285, 235	609	301[hesp] ($[M-H]^-$ − neohesp)
19	39.7	nd	nd	diosmetin-*O*-rutinoside	345, 250	607	299 ($[M-H]^- - rut$)

[a] Peak numbers and HPLC retention times. ($[M-H]^-$, negatively charged molecular ion; erid, eriodictyol; glu, glucoside; rut, rutinoside; hesp, hesperetin; neohesp, neohesperidoside; t_R, retention time; nd, not detected. [b] Samples were analyzed using 10−90% acetonitrile in 0.1% formic acid for 80 min. [c] Samples were analyzed using 5−30% acetonitrile in 0.1% formic acid for 70 min. [d] Samples were analyzed using 10−60% acetonitrile in 0.1% formic acid for 50 min.

Peak 1 (λ_{max} = 335, 270 nm) had a negatively charged molecular ion ($[M-H]^-$) at m/z 593, which yielded secondary fragments at m/z 473 ($[^{0,2}X_0]^-$; $[M-H]^- - 120$), m/z 503 ($[^{0,3}X_0]^-$; $[M-H]^- - 90$), and m/z 533 ($[^{1,3}X_0]^-$; $[M-H]^- - 60$), and was tentatively identified as apigenin-6,8-di-*C*-glucoside. Although an authentic standard was not available, the MS spectrum showed secondary fragments indicative of cleavage of the saccharide residues, and the spectrum is in keeping with that of apigenin-6,8-di-*C*-glucoside. A similar compound was detected in southern Italian citrus juices from orange, lemon, bergamot, citron, mandarin, and Clementine.[8] The absorbance spectra also corresponded with apigenin-6,8-di-*C*-glucoside with the λ_{max} at 335 nm.

Peak 2 (λ_{max} = 345, 270 nm) had a $[M-H]^-$ at m/z 623 and MS^2 ions at m/z 503, 383, 413, 533, and 605. Fragments at m/z 503 found after cleavage at ($[^{0,2}X_0]^-$; $[M-H]^- - 120$) followed by m/z 533 ($[^{0,3}X_0]^-$; $[M-H]^- - 90$), and m/z 383 ($[^{0,2}X_0\ ^{0,2}X_1]^-$; ($[M-H]^- - 120 - 120$) were related to the fragmentation of a second glucose unit. This compound is identified as diosmetin-6,8-di-*C*-glucoside. The presence of diosmetin-6,8-di-*C*-glucoside has been reported in southern Italian citrus[9] with the mass spectrum very similar to that obtained in the present study.

Peak 3 (λ_{max} = 335, 270 nm) had a $[M-H]^-$ at m/z 623 and MS^2 ions at m/z 605, 533, 503, 413, and 383 and could be chysoeriol-6,8-di-*C*-glucoside. Fragment ions observed at m/z 413 along with the m/z 383 characterize the aglycones as trihydroxymethoxy flavones.

Peak 4 (λ_{max} = 285, 230 nm) had a $[M-H]^-$ at m/z 595. The MS^2 spectrum had an eriodictyol fragment at m/z 287 resulting from a 308 amu cleavage of rutinose. This peak also coeluted with, and had the same mass spectrum as, a standard of eriodictyol-7-*O*-rutinoside (eriocitrin). Eriodictyol-7-*O*-rutinoside has been detected previously in citrus.[10,11]

Peak 5 (λ_{max} = 285, 230 nm) had a $[M-H]^-$ at m/z 595 and MS^2 ions at m/z 459 ($[^{1,3}A]^-$; $[M-H]^- - 136$), m/z 286 ($[Y_0]^-$ $[M-H]^- - 309$), and m/z 235 ($[^{1,3}X_0]$; $[M-H]^- - 360$). On the basis of having the same mass spectral and chromatographic properties as a reference compound, this peak was eriodictyol-7-*O*-neohesperidoside (see Figure 4).

Peak 6 (λ_{max} = 340, 265 nm) had a $[M-H]^-$ at m/z 577 and MS^2 ions at m/z 413 and 293. The $[^{0,2}X_1]^-$ ion at m/z 457 and $[Z_1]^-$ ion at m/z 413 showed the terminal rhamnose residue and the *C*-linked glucoside were connected by the 1,2-interglycosidic linkage. On the basis of the ESI-MS data and cochromatography with a standard, this compound is apigenin-8-*C*-glucoside-2″-*O*-rhamnoside (also known as vitexin-2″-*O*-rhamnoside). This flavone has been detected in *Crataegus monogyna* (hawthorn),[12] but not in citrus species.

Peak 7 (λ_{max} = 340, 270 nm) had a $[M-H]^-$ at m/z 577 and MS^2 ions at m/z 413 and 293 and is thus an unknown apigenin conjugate.

Peak 8 (λ_{max} = 285, 235 nm) was tentatively identified as phloretin-3′,5′-di-*C*-glucoside and had a $[M-H]^-$ at 597 and MS^2 ions at m/z 477, 387, 357, and 417. The MS spectrum at m/z 477 ($[^{0,2}X_1]^-$; $[M-H]^- - 120$), m/z 417 ($[^{0,3}X_1\ ^{0,3}X_0]^-$; $[M-H]^- - 90 - 90]$), m/z 387 ($[^{0,2}X_1\ ^{0,3}X_0]^-$; $[M-H]^- - 120 - 90]$), and m/z 357 ($[^{0,2}X_1\ ^{0,2}X_0]^-$; $[M-H]^- - 120 - 120$) showed that most of the fragments were obtained from loss of the saccharide residues (Figure 4) with many originating from cleavage of the glucosyl unit from the hydrochalcone structure. The fragment ion at m/z 579 ($[M-H]^- - 18$) may be due to sugar structures linked directly to the aglycone. This compound has also been found in *Fortunella* species, including *F. japonica*,

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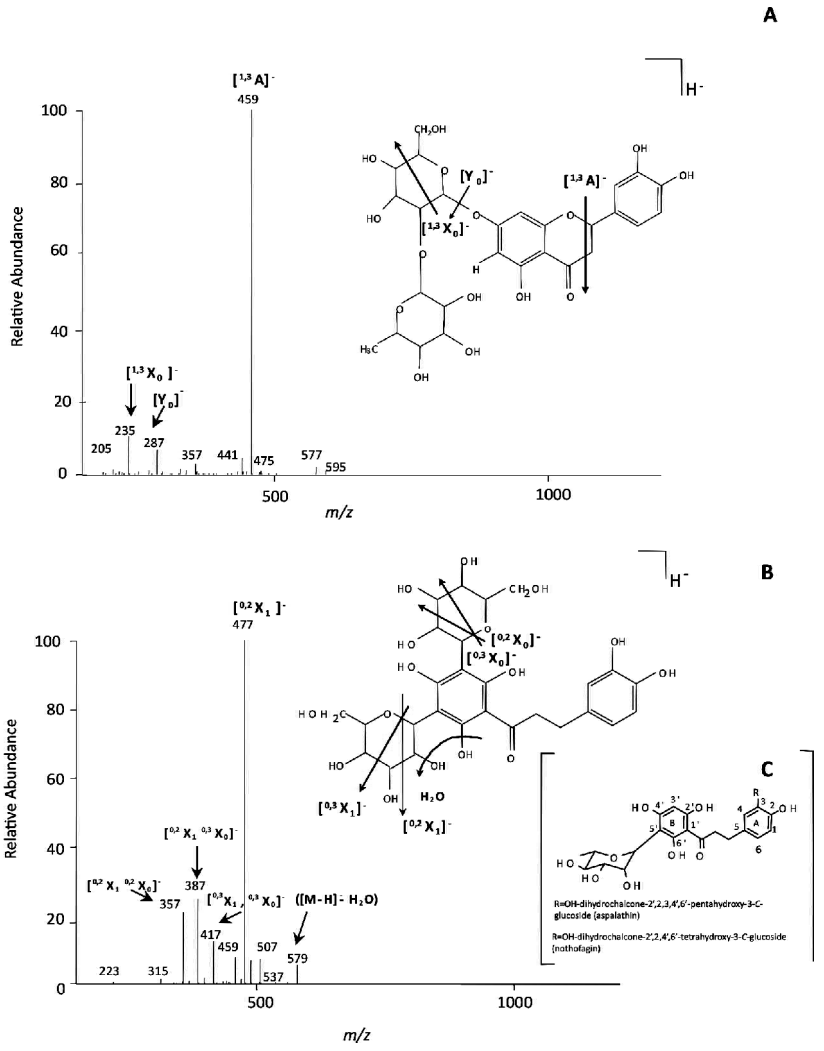

Figure 4. Nomenclature and diagnostic product ions of (A) deprotonated eriodictyol-7-*O*-neohesperidoside and (B) deprotonated phloretin-3′,5′-di-*C*-glucoside analyzed using HPLC-MS-ESI. (C) Structures of aspalathin and nothofagin found in rooibos tea.

F. margarita, *F. polyandra*, and *F. hindsii*, a genus that is taxonomically close to the genus *Citrus*.[13,14]

Peak 9 (λ_{max} = 350, 230 nm) had a $[M - H]^-$ at *m*/*z* 607 and MS^2 ions at *m*/*z* 443 and 323 (diosmetin). The fragment ions at *m*/*z* 443 and 323 could not be identified but may be an unknown diosmetin conjugate.

Peak 10 (λ_{max} = 330, 275 nm) had a $[M - H]^-$ at *m*/*z* 579, which, with a 308 amu loss corresponding to cleavage of a rutinoside moiety, produced an MS^2 naringenin fragment at *m*/*z* 271. Cochromatography with a reference compound established the identity of this component as naringenin-7-*O*-rutinoside.

Peak 11 (λ_{max} = 280, 230 nm) may be quercetin-3-*O*-rutinosyl-7-*O*-glucoside, which has been identified in *Citrus limon*.[15] It yielded a $[M - H]^-$ at *m*/*z* 771 and MS^2 fragments at *m*/*z* 609, 463, and 301 (quercetin). This was due to the losses of 308 and 162 amu corresponding to respective cleavages of glucose and rutinose units.

Peak 12 (λ_{max} = 350, 255 nm) had a $[M - H]^-$ ion at *m*/*z* 609, which with neutral loss of 308 amu corresponding to cleavage of glucose and rhamnose units, yielded an MS^2 quercetin fragment at *m*/*z* 301. This compound was identified as quercetin-3-*O*-rutinoside and was confirmed by coelution with a standard.

Peak 13 had a λ_{max} = 340 and 230 nm and yielded a $[M - H]^-$ at *m*/*z* 593 and with a 308 amu loss (cleavage of rutinose) yielded a luteolin MS^2 fragment at *m*/*z* 285. On the basis of this spectrum and its previous detection in *Taraxacum officinale*,[16] this peak is tentatively identified as luteolin-7-*O*-rutinoside.

Peak 14 (λ_{max} = 330, 245 nm) had a $[M - H]^-$ at *m*/*z* 461 and MS^2 ions at *m*/*z* 341 ($[^{0,2}X_0]^-$; $[M - H]^- - 120$), *m*/*z* 371 ($[^{0,3}X_0]^-$; $[M - H]^- - 90$]), and *m*/*z* 383 ($[M - H]^- - 60 - H_2O$) and was tentatively identified as diosmetin-6-*C*-glucoside, which has been detected in citrus.[11]

Peak 15 (λ_{max} = 285, 230 nm) had a $[M - H]^-$ at *m*/*z* 609, which on MS^2 with a 308 amu loss of rutinoside yielded a hesperetin fragment ion at *m*/*z* 301. It was identified as hesperetin-7-*O*-rutinoside (hesperidin) and confirmed by cochromatography with the reference compound.

Peak 16 (λ_{max} = 280, 230 nm) had a $[M - H]^-$ at *m*/*z* 593 and MS^2 ions at *m*/*z* 285 [M − 308] due to cleavage of rutinose. This compound was identified as isosakuranetin-7-*O*-rutinoside, a known constituent of citrus fruit.[17] The identification was confirmed by cochromatography with a reference compound.

Peak 17 (λ_{max} = 345, 250 nm) had a $[M - H]^-$ at *m*/*z* 607 and a diosmetin MS^2 ion at *m*/*z* 299 as the result of a 308 amu loss of

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Table 4. Quantification of Flavonoids in Tissues of Tropical Citrus Species[a]

peak	compound	CSP	CSF	CMP	CMF	CMe1P	CMe1F	CMe2P	CMe2F	CHL	CHP	CHF
1	apigenin-6,8-di-*C*-glucoside	102 ± 1	95 ± 3	nd	200 ± 30	nd	nd	nd	nd	143 ± 22	nd	nd
2	diosmetin-6,8-di-*C*-glucoside	472 ± 2	nd	nd	nd	nd	nd	38 ± 1	301 ± 5	nd	nd	nd
3	chysoeriol-6,8-di-*C*-glucoside	252 ± 7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4	eriodictyol-7-*O*-rutinoside	nd	nd	nd	nd	499 ± 17	824 ± 15	340 ± 36	462 ± 5	nd	nd	3868 ± 157
5	eriodictyol-7-*O*-neohesperidoside	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	144 ± 8
6	apigenin-8-*C*-glucosyl-2″-*O*-rhamnoside	nd	nd	219 ± 24	570 ± 39	nd	nd	nd	nd	nd	nd	nd
7	unknown apigenin conjugate	nd	nd	288 ± 42	681 ± 48	nd	nd	nd	nd	nd	nd	nd
8	phloretin-3′,5′-di-*C*-glucoside	nd	nd	445 ± 60	675 ± 38	169 ± 12	211 ± 36	nd	nd	nd	nd	665 ± 25
9	unknown diosmetin conjugate	nd	nd	276 ± 60	256 ± 60	nd	nd	nd	nd	nd	nd	nd
10	naringenin-7-*O*-rutinoside	nd	13129 ± 266	nd	nd	nd	nd	nd	nd	nd	nd	nd
11	quercetin-3-*O*-rutinosyl-7-*O*-glucoside	nd	nd	nd	nd	nd	nd	nd	nd	145 ± 23	nd	nd
12	quercetin-3-*O*-rutinoside	nd	nd	nd	nd	nd	nd	nd	nd	565 ± 112	nd	nd
13	luteolin-7-*O*-rutinoside	nd	nd	nd	nd	nd	nd	nd	nd	113 ± 16	nd	nd
14	diosmetin-6-*C*-glucoside	nd	nd	nd	nd	nd	nd	nd	95 ± 3	nd	nd	nd
15	hesperetin-7-*O*-rutinoside	2769 ± 19	2742 ± 51	318 ± 54	389 ± 9	344 ± 26	404 ± 5.0	545 ± 28	610 ± 15	845 ± 161	1473 ± 5	823 ± 64
16	isosakuranetin-7-*O*-rutinoside	108 ± 1	387 ± 6	nd	nd	nd	nd	nd	nd	nd	nd	nd
17	diosmetin-7-*O*-rutinoside	nd	nd	nd	nd	68 ± 4	nd	219 ± 6	150 ± 3	699 ± 125	122 ± 3	nd
18	hesperetin-7-*O*-neohesperidoside	nd	nd	314 ± 63	296 ± 34	164 ± 10	194 ± 4	87 ± 5	57 ± 2	2831 ± 470	1533 ± 16	1245 ± 85
19	diosmetin-*O*-rutinoside	nd	nd	nd	nd	nd	nd	nd	nd	1061 ± 183	nd	nd
	total	3703	16353	1860	3068	1243	1632	1229	1674	6402	3128	6745

[a] CSP, *C. suhuiensis* peel; CSF, *C. suhuiensis* fruit flesh; CMP, *C. microcarpa* peel; CMF, *C. microcarpa* fruit flesh; CMe1P, *C. medica* var. 1 peel; CMe1F, *C. medica* var. 1 flesh; CMe2P, *C. medica* var. 2 peel; CMe2F, *C. medica* var. 2 flesh; CHL, *C. hystrix* leaf; CHP, *C. hystrix* peel; CHF, *C. hystrix* flesh; nd, not detected. Data for the individual compounds are expressed as μg/g dry weight ± standard error ($n = 3$).

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a rutinosyl group. It was identified as diosmetin-7-*O*-rutinoside, also a known constituent in citrus fruits.[15] This peak cochromatographed with a diosmetin-7-*O*-rutinoside standard, and both compounds had the same MS fragmentation pattern.

Peak 18 (λ_{max} = 285, 235 nm) had a $[M - H]^-$ at *m/z* 609 and ionized, yielding an MS^2 hesperetin ion at *m/z* 301 (M − 308, loss of neohesperidose). This compound was identified as hesperetin-7-*O*-neohesperidoside, and this was confirmed by reference to a standard. Hesperetin-7-*O*-neohesperidoside has been reported to occur in *C. microcarpa*[18] and in other citrus species.[11,14,19]

Peak 19 (λ_{max} = 345, 250 nm) had a $[M - H]^-$ at *m/z* 607, which produced a diosmetin MS^2 daughter ion at *m/z* 299 due to the loss of a 308 amu rutinoside group. It is an undesignated diosmetin-*O*-rutinoside conjugate as it was chromatographically distinct from the earlier eluting peak 17, diosmetin-7-*O*-rutinoside.

With most of the flavonoid-*C*-glycosides such as apigenin-6,8-di-*C*-glucoside and diosmetin-6,8-di-*C*-glucoside, MS fragments are formed from cleavage at the 6-*C*-glucosyl group. This, according to the proposal by Cuyckens and Claeys,[20] is due to cleavage at $[^{0,2}X_0]^-$, $[^{0,3}X_0]^-$, and $[^{0,4}X_0]^-$, typically at the 6-*C*- rather than 8-*C*-position. The fragment ion at *m/z* 575 ($[M - H]^- - 18$) (for apigenin-6,8-di-*C*-glucoside) and *m/z* 605 ($[M - H]^- - 18$) (for diosmetin-6,8-di-*C*-glucoside) is due to loss of water from the 6-*C*-glucosyl and/or 8-*C*-glucosyl of the flavonoid ring. Phloretin-3′,5′-di-*C*-glucoside also has a sugar structure linked directly to the aglycone and also shows MS fragmentation pattern inside the glycosyl units (Figure 4).

The fragmentation of eriodictyol-7-*O*-neohesperidoside, as reported by Shi et al.,[21] is slightly different from that of dihydrochalcone *C*-diglycosides, as it leads to *C*-ring cleavage at $^{1,3}A$ which, by providing the substitution pattern in the A ring illustrated in Figure 4, yields a major ion at *m/z* 459 rather than *m/z* 477.

The *O*- and *C*-sugar structure of the flavonoids in the *C. microcarpa* flesh extract was further confirmed after refluxing with an HCl solution for 2 h. The apigenin and diosmetin-*C*-glycosides were resistant to hydrolysis, whereas the naringenin, hesperetin, and isosakuranetin *O*-glycosides were not.

Most of the tropical citrus flavones occurred as either rutinosides or neohesperidosides (Table 4). Neohesperidosides were found in most of the tropical citrus fruits except *C. suhuienis*. In this study, *C. microcarpa*, *C. suhuiensis*, and *C. hystrix* can also be characterized by their flavonoids, for example, apigenin-8-*C*-glucosyl-2″-*O*-rhamnoside and phloretin-3′,5′-di-*C*-glucoside in *C. microcarpa*; naringenin-7-*O*-rutinoside in *C. suhuiensis*; and eriodictyol-7-*O*-rutinoside in *C. hystrix*.

The dihydrochalcone phloretin-3′,5′-di-*C*-glucoside is structurally very similar to rooibos tea dihydrochalcones aspalathin and nothofagin (Figure 4) and is seldom found in the genus *Citrus*. It was, however detected in *C. microcarpa*, *C. hystrix*, and *C. medica* var. 1 and was present in highest amount, 675 ± 38 μg/g dry wt, in *C. microcarpa* flesh and also occurred in high quantity in the peel (Table 4).

An earlier HPLC-based study reported the occurrence isosakuranetin-7-*O*-rutinoside, eriodictyol-7-*O*-rutinoside, hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, eriodictyol-7-*O*-neohesperidoside, hesperetin-7-*O*-neohesperidoside, and diosmetin-7-*O*-rutinoside in *C. microcarpa* using HPLC.[6] Of these seven compounds, the current HPLC-PDA-MS^2- based investigation detected only three, hesperetin-7-*O*-rutinoside, hesperetin-7-*O*-neohesperidoside, and diosmetin-7-*O*-rutinoside. This marked difference in flavonoid content may be due to variability of the cultivar, or *C. microcarpa* may have undergone natural crossing. Alternatively, the HPLC-based identifications may have been inaccurate.

The levels of *O*-glycosylated flavonoids in all tested citrus fruits (Table 4) varied somewhat from those previously reported.[18,22−24] The highest concentration of hesperetin-7-*O*-rutinoside was found in *C. suhuiensis* peel at 2769 ± 19 μg/g dry wt. Hesperetin-7-*O*-neohesperidoside, which gives the bitter taste to lime, was found in most of the tropical citrus fruits, except *C. suhuiensis*, occurring in amounts ranging from 90 to 2900 μg/g dry wt. The highest concentration of hesperetin-7-*O*-neohesperidoside was found in *C. hystix* leaf at 2831 ± 470 μg/g dry wt. Hesperetin-7-*O*-rutinoside was detected in all the citrus tissues in concentration ranging from 344 ± 28 to at 2769 ± 19 μg/g dry wt. Naringenin-7-*O*-rutinoside was detected in *C. suhuiensis* flesh at 13129 ± 266 μg/g dry wt, but was not found in any of the other samples (Table 4). The USDA has reported that hesperetin-7-*O*-rutinoside or a combination of hesperetin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside is the major flavonoid in most citrus fruits.[18] Eriodictyol-7-*O*-rutinoside, which is a minor flavanone in orange, showed the highest concentration in *C. hystrix* flesh at 3868 ± 157 μg/g dry wt (Table 4).

The *C*-glycosylated flavones found in *C. microcarpa*, *C. suhuiensis*, *C. hystrix*, and *C. medica* var. 2 were apigenin-6,8-di-*C*-glucoside and diosmetin-6,8-di-*C*-glucoside. These compounds were found to be highest in *C. microcarpa* flesh and *C. suhuiensis* peel at 200 ± 30 and 472 ± 2 μg/g dry wt, respectively. Apigenin-8-*C*-glucosyl-2′-*O*-rhamnoside was found only in *C. microcarpa* flesh and peel. A trace quantity of diosmetin-6-*C*-glucoside was found in *C. medica* var. 2 flesh (Table 4). The *C*-glycosylated flavonoids were found in *C. hystrix* and *C. medica* var. 1 flesh.

In conclusion, this study provided information on the distinct spectrum of flavonoids in tropical citrus species. Most of the flavanones were neohesperidoside conjugates, which contribute to the bitter taste of the fruits. A compound tentatively identified as the dihydrochalcone phloretin-3′,5′-di-*C*-glucopyranoside, which had not previously been detected in citrus species, was found in *C. microcarpa* and *C. hystrix*.

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