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Flavonoid Profiling in Leaves of Citrus Genotypes under Different Environmental Situations

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Citrumelo CPB 4475 and Carrizo citrange are two citrus genotypes that differ in their tolerance to abiotic stress. Little information is found on flavonoid content in citrus leaves and even less on the role of flavonoids on citrus tolerance to environmental stress. This work developed a multiplexed approach utilizing HPLC-DAD-ESI-MS and NMR for the identification and profiling of flavonoids in citrus leaves. A total of 47 phenolic compounds were detected and 45 tentatively identified. Eleven of them were acylated with hydroxycinnamic acid derivatives. Some of the compounds identified were not reported in citrus and two of them not even in the plant kingdom. In response to soil flooding, the relative reduction in leaf flavonoids was more important in citrumelo, which can give additional insights to the low tolerance of this genotype to flooding. The method reported could serve as an effective tool for profiling flavonoids in leaves of different genotypes under distinct environmental situations.

KEYWORDS: Citrange Carrizo; citrumelo CPB4475; flooding; HPLC-DAD-ESI-MS/MS; NMR; polyphenols

INTRODUCTION

Citrumelo CPB 4475 (*Citrus paradisi* L. Macf. × *Poncirus trifoliata* L. Raf.] and Carrizo citrange (*Citrus sinensis* L. Osb. × *P. trifoliata* L. Raf.) are two hybrid citrus genotypes widely used as rootstocks because of their tolerance to the citrus tristeza virus and other diseases (*1*). However, these genotypes differ in their tolerance to abiotic stress (*2, 3*). We have previously demonstrated that specific but probably connected mechanisms could be the basis of this differential tolerance. A precise adjustment of the photosynthetic machinery to adverse environmental conditions (*2*), specific hormonal signals (*4*), and, particularly, a capability to prevent stress-induced oxidative damage through enhanced enzymatic activities and accumulation of antioxidant metabolites (*3*) seems to be crucial for citrus tolerance to abiotic stress.

Flavonoids are among the most bioactive plant secondary metabolites and can be subdivided into different classes including flavones, flavanones, flavonols, flavonones, isoflavones, flavans, and anthocyanins (*5, 6*). Among these, flavanones and flavones are less represented in plants with the exception of citrus species. Flavanones, in fact, are the most important secondary metabolites of citrus juices (*7*). Naringenin and hesperetin are the most abundant aglycone forms among citrus flavanones, although they appear almost exclusively as polar glycoside derivatives, neohesperidosides and rutinosides being the two main types of glycosides. Flavones are mainly present as glycoside derivatives in citrus. In addition, *C*-glycoside flavone derivatives, less widespread in plant species, can also be found (*8*).

The continuous interest in natural flavonoids is due to different reasons. For example, flavanone chemical structures are specific for every genotype, which make them suitable as markers of adulteration in commercial juices (*9*). In this sense, naringin is never present in sweet orange juice, and its presence is used to detect adulteration (*10*). The potential industrial exploitations of natural flavonoids are also attractive aspects. Among these, their pharmacological and nutraceutical features are most intriguing for humans. Their presence in human diet is associated with protective effects against some chronic-degenerative diseases related to oxidative stress (*11*). Flavonoids have also potent antiatherogenic activities (*12*). Naringenin, the most abundant flavanone in citrus, has been associated with DNA repair following oxidative damage in prostate cancer cells (*13*) and with inhibition of human tumor growth cells implanted in mice (*14*). Many citrus flavones have also demonstrated an antiproliferative effect in carcinogenic cells (*15*).

All of this active research is mainly focused on fruits, and considerably less information on flavonoid content in citrus leaves is available. Kawaii et al. (*16*) performed a quantitative study of flavonoids in the leaves of 68 citrus species with a chemotaxonomic objective. Only a few references on the role of flavonoids in citrus tolerance to environmental stress could be found. Manthey et al. (*17*) studied the changes in leaf concentration of 11 flavonoids under biotic stress. In grapevine,

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Castellarin et al. (*18*) found an increased anthocyanin content in fruits under water stress. Anthocyanins could be also implicated in tolerance to drought, heavy metals, and even resistance to herbivore and pathogen attacks (*19*). In different plant systems, flavonoids have been proposed as a mechanism for aluminum detoxification in plant cells (*20*). In contrast, there is still a matter of controversy whether the ability to accumulate flavonoids, particularly flavonoids with ortho-dihydroxylated B-rings, and "tolerance" to UV radiation stress are correlated (*21*).

The purpose of this work was to develop a multiplexed approach using HPLC-DAD-ESI-MS for the identification and profiling of flavonoids in citrus leaves. Mass spectrometry and NMR were performed to assist in the structural elucidation of both aglycones and glycosidic conjugates. Furthermore, MS-based methods allowed us to differentiate the flavonoid composition of two citrus genotypes. The hypothesis to test was whether there was a differential accumulation of these compounds in control or stress conditions. The variations in flavonoid content were compared with the well-known distinct tolerance of the two genotypes to soil flooding, Carrizo being more tolerant than citrumelo to this environmental stress.

MATERIALS AND METHODS

General Experimental Procedures. Fractionation and purification of extracts were performed on columns of Sephadex LH-20 (Fluka, Barcelona, Spain) and 32−63 Å silica gel (Brunschwig Chemie, Hexaanweg, The Netherlands). The HPLC instrument used was an Agilent 1100 series (Agilent Technologies Inc., Santa Clara, CA) equipped with a G1312A binary pump, a G1313A autosampler, and a G1322A degasser, connected in series with a G1315B diode array detector (DAD). An LCQ (Thermo-Finnigan, San Jose, CA) ion trap mass spectrometer with an electrospray ionization source (ESI) coupled to the outlet of the DAD was selected for its high sensitivity in full-scan mode and the possibility of performing several fragmentations on a single compound. For multiresidual analyses (MRM), a Waters Alliance 2695 HPLC pump attached to a triple-quadrupole mass spectrometer (Quattro LC, Micromass Ltd., Manchester, U.K.) was chosen due to the higher specificity and sensitivity demands of the multiresidual method that cannot be fulfilled with our ion trap system. The 1D and 2D NMR spectra were recorded on a Varian Innova 500 MHz instrument (Varian Inc., Palo Alto, CA), using MeOH-d_4 as a solvent. NMR was used to elucidate the position of certain functional groups when it was not evident from mass spectrometry experiments.

Hydrochloric acid, sodium hydroxide, acetic acid, and HPLC grade acetonitrile and methanol were purchased from Scharlab (Barcelona, Spain). Biochanin A, naringin, and diosmin standards were purchased from Sigma (Madrid, Spain).

Plant Material, Treatments, and Sample Collection. For flavonoid identification and standard isolation, 5-year-old trees of citrumelo CPB 4475 (*C. paradisi* L. Macf. × *P.trifoliata* L. Raf.) and Carrizo citrange (*C. sinensis* L. Osb. × *P. trifoliata* L. Raf.) grown in our experimental field at Campus Riu Sec, Castelló, Spain, were used. For stress studies, 2-year-old seedlings of the same genotypes were grown in a greenhouse with natural photoperiod and average day and night temperatures of 25 ± 3.0 and 18 ± 2.0 °C, respectively. Flooding experiments were performed as described previously (*3, 4*). Briefly, for each genotype, 12 plants were maintained under regular watering, whereas another 12 were continuously flooded for 6 days.

In both cases, mature leaves were sampled and immediately frozen in liquid nitrogen. The frozen material was ground to fine powder, lyophilized, and stored at 4 °C until analyses.

Sample Preparation. Lyophilized tissue (600 g) was extracted with a mixture of CH_2Cl_2/MeOH (1:1). Most of the compounds studied in this work were in a range of concentration too low to be reliably detected and identified in a nontargeted manner. Therefore, high amounts of raw material were needed to find significant amounts of analytes. After filtration and solvent evaporation, 40 g of crude extract was obtained. The crude extract was dissolved in water and sequentially partitioned against hexane, diethyl ether, and ethyl acetate to give 8, 3, and 4 g of extract, respectively. The evaporation of the water layer gave 20 g of extract. HPLC-DAD analyses indicated that only the ethyl acetate extract and the water layer contained phenolic compounds.

Purification. The ethyl acetate extract was subjected to repeated Sephadex chromatography on a 70 × 2 cm column with MeOH. Fractions (5 mL each) were collected and grouped in four blocks by RP-10 (Merck, Barcelona, Spain) TLC profiling [TLC was performed on 10 cm height plates using a mixture of MeOH/H_2O (8:2) as a solvent]. In the second and third groups, different amounts of powder precipitated containing mixtures of compounds. The solid mixtures were purified with a 250 mm × 8.0 mm i.d., 5 μm, Kromasil 100 C18 semipreparative column (Scharlab, Barcelona, Spain) using MeOH and water as solvents. The aqueous extract was fractionated by column chromatography using 300 g of silica gel and vacuum. The solvent gradient started with ethyl acetate/MeOH/H_2O (85:10:5) to progressively increase the percentage of MeOH. Forty fractions of 250 mL were collected and grouped in eight fractions according to TLC (RP-18) profiling. After repeated column chromatography followed by separation on the semipreparative column, fractions 2 and 6 yielded 3 and 10 compounds, respectively.

Alkaline and Acid Hydrolyses. Alkaline hydrolysis was performed by adding 1 mL of 4 N NaOH to 1 mL of the hydroalcoholic leaf extracts in sealed test tubes at room temperature. After 24 h of incubation, extracts were acidified with concentrated HCl (pH ∼2) and directly analyzed by HPLC-DAD-MS/MS. Acid hydrolysis was carried out by adding 1 mL of 4 N HCl to 1 mL of the leaf extract in sealed test tubes at 90 °C. After 30 min, extracts were directly analyzed by HPLC-DAD-MS/MS.

The use of a multiresidual analytical approach increased sensitivity and specificity to a great extent. Therefore, for abiotic stress experiments, only 100 mg of plant material was double-extracted with 200 μL of MeOH/H_2O (8:2) by gentle sonication in an ultrasonic bath for 10 min. The mixture was then centrifuged at 9200*g* for 20 min at 4 °C and supernatant recovered and filtered through 0.45 μm pore size nylon syringe filters (Whatman Inc., Clifton, NJ) for direct injection into the HPLC.

Identification of Flavonoid Compounds by HPLC-DAD-ESI-MS/MS. Separation of flavonoids was achieved on a 100 mm × 2.1 mm i.d., 5 μm, Kromasil column (Scharlab). The mobile phase was (A) water/acetic acid (99.99:0.01) and (B) acetonitrile. The linear gradient started with 5% B to reach 15% B after 15 min, 30% B at 30 min, 70% B at 40 min, and, finally, 100% B at 45 min. The flow rate was set at 0.3 mL/min. The UV spectra were recorded between 200 and 450 nm, and simultaneous detection by diode array was performed at 278 and 325 nm.

For the ion trap, the capillary temperature was set at 300 °C; spray needle voltage was at −3.5 kV, and nitrogen was used as sheath gas with a flow of 60 arbitrary units. Mass analysis was carried out in full-scan mode from 50 to 1500 amu in negative mode. The MS/MS spectra were obtained by applied collision energies of 30−40% of the instrument maximum. Samples were tested three times and gave superimposable chromatograms.

Relative Quantification of Flavonoids by HPLC-ESI-MS/MS. The information obtained from the above tests was compiled in a multiresidual analysis in a triple-quadrupole mass spectrometer (Quattro LC, Micromass Ltd.). The mass spectrometer was operated in negative electrospray mode, and only the selected flavonoid transitions were recorded over the chromatogram run. Nebulizer and desolvation gas was nitrogen, and flow rates were set to 120 and 800 arbitrary units, respectively. Source block temperature was set to 120 °C and desolvation gas temperature to 350 °C. Capillary, cone, and extractor voltages were set to 4 kV, 25, and 3 eV, respectively. Cone voltage was optimized to reduce adduct formation and to prevent source fragmentation of analytes. Biochanin A (Sigma-Aldrich) was added (1 ng/mL) as an internal standard for relative quantification. Separation of samples was carried out using the column, gradient, and flow rate described above but setting an initial 4 min solvent delay to prevent buildup of salts in the source. Samples were injected in triplicate to ensure statistical validity of analyses. Average recovery values ranged

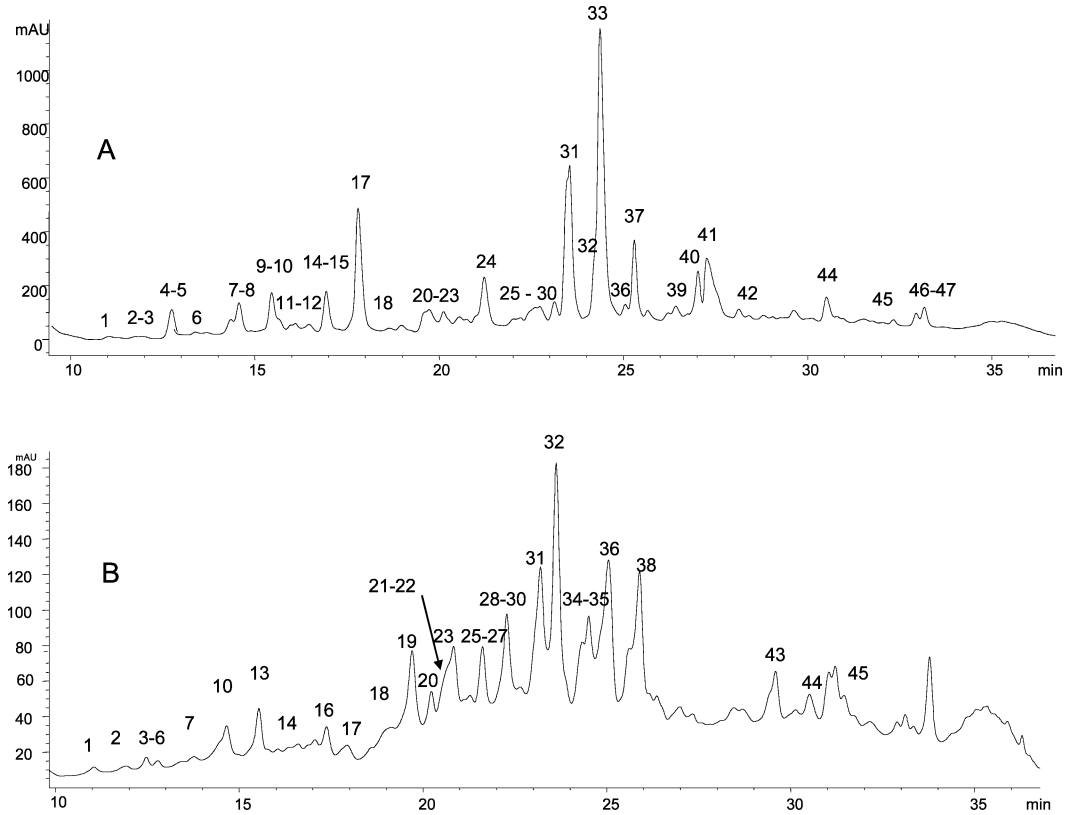

Figure 1. Separation of flavonoids and derivatives from leaves of (**A**) citrumelo CPB4475 and (**B**) Carrizo citrange by HPLC-DAD. For peak assignment see **Tables 1**−**3**.

Table 1. Retention Time (t_R) and UV and MS^n Spectra of Flavonoid 6,8-Di-*C*-glycoside

				− MS^2 [M − H]$^-$ (%)							
compound	t_R (min)	[M − H]$^-$ (*m*/*z*)	UV λ (nm)	−18 − (H_2O)	−90 ($^{0,3}_6$X)	−120 ($^{0,2}_6$X)	[A + 83]$^-$ (−240)	[A + 113]$^-$ (−210)	−206 (−syn)	−176 (−fer)	−162 (−Glc)
1, eriodictyol 6,8-di-*C*-Glc[a,b,c]	10.7	611	288	593.1 (5)	521.1 (20) (100)	491.1	370.9 (60)	401.2 (40)			
4, naringenin 6,8-di-*C*-Glc[a,b,c]	12.3	595	284	576.9 (6)	505.5 (25)	475.2 (100)	355.1 (70)	385.0 (60)			
5, leucenin-2[a,b]	12.6	609	270, 349	591.0 (8)	519.0 (28)	489.0 (100)	369.0 (28)	399.0 (17)			
10, apigenin 6,8-di-*C*-Glc[a,b,c]	15.3	593	271, 335	575.1 (11)	503.0 (25)	473.0 (100)	353.1 (10)	383.0 (15)			
12, leucenin-2,4′-methyl ether[a,b]	15.9	623	271, 348	605.2 (6)	533.0 (17)	503.0 (100)	383.0 (22)	413.0 (10)			
24, vicenin-2,4′-methyl ether[a]	20.7	607	272, 345	589.1 (5)	517.0 (30)	487.0 (100)	367.0 (28)	397.0 (15)			
derivatives of **10**, apigenin 6,8-di-*C*-glucoside											
8, 10-glucosyl[a]	14.2	755	269, 334	737.2 (4)	665.0 (5)	635.2 (10)	352.9 (10)				593(100)
		[MS^3→593]$^-$		575.1 (12)	503.2 (30)	473.1 (100)	353.2 (18)	383.1 (10)			
40, 10-sinapoyl[a]	27.1	799	270, 336	781.1 (10)	709.2 (100)	679.0 (80)	353.1 (37)	383.2 (31)	593(35)		
		[MS^3→593]$^-$		575.1 (25)	503.4 (70)	473.0 (100)	353.3 (45)	382.9 (38)			
41, 10-feruloyl[a]	27.6	769	271, 335	751.2 (12)	679.1 (70)	649.1 (100)	353.0 (30)	383.0 (27)		593(30)	
		[MS^3→593]$^-$		575.3 (27)	503.2 (70)	473.2 (100)	353.1 (40)	383.3 (30)			

[a] Compounds isolated from citrumelo CPB4475. [b] Compounds isolated from Carrizo citrange. [c] Glc, glucosyl.

between 80 and 85%, and relative standard deviation (calculated as sd/mean × 100, RSD) of area and retention time values varied between 4.2 and 15.3% and between 0.2 and 0.5%, respectively.

Statistical Analyses. Data mean comparisons were performed with Statgraphics Plus V.5.1. software (Statistical Graphics Corp., Herndon, VA). One-way analysis of variance (ANOVA) was performed to assess differences between control and flooded plants considering a significance value of 0.05.

RESULTS AND DISCUSSION

Flavonoid Identification in Leaves of Citrumelo and Carrizo. Citrumelo and Carrizo leaf extracts were analyzed by HPLC-DAD-MS, and the chromatogram profiles are shown in **Figure 1**. A total of 47 phenolic compounds were detected, and 45 were tentatively identified. Eleven of them were acylated with hydroxycinnamic acid derivatives. For characterization and identification of native compounds, extract saponification followed by the analysis of deacylated derivatives was performed. After hydrolysis, all identified flavonoids described in this work gave glucose and rhamnose as sugar moieties and apigenin, kaempferol, eriodictyol, and quercetin as aglycones.

Flavonoid *C*-Glycosides. This group of flavonoids is resistant to acid hydrolysis. By comparison of DAD chromatograms at 325 nm from crude extracts with those previously hydrolyzed, it was observed that peaks for compounds **1**, **4**, **5**, **10**, **12**, and **24** appeared unchanged. This behavior, together with the absence of an aglycone ion in the MS/MS spectra, suggested that these compounds were flavonoid *C*-glycosides.

The MS/MS spectra obtained by focusing on each [M − H]$^-$ ion of these compounds exhibited the same pattern of fragmentation ([(M − H) − 18]$^-$, [(M − H) − 90]$^-$, [(M − H) − 120]$^-$,

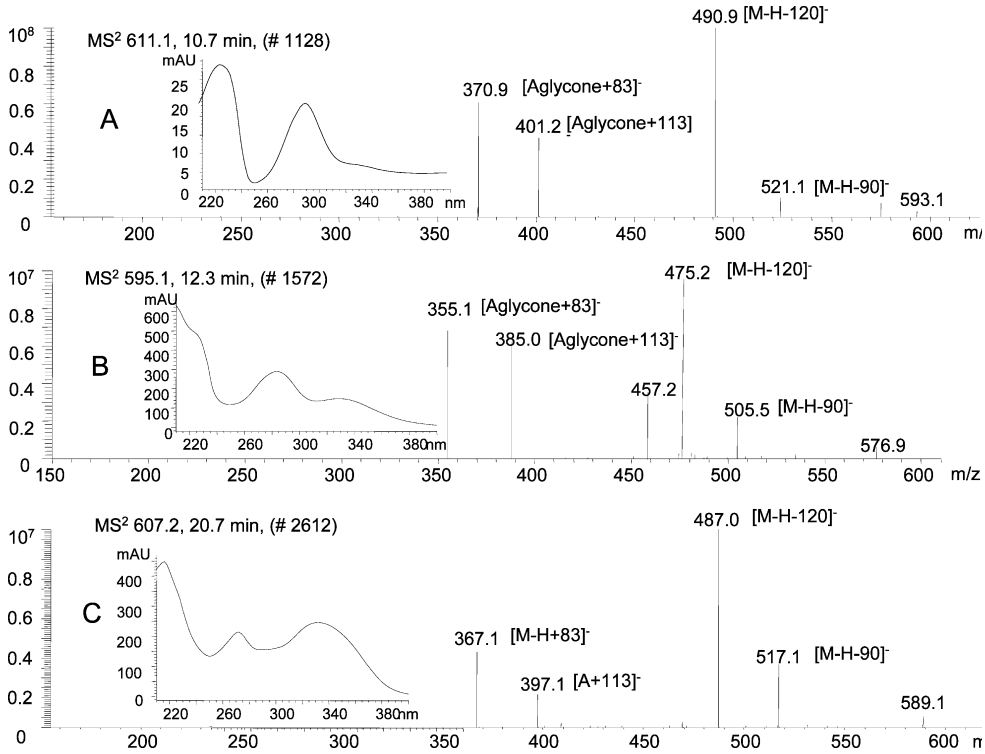

Figure 2. DAD and MS^2 spectra of (**A**) eriodictyol 6,8-di-*C*-glucosyl, (*B*) naringenin 6,8-di-*C*-glucosyl, and (**C**) vicenin-2,4′-methyl ether.

$[A + 83]^-$, and $[A + 113]^-$), typical of di-*C*-glycosylflavonoids. The maximum of band II in the UV spectrum was around 270 nm or higher in case of compounds **5**, **10**, **12**, and **24**, suggesting that they were substituted at positions 6 and 8 (*22*). DAD chromatograms of compounds **1** and **4** gave only one maximum at 288 nm, suggesting the presence of a flavanone skeleton. The presence of the $[(M - H) - 120]^-$ (base peak) and $[(M - H) - 90]^-$ peaks confirmed that the sugar substituent was a hexose (*23, 24*). Ions at *m/z* $[A + 113]^-$ and $[A + 83]^-$ correspond to the aglycone bearing sugar fragments, and they are particularly important for aglycone identification (**Table 1**).

Compound 1. This compound was present in trace amounts at a t_R of 10.7 min. The UV spectrum showed a λ_{max} at 288 nm and a shoulder at 333 nm typical of a flavanone skeleton. Full mass spectra gave a signal at *m/z* 611.1 identified as the $[M - H]^-$ ion peak. The MS^2, particularly ions at *m/z* 593 $[(M - H) - 18]^-$, 521 $[(M - H) - 90]^-$, and 491 $[(M - H) - 120]^-$, characterized the occurrence of a 6,8-di-*C*-glucoside (*22*). The ions at *m/z* 401 [aglycone + 113]$^-$ and 371 [aglycone + 83]$^-$ correspond to the aglycone-bearing sugar fragments (**Figure 2**). Therefore, compound **1** was tentatively identified as eriodictyol 6,8-di-*C*-glucoside.

Compound 4. It was present in trace amounts at a t_R of 12.3 min. Its UV spectrum was similar to that of compound **1**. Full mass scan analyses showed a signal at *m/z* 595.1 corresponding to a deprotonated ion $[M - H]^-$. MS/MS spectra exhibited a fragmentation pattern similar to that of compound **1**. The presence of peaks $[(M - H) - 90]^-$ and $[(M - H) - 120]^-$ (base peak) suggested that the sugar substituents were hexoses (*23*). Peaks at *m/z* 385 $[A + 113]^-$ and 355 $[A + 83]^-$ indicated the mass of the aglycone. This compound was tentatively identified as naringenin 6,8-di-*C*-glucoside.

Compound 24 was detected at 20.7 min. The UV spectra showed maxima at 272 and 345 nm corresponding to bands II and I, respectively, and characteristic of a flavone (*25*). The MS/MS spectra focused on the pseudomolecular ion (*m/z* 607 $[M - H]^-$) showed fragments generated by typical losses of 18, 90, and 120 amu characteristic of the 6,8-di-*C*-glucosides. In fact, this fragmentation is related to a sugar moiety rather than to the loss of a whole sugar unit as in the *O*-glucosides. The ions at *m/z* 367 [aglycone + 83]$^-$ and 397 [aglycone + 113]$^-$ indicated the aglycone as a methyl ether trihydroxyflavone. Therefore, this compound could be 4′-methoxy-5,7-dihydroxyflavone 6,8-di-*C*-glucoside (vicenin-2,4′-methyl ether).

To confirm these structures, 1 mg of compound **1** was isolated, and the ^1H NMR spectrum presented characteristic resonance of aromatic proton of ring B [δ 8.09 (d, 2H, $J_{2'6'}$ = 8.00 Hz, H2′ and H6′) and δ 7.04 (d, 2H, $J_{3'5'}$ = 8.00 Hz, H3′ and H5′)]. The proton at position 3 (H-3) appeared as a singlet at δ 6.66, and a singlet at δ 3.45 (3H) represented a methyl group. The two anomeric protons of both glucose units appeared as a singlet at δ 5.48.

According to our knowledge, this is the first time that compounds **1** and **4** have been described in the plant kingdom. In addition, compounds **2** and **24** are detected for the first time in the *Citrus* genus and relatives.

Compounds 5, 10, and 12. Compounds **5** (*m/z* 609), **10** (*m/z* 593), and **12** (*m/z* 623) were identified as luteolin 6,8-di-*C*-glucoside (leucenin-2), apigenin 6,8-di-*C*-glucoside (vicenin-2), and diosmetin 6,8-di-*C*-glucoside (leucenin-2,4-dimethyl ether), respectively, by comparison with previously reported spectroscopic data. These compounds have been previously described in *Citrus limon* and *Citrus bergamia* (*26, 27*). Compound **10** was isolated (1.6 mg), and the ^1H NMR spectrum presented characteristic resonance of aromatic protons of ring B [δ 7.98 (2H, d, $J_{2'6'}$ = 8.4 Hz, H-2′ and H-6′); δ 6.95 (2H, d, $J_{3'5'}$ = 8.4 Hz, H-3′ and H-5′)], and the H-3 proton appeared as a singlet at 6.62. The two anomeric protons of both glucose units appeared as a based singlet at δ 5.01. Both UV and ^1H NMR data were superimposable with those previously reported (*8, 28*).

Flavonoid *O*-Glycosides. Flavonoid *O*-glycosides are easily hydrolyzed in acid medium, which helps to define this group

Table 2. Retention Time and UV and MS^n Spectra of Flavonoid Compounds

compound	t_R (min)	$[M - H]^-$ (*m/z*)	λ (nm)	$-MS^n$ $[M - H]^-$ (%)
2, quercetin 3,7-*O*-diglucoside[a,b]	11.6	625	255, 266sh, 353	463 (100) MS^3 [625→463]: 301 (100)
3, quercetin7-*O*-glucosyl-3-*O*-rutinoside[a,b]	12.0	771	257, 267sh, 355	609 (100) MS^3 [771→609]: 301 (100)
6, quercetin 3,7-triglucoside[a,b]	12.9	771	256, 354	609 (100), 463 (10) MS^3 [771→609]: 463 (100), 301 (40)
7, kaempferol-3,7-*O*-diglucoside[a,b]	13.8	609	253, 315sh, 350	489 (28), 447 (100), 301 (25)
9, kaempferol-3-*O*-rutinoside-7-*O*-glucosyl[a,b]	15.2	755	254, 318sh, 349	593 (100) MS^3 [755→593]: 285 (100)
11, apigenin-7-*O*-glucosyl-6-*C*-glucoside[a]	15.5	593	269, 337	575 (5), 503 (8), 473 (60), 431 (5), 311 (30)
13, eriodictyol-7-*O*-rutinoside-4′-*O*-glucoside[b]	15.9	757	283	595 (30), 449 (100), 287 (12) MS^3 [757→449]: 287 (100)
14, quercetin-3-*O*-sophroside[a,b]	16.1	625	258, 268sh, 355	505 (25), 463 (30), 445 (60), 301 (100)
15, quercetin-7-*O*-glucosyl-3-*O*-diglycoside[a]	16.7	757	255, 265sh, 356	595 (100), 301 (8) MS^3 [757→595]: 463 (12), 301 (100)
16, eriodictyol-7-*O*-neohesperidoside-4′-*O*-glucoside[b]	17.3	757	283	595 (100), 459 (15) MS^3 [757→595]: 459 (100), 235 (22)
17, unknown[a,b]	17.5	783		621 (100) MS^3 [783→621]: 559 (25), 519 (55), 477 (100), 315 (20)
18, kaempferol-3-*O*-sophroside[a,b]	18.4	609	266, 295sh, 351	489 (10), 447 (15), 429 (75), 285 (100)
19, eriodictyol-7-*O*-rutinoside[b]	19.4	595	284, 332 (low)	287 (100)
20, quercetin 3-*O*-rutinoside[a,b]	19.6	609	255, 266sh, 355	301 (100)
21, quercetin 3-*O*-glucoside[a,b]	20.2	463	255, 266sh, 355	301 (100)
22, kaempferol 3-*O*-glucoside[a,b]	20.4	447	266, 295sh, 348	285 (100)
23, eriodictyol 7-*O*-neohesperidoside[b]	20.5	595	284	459 (100)
25, apigenin 7-*O*-rutinoside[a,b]	21.1	577	267, 337	269 (100)
26, kaempferol 7-*O*-rhamnosyl 3-*O*-glucoside[a,b]	21.5	593	266, 349	447 (100), 285 (10)
27, quercetin 3-*O*-(acetyl)glucoside[a,b]	21.7	505	255, 355	463 (40), 301 (100)
28, narirutin[a,b]	22.6	579	284, 330 (low)	271 (100)
29, kaempferol-3-*O*-rutinoside[a,b]	22.7	593	265, 294sh, 348	285 (100)
30, isorhamnetin 3-*O*-rutinoside[a,b]	22.9	625	255, 268sh, 354	315 (100), 300 (25)
31, unknown[a,b]	23.3	471	228, 332	265 (100)
32, naringin[a,b]	23.5	579	284, 324 (low)	459 (100), 373 (8), 313 (15), 271 (28), 235 (12)
33, apigenin 7-*O*-neohesperidoside[a]	23.9	577	267, 337	269 (100)
36, diosmin[a,b]	24.8	607	284, 325 (low)	299 (100), 284 (34)
42, quercetin 3-*O*-(acetyl)rutinoside[a,b]	28.0	651	250, 349	609(100), 301 (15)
44, poncirin[a,b]	31.1	593	284,	473(28), 325(25), 285(100)

[a] Compounds isolated from citrumelo CPB4475. [b] Compounds isolated from Carrizo citrange.

Table 3. Retention Time and UV and MS^n Spectra of Acylated Flavonoid 3,7-*O*-Glycosides

				$-MS^2$ $[M - H]^-$ (%)					
	t_R (min)	$[M - H]^-$ (*m/z*)	λ (nm)	−Glc −162	MeOCaf −192	−Sin −206	−(Glc + Rham)−308	−(Glc + Sin) −368	$-MS^3$ $[M - H]^-$ (%)
34, quercetin 3-*O*-(sin)Glc 7-*O*-rutinoside[b]	24.7	977	278, 340				669 (92)		[977→669]: 353 (60), 301 (100)
35, quercetin 3-*O*-rutinoside 7-(sin)glucoside[b]								609 (88)	[977→609]: 301 (100)
37, apigenin 7-*O*-(sin)triglycoside[a]	25.0	945	270, 325				637 (20)	577 (100)	[945→577]: 457 (30), 413 (40), 311 (20), 269 (100)
38, eriodyctiol(sin) neohesperidoside[b]	26.0	963	385, 333			757 (15)		595 (100)	[963→595]: 459 (100), 441 (12), 287 (5), 235 (20)
39, naringenin 7-*O*-(sin)triglycoside[a]	26.6	947	283, 332	785 (65)			639 (65)	579 (100)	[947→579]: 459 (100), 441 (40), 353 (30), 271 (80)
43, eriodyctiol 7-*O*-(sin) neohesperidoside[b]	30.5	801	282, 334			595 (100)			[801→595]: 459 (100), 441 (15), 235 (25)
45, naringenin 7-*O*-(sin)neohesperidoside[a,b]	32.6	785	282, 332			579 (100)			[785→579]: 459 (100), 313 (15), 271 (50)
46, kaempferol (methoxycafeoyl) rutinoside[a]	32.7	785	283, 385		593 (100)				[785→593]: 285 (100)
47, quercetin 7-*O*-sinapoyl 3-*O*-rutinoside[a]	33.0	815	268, 328			609 (100)			[815→609]: 301 (100)

[a] Compounds isolated from citrumelo CPB4475. [b] Compounds isolated from Carrizo citrange.

of flavonoids in an extract by comparing DAD chromatograms at 325 nm before and after hydrolysis. The assignation of different sugar substitutions (mono-, di-, or triglycosides) on the flavonoid hydroxyls has been carried out in accordance with previous studies focused on the MS analyses of similar compounds from citrus juice and other plant species (*29*).

Flavanol-3-*O*-rutinoside-7-*O*-glucoside. Previous studies showed that the first fragmentation of the $[M - H]^-$ ion is always due to the breakdown of the *O*-glycosidic bond at position 7, leading to a base peak ion Y_0^{7-} $[M - H - 162]^-$ (*30*). The remaining sugars on the flavonoid molecule should be linked to the hydroxyl group at position 3 of the flavonol nucleus. The interglycosidic linkages were identified as (1→6) by the MS fragmentation in many cases at position 3.

Compound 9. The UV spectroscopic data of this compound provided three maxima at 256, 318sh, and 349 nm, compatible with a flavonol glycosylated at position 3 (*8, 25*). Mass analyses in negative mode provided a deprotonated molecule at *m/z* 755.2. Its MS^n fragmentation revealed a base peak at *m/z* 593.1 $[(M - H) - 162]^-$ and an additional peak at *m/z* 285.2 with a relative abundance of 10%, which corresponded to $[(M - H) - 162 - 146]^-$. This type of fragmentation is characteristic of 3,7-di-*O*-glucosylflavonols. Previous studies that isolated similar flavonoids from cauliflower and lemon juice indicated that the first loss by HPLC-ESI-MS^n was due to a glucosylation at position 7 and then in position 3 (*26, 29*). The ion at *m/z* 593.1 indicated the occurrence of kaempferol rhamnoglucoside. In addition, the absence of an intermediate ion in the MS^3 spectra

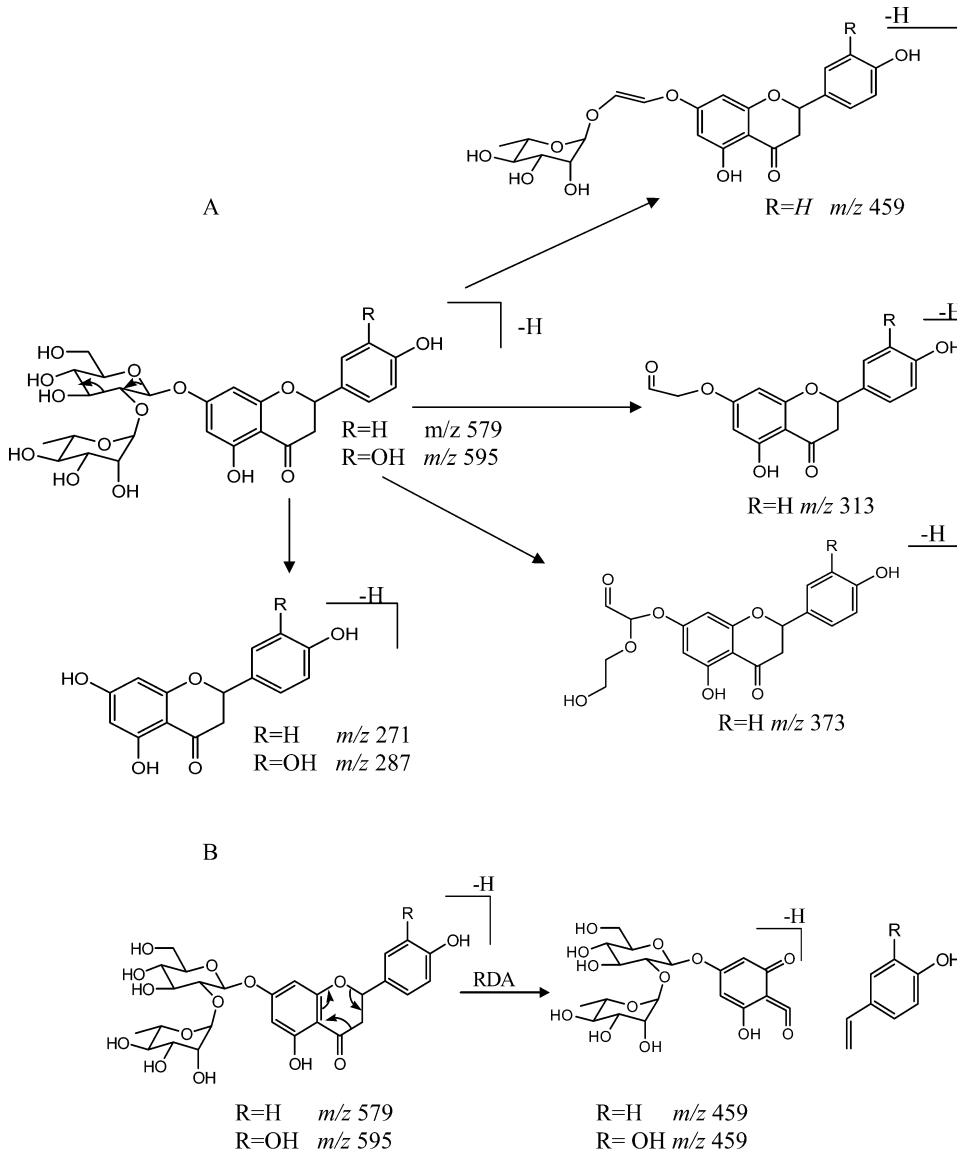

Figure 3. (**A**) Proposed MS^2 pathway for naringin and neoeriocitrin. (**B**) Retrocyclization of naringin and neoeriocitrin.

between the ions at *m/z* 593.1 and 285.2 showed a (1→6) interglycosidic linkage (rutinoside). Therefore, the compound was tentatively identified as kaempferol-3-*O*-rutinoside-7-*O*-glucoside (**Table 2**).

Flavonol 3,7-Di-*O*-glucosides. Fragmentation of the parent ion $[M - H]^-$ of these compounds is characterized by the presence of a base peak ion at $[M - H - 162]^-$, mainly due to the loss of glucose at position 7, which is considered as the easiest breakdown position (*31*). In addition, the low occurrence of the fragment ion $[M - H - 120]^-$ ($^{0,2}X^-$, due to the partial sugar breakdown) and the ion that characterizes the aglycone $[M - H - 324]^-$ together with the absence of the ion $[M - H - 180]^-$ (Z_1^-) complete the differentiation (*31*). In this group, quercetin and kaempferol appeared to be the aglycones **2** and **7** respectively, (**Table 2**).

Flavonoid 7-Di-*O*-glycosides. The DAD spectra of compounds **19**, **23**, **28**, and **32** showed a maximum absorption around 284 nm corresponding to a flavanone structure (**Table 2**).

Compounds 19 and 28. Negative mode MS/MS focused on *m/z* 595 (t_R 19.4 min) and 579 (t_R 22.6 min) $[M - H]^-$ rendered peaks at *m/z* 287 and 271, respectively, corresponding to a base peak at $[M - H - 308]^-$, associated with a loss of a disaccharide. The absence in both cases of a $[M - H - 120]^-$ fragment indicated a (1→6) interglycosidic linkage of a disaccharide that was identified as rutinoside (*32*). Compounds **19** and **28** were tentatively identified as eriodictyol 7-*O*-rutinoside (eriocitrin) and naringenin 7-*O*-rutinoside (narirutin), respectively (*32*).

Compounds 23 and 32. Full mass scan analysis of compound **32** showed a signal at *m/z* 579 $[M - H]^-$. The MS^2 spectrum provided fragments at *m/z* 459 $[M - H - 120]^-$ (base peak), 313 $[M - H - 146 - 120]^-$, and 271 $[M - H - 308]^-$. The difference of 120 amu can be due to a partial loss of a glucose unit (*31*) as a typical transition of neohesperidoside (**Figure 3**). This compound was identified as naringenin 7-*O*-neohesperidoside (naringin) according to spectroscopic data and confirmation with a commercial standard (*33*).

The MS/MS spectrum of compound **23** in negative ion mode focused on *m/z* 595.1 and showed a base peak at *m/z* 459.0 corresponding to an unexpected loss of 136 amu. This would represent an alternative pathway to the loss of the entire disaccharide (*m/z* 287) $[M - H - 308]^-$ that was detected with a very low intensity (<10%). According to Gattuso et al. (*22*), the base peak at *m/z* 459 can be due to a retro-Diels−Alder reaction in the C-ring of the flavanone (**Figure 3**). The presence of fragment at *m/z* 287 indicated eriodictyol as aglycone. It could also be observed that the difference between compounds **23** and

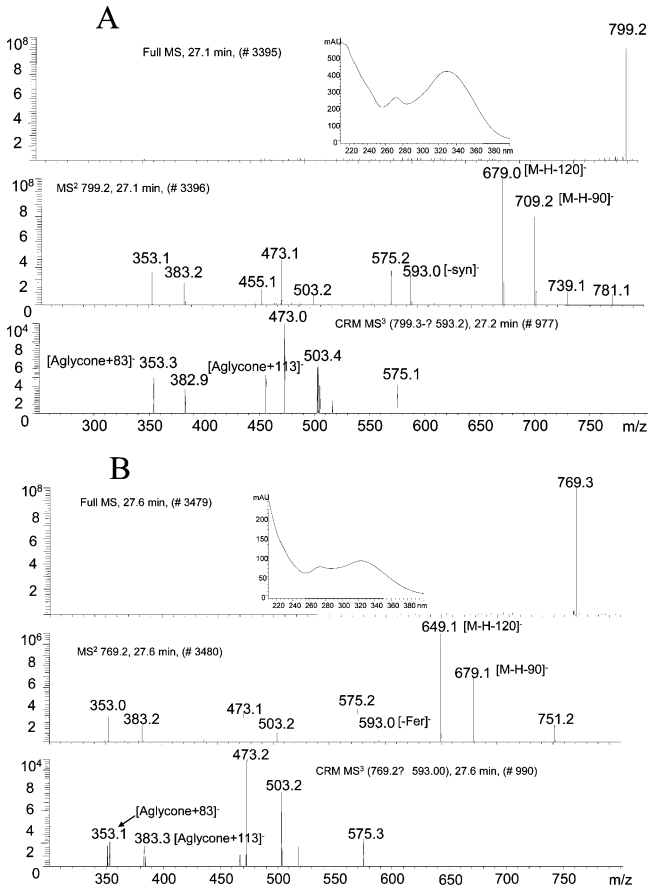

Figure 4. DAD and MS^n spectra of (**A**) 10-sinapoyl and (**B**) 10-feruloyl derivatives of the apigenin 6,8-di-*C*-glucoside.

32 was one oxygen atom, which suggested that two fragmentation pathways can compete in the fragmentation of flavanone 7-*O*-neohesperidosides (**Figure 3**). According to our results, retrocyclization could be the primary pathway. This suggestion is in accordance with previous results (*33, 34*) that indicate that under low-energy CID conditions, a retro-Diels−Alder fragmentation in the C-ring involving a cleavage of aglycone bonds 1 and 3 was likely to occur. Compound **23** was identified as eriodictyol 7-*O*-neohesperidoside (neoeriocitrin).

Compounds 25 and 33. The UV spectra presented maxima at 270 and 345 nm corresponding to bands II and I of a flavone structure. Both of them had the same molecular ion. The MS/MS spectra of the pseudomolecular ion at *m/z* 577 of compound **33** showed a base peak at *m/z* 269 corresponding to the aglycone apigenin. This compound (25 mg) was isolated and the NMR analysis performed. The ^{1}H NMR spectrum presented a characteristic resonance of aromatic protons of ring A [δ 6.76 (1H, d, $J = 1$ Hz, H-6), δ 6.45 (1H, d, $J = 1$ Hz, H-8)], ring B [δ 7.98 (2H, d, $J_{2'6'} = 8.1$ Hz, H-2′ and H-6′), δ 6.95 (2H, d, $J_{3'5'} = 8.1$ Hz, H-3′ and H-5′)], and the H-3 proton appeared as a singlet at δ 6.65. The two anomeric protons of both glucose and rhamnose units appeared at δ 5.18 (1H, d, $J = 7.6$ Hz, H-1″ and δ 5.30 (1H, s, H-1‴). The proton of methyl group appears at δ 1.35 (3H, d, $J = 7$ Hz, H-6‴). The COSY spectrum showed correlations between H-6, H-2′ and H-8, H-3′, respectively. The ^{13}C NMR {184.0 (C-4), 166.7 (C-2), 164.4 (C-7), 162.9 (C-4′), 162.8 (C-10), 158.4 (C-5), 129.6 (C-3′, C-5′), 123.0 (C-1′), 117.0 (C-2′, C-6′), 107.0 (C-9), 104.1 (C-3), 102.5(C-1‴), 101.0 (C-8), 99.8 (C-1″), 95.9 (C-6), 18.2(C-6‴), and [79.1; 79.0; 78.3; 74.0; 72.3; 72.2; 71.4; 70.0; 62.4], corresponding to hexose carbons} confirmed the presence of 27 atoms of carbon. The 2D NMR spectra (HMBC) showed correlation between the proton of rhamnose H-1‴ (δ 5.3) and the carbon of glucose C-2″ (δ 79.1), indicating the (1→2) interglycosidic linkage. It is worth noting that the transition ion was not detected in the MS/MS spectrum. This can be due to the high lability of the glycosidic linkage at position 7 of the flavone (*31*). Compound **33** was tentatively identified as apigenin-7-*O*-neohesperidoside. The MS/MS spectrum of compound **25** was superimposable with that of compound **33**. According to Dugo et al. (*35*), rutinosides always elute before neohesperidosides. Therefore, this compound was tentatively identified as apigenin 7-*O*-rutinoside. Compounds **25** and **33** were previously identified in lemon, grapefruit, bergamot, orange, and mandarin juices (*35*).

Acylated Glycosides. The presence of 11 hydroxycinnamic acid derivatives of flavonoids was also detected in this analysis of citrus leaves (**Tables 1** and **3**). The UV spectra of flavonoid glycosides and acids overlap and, therefore, this kind of compound can be characterized by a high absorption maximum at 333 nm (330−335 nm) and a less intense second peak coincident with flavonoid band II (255−265 nm). Most of the characterized compounds were acylated with sinapic acid. However, the MS fragmentation of compounds **41** and **46** showed losses at $[M - H - 176]^-$ and $[M - H - 192]^-$ that were identified as ferulic and methoxycaffeic acids, respectively (*29*).

***C*-Glycoside Derivatives.** *Compounds 40 and 41.* The MS^3 fragmentation of the ion at *m/z* 593, corresponding to losses of 206 and 176 amu for compounds **40** and **41**, respectively, rendered in both cases fragments generated by losses of 18, 90, and 120 (base peak) amu typical of *C*-glycosides. The presence of ions at *m/z* 383 [aglycone + 113]$^-$ and at *m/z* 353 [aglycone + 83]$^-$ in both compounds indicated the aglycone as trihydroxyflavone (**Figure 4**). Taking into consideration that without acylation, mass spectra of both compounds were coincident with apigenin-6,8-di-*C*-glycoside, compounds **40** and **41** were tentatively identified as apigenin-6,8-di-*C*-(sinapoyl) glycoside and apigenin-6,8-di-*C*-(feruloyl) glycoside, respectively. Compound **40** was isolated (1.9 mg), and the ^{1}H NMR spectrum showed all peaks present in compound **10**. In addition, two signals at δ 7.53 and 6.26 (d, $J_{\alpha,\beta} = 15.5$ Hz) that can be attributed to free trans olefinic protons were found (*26*). Two more signals at δ 6.68 (s, 2H) and 3.35 (s, 6H) were observed. These chemical shifts are characteristic of the sinapic acid (*36*).

***O*-Glycoside Derivatives.** *Compounds 37 and 39.* The UV spectroscopic analysis of these compounds showed that they were flavonoids acylated with hydroxycinnamic acid derivatives. The MS/MS spectrum of compound **37**, focused at *m/z* 945, rendered ions at *m/z* 637 $[M - H - 308]^-$ (low intensity) and at *m/z* 577 $[M - H - 368]^-$ (base peak) corresponding to the losses of diglycoside and sinapoyl-glucose units, respectively. The fragmentation spectrum of the ion at *m/z* 577 was superimposable with compound **33**; therefore, compound **37** was tentatively identified as apigenin 7-*O*-(sinapoyl) triglycoside. The MS/MS analysis of compound **39** with a pseudomolecular ion at *m/z* 947 showed ions at *m/z* 785, 639, and 579 (base peak) corresponding to the losses of glucose, diglycoside, and sinapoyl-glucose units, respectively. MS^3 of pseudomolecular ion (947→579) showed a spectrum identical to that of compound **32**. In the MS/MS analysis of the saponified crude extract, the triglycoside derivatives of compounds **37** and **39** were not detected as they were compounds **32** and **33**. This observation suggests that one hexose was linked to the sinapoyl unit (**Table 3**).

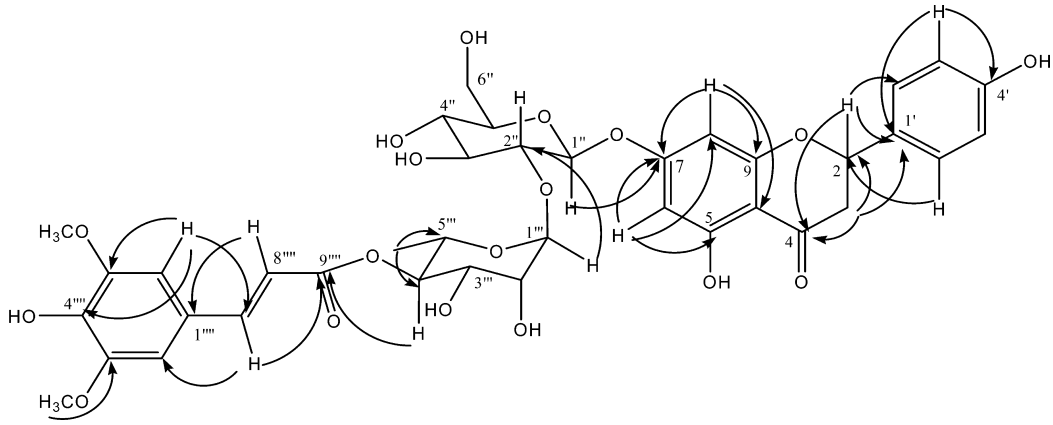

Figure 5. Significant HMBC correlations of naringenin 7-*O*-(sinapoyl)neohesperidoside.

Table 4. ^{1}H NMR (500 Hz) and ^{13}C NMR (125 Hz) Spectral Data of Compound **45** in MeOH d_4

atom	**45** δ_H (J in Hz)	**45** δ_c	atom	**45** δ_H (J in Hz)	**45** δ_c
2	5.23 (dd, 1H, J = 12.8, 2.8 Hz, H-2)	80.6	rhamnose		
3	3.15 (dd, 1H, J = 17.2, 12.8 Hz, H-3) 272 (dd, 1H, J = 17.2, 2.8 Hz, H-3)	43.9	1‴	5.4 (s, 1H)	101.6
4		198.7	2‴	3.80 (m, 1H)	70.3
5		164.8	3‴	3.70 (m, 1H)	71.3
6	6.19 (d, 1H, J = 2.5 Hz, H-6)	98.0	4‴	5.08 (m, 1H)	75.7
7		164.8	5‴	4.2 (m, 1H)	67.7
8	6.21 (d, 1H, J = 2.5 Hz, H-8)	96.7	6‴	1.21 (d, 3H, J = 6.2 Hz)	18.1
9		164.9	sinapoyl		
10		105.5	1⁗		126.5
1′		130.8	2⁗, 6⁗	6.86 (s, 2H)	107.0
2′, 6′	7.21 (d, 2H, J = 8.0 Hz, H-2′, H-6′)	129.1	3⁗, 5⁗		149.5
3′, 5′	6.76 (d, 2H, J = 8.0 Hz, H-3′, H-5′)	116.4	6⁗		140.1
4′		159.1	CH_3O−C-3⁗	3.86 (s, 6H)	56.9
glucose			CH_3O−C-5⁗		
1″	5.11 (d, 1H, J = 8 Hz)	100.0	7⁗	7.56 (d, 1H, J = 15.5 Hz)	147.3
2″	3.62 (m, 1H)	79.1	8⁗	6.76 (d, 1H, J = 15.5 Hz)	116.5
3″	3.97 (br, 1H)	72.2	9⁗		168.8
4″	3.65 (m, 1H)	77.5			
5″	3.54 (m, 1H)	78.3			
6″	3.72 (m, 1H) 3.88 (m, 1H)	62.3			

Compound 45 was isolated as a yellow powder. The UV spectra showed two maxima at 282 and 332 nm. The MS/MS spectrum of the pseudomolecular ion at m/z 785 showed a base peak at m/z 579 $[M - H - 206]^-$ corresponding to the loss of a sinapoyl unit. The MS^3 spectrum of $[785\rightarrow579]^-$ presented the same pattern of fragmentation as compound **32** (**Table 3**). Analyses of the ^{13}C NMR (**Table 4**) and DEPT spectra indicated the presence of naringenin as aglycone together with two sugar residues and a sinapoyl unit. The ^{1}H NMR spectra showed signals of 1,4-disubstituted aromatic ring system at δ_H 7.21 (2H, d, J = 8 Hz, H-2′, H-6′) and 6.76 (2H, d, J = 8 Hz, H-3′, H-5′), revealing the A_2B_2 system of the B-ring of a flavanone. The aromatic signal at δ_H 6.21 (d, 1H, J = 2.5 Hz, H-6) and 6.19 (d, 1H, J = 2.5 Hz, H-8) showed the presence of the A-ring of a flavanone system. Signals at 5.23 (dd, 1H, J = 12.8, 2.8 Hz, H-2), 3.15 (dd, 1H, J = 17.2, 12.8 Hz, H-3 and; dd, 1H, J = 17.2, 2.8 Hz, H-3) were characteristic of a flavanone skeleton; moreover, signals of the two anomeric protons appeared at δ_H 5.11 (d, 1H, J = 8 Hz, H-1″) and 5.40 (s, 1H, H-1‴) (*37*). In addition, two olefinic protons with a trans configuration were observed at δ_H 6.76 (d, 1H, J = 15.5 Hz, H-8″) and 7.56 (d, 1H, J = 15.5 Hz, H-7‴) together with a proton at 6.86 (2H, s, H-2⁗, H-6⁗).

The rhamnose unit of the glycoside part was determined through analysis of the chemical shifts of its proton signals, which were connected in the ^{1}H−^{1}H COSY spectra.

The sinapoyl position was established through pulse field gradient HMBC spectra data (**Figure 5**). ^{1}H−^{13}C long-range correlations were observed between the methoxyl protons (δ_H 3.86) and C-3⁗ (δ_c 149.5),; the first olefinic H-7⁗ (δ_H 7.56) and the ester carbonyl atom C-9⁗ (δ_c 168.8) and C-2⁗ (δ_c 107.0); the second olefinic proton H-8⁗ (δ_H 6.76) correlated with the carbon C-1⁗ (δ_c 126.5) and C-9⁗; the aromatic proton H-2⁗ (δ_H 6.86) correlated with the olefenic carbon C-7⁗ (δ_c 147.3); and the two quaternary carbon atoms C-3⁗ (δ_c 149.5) and C-4⁗ (140.1). In the sugar moiety, the ^{1}H−^{13}C long-range correlation of the two anomeric protons H-1″ (δ_H 5.11) and H-1‴ (δ_H 5.40) with C-7 (δ_c 164.8) and C-2″ (δ_c 77.5), respectively, indicated that a glucose (H-1″) was attached to the aglycone at position 7 and a rhamnose was linked to the glucose at the position 2″. The correlation of proton H-4‴ (δ_H 5.08) of the rhamnose with the ester carbonyl C-9⁗ (δ_c 168.8) showed that the sinapoyl residue was attached to the rhamnose unit at position 4‴. Therefore, compound **45** was tentatively identified as 5,4′-dihydroxyflavanone-7-*O*-[β-glycopyranosyl-(1→2)-α-rhamnopyranosyl-4‴-sinapoyl].

As a general observation, flavanone 7-*O*-rutinosides were less retained than flavanone 7-*O*-neohesperidosides with reversed phase HPLC under the same chromatographic conditions. Therefore, eriocitrin (t_R 19.4 min) and narirutin (t_R 22.6 min) eluted slightly more rapidly than neoeriocitrin (t_R 20.5 min) and naringin (t_R 23.5 min), respectively. In addition, we observed that acylated flavonoids eluted later than deacylated derivatives

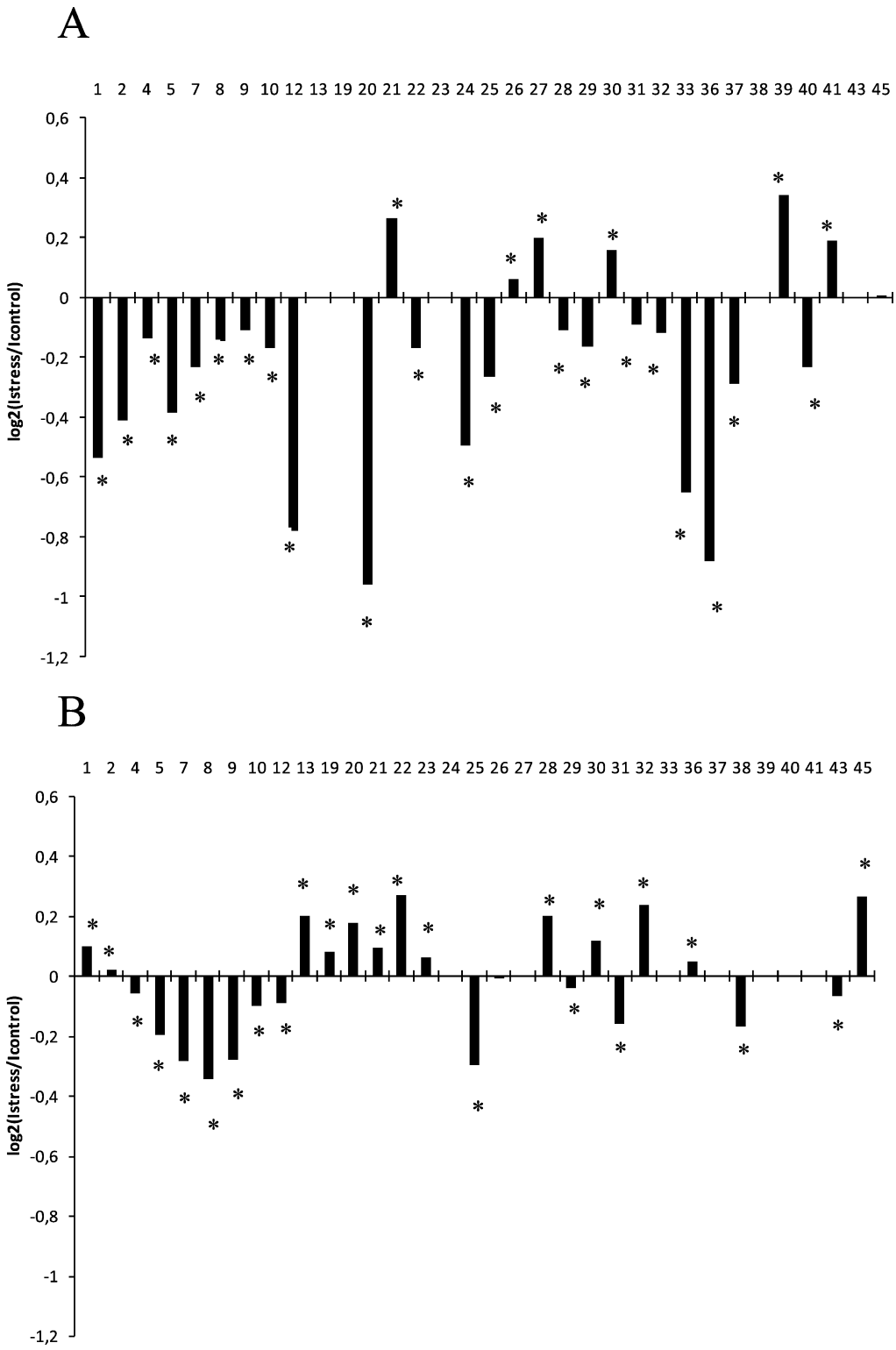

Figure 6. Flavonoid profiling in leaves of (**A**) citrumelo CPB4475 and (**B**) Carrizo citrange under flooding conditions. Data were normalized to $\log_2$ (stress/control) to better depict up- or down-regulation. Numbers in the upper part correspond to the compounds listed in **Tables 1**−**3**. Asterisks denote significant differences in area values between control and stressed plants at $p < 0.05$.

even after saponification. Within acylated flavonoids, compound **41** linked to sinapic acid was less retained than compound **40**, linked to ferulic acid. These data are in accordance with the literature (*29*). However, an unexpected behavior in the chromatographic mobility was observed when the elution orders of flavonoid glucosides and their corresponding acylated derivatives were compared (considering that acylation was always on the same position). Under the chromatographic gradient tested, naringin eluted at 23.5 min, whereas apigenin-7-*O*-neohesperidoside eluted slightly later (at 23.9 min). In contrast, the elution order of the corresponding acylated compounds was inverted, apigenin 7-*O*-(sinapoyl)triglycoside eluted at 25.0 min and naringenin 7-*O*-(sinapoyl)triglycoside at 26.6 min.

Flavonoid Profiling in Leaves of Citrumelo and Carrizo under Abiotic Stress. Flavonoid content in leaves of plants cultivated under soil flooding conditions was determined and expressed relative to the control levels. Data normalization was accomplished by using the $\log_2$ of stress/control area values

(corrected by using biochanin A as an internal standard). This normalization allowed the comparison among flooded plants from the two different genotypes. Therefore, **Figure 6** shows the flavonoid profiling in leaves of citrumelo and Carrizo. In citrumelo, the content of 21 flavonoids was relatively lower in leaves of stressed plants when compared with controls, whereas in Carrizo, only the content of 12 flavonoids relatively decreased. Not only the number of compounds affected by flooding differed between genotypes but also the absolute level of decrease. In citrumelo, $\log_2$ (stress/control) for most of the compounds was lower than in Carrizo. These results could sum to the previously described physiological mechanisms that explain the higher tolerance of Carrizo to flooding stress (*3, 4*). In particular, the higher stability of flavonoids could help enzymatic mechanisms (*3*) to keep a higher antioxidant activity under stress. In addition, it should be noted that most of the compounds with relatively increased leaf concentrations under soil flooding had quercetin, kaempferol, and eriodictyol as aglycones, whereas endogenous levels of apigenin-based flavonoids were reduced in citrus leaves under stress. The biological relevance of these findings and the involvement of flavonoid content in the responses of different citrus genotypes to biotic and abiotic stress will be the subject of future analyses. The presented results are an example of the kind of work that can be accomplished with the method described. The results of this work could also be implemented in metabolomics platforms to detect, identify, and quantify flavonoids and other polyphenolic compounds in different plant extracts.

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Supporting Information Available: DAD and MS^n spectra of kaempferol-3-*O*-rutinose-7-*O*-glucoside, DAD and MS^2 spectra of naringin and neoeriocitrin, and DAD and MS^n spectra of apigenin 7-*O*-(sinapoyl) triglycoside and naringenin 7-*O*-(sinapoyl)triglycoside. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Moreno, P.; Ambrós, S.; Albiach-Martí, M. R.; Guerri, J.; Peña, L. *Citrus tristeza* virus: a pathogen that changed the course of the citrus industry. *Mol. Plant Pathol.* **2008**, *9*, 251–268.

(2) Lopez-Climent, M. F.; Arbona, V.; Pérez-Clemente, R. M.; Gómez-Cadenas, A. Relationship between salt tolerance and photosynthetic machinery performance in citrus. *Environ. Exp. Bot.* **2008**, *62*, 176–184.

(3) Arbona, V.; Hossain, Z.; Lopez-Climent, M. F.; Perez-Clemente, R. M.; Gomez-Cadenas, A. Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. *Physiol. Planta.* **2008**, *132*, 452–466.

(4) Arbona, V.; Gómez-Cadenas, A. Hormonal modulation of citrus responses to flooding. *J. Plant Growth Regul.* **2008**, *27*, 241–250.

(5) Tripoli, E.; La Guardia, M.; Giammanco, S.; Di Majo, D.; Giammanco, M. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: a review. *Food Chem.* **2007**, *104*, 466–479.

(6) Tusa, N.; Abbate, L.; Renda, A.; Ruberto, G. Polyphenols distribution in juices from *Citrus* allotetraploid somatic hybrids and their sexual hybrids. *J. Agric. Food Chem.* **2007**, *55*, 9089–9094.

(7) Robards, K.; Antolovich, M. Analytical chemistry of fruit bioflavonoids —a review. *Analyst* **1997**, *122*, R11−R34.

(8) Caristi, C.; Bellocco, E.; Gargiulli, C.; Toscano, G.; Leuzi, U. Flavone-di-C-glucosides in citrus juices from southern Italy. *Food Chem.* **2006**, *95*, 431–437.

(9) Ooghe, W. C.; Detavernier, C. M. Detection of the addition of *Citrus reticulata* and hybrids to *Citrus sinensis* by flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 1633–1637.

(10) Mouly, P. P.; Arzouyan, C. R.; Gaydou, E. M.; Estienne, J. M. Differentiation of citrus juices by factorial discriminant analysis using liquid chromatography of flavanone glycosides. *J. Agric. Food Chem.* **1994**, *42*, 70–79.

(11) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S.

(12) Kim, J.-D.; Liu, L.; Guo, W.; Meydani, M. Chemical structure of flavonols in relation to modulation of angiogenesis and immune-endothelial cell adhesion. *J. Nutr. Biochem.* **2006**, *17*, 165–176.

(13) Gao, K.; Henning, S. M.; Niu, Y.; Youssefian, A. A.; Seeram, N. P.; Xu, A.; Heber, D. The citrus flavonoid naringenin stimulates DNA repair in prostate cancer cells. *J. Nutr. Biochem.* **2006**, *17*, 89–95.

(14) Kanno, S.; Tomizawa, A.; Hiura, T.; Osanai, Y.; Shouji, A.; Ujibe, M.; Ohtake, T.; Kimura, K.; Ishikawa, M. Inhibitors effects of naringenin on tumor growth in human cancer cell lines and sarcoma s-180-implanted mice. *Biol. Pharm. Bull.* **2005**, *28*, 527–530.

(15) Manthey, J. A.; Guthrie, N. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. *J. Agric. Food Chem.* **2002**, *50*, 5837–5843.

(16) Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M.; Koizumi, M.; Ito, C.; Furukawa, H. Quantitative study of flavonoids in leaves of citrus plants. *J. Agric. Food Chem.* **2000**, *48*, 3865–3871.

(17) Manthey, J. A.; Grohmann, K.; Berhow, M. A.; Tisserat, B. Changes in citrus leaf flavonoid concentrations resulting from blight-induced zinc-deficiency. *Plant Physiol. Biochem.* **2000**, *38*, 333–343.

(18) Castellarin, S. D.; Pfeiffer, A.; Sivilotti, P.; Degan, M.; Peterlunger, E.; Di Gaspero, G. Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under seasonal water deficit. *Plant Cell Environ.* **2007**, *30*, 1381–1399.

(19) Gould, K. S. Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *J. Biomed. Biotechnol.* **2004**, *5*, 314–320.

(20) Barceló, J.; Poschenrieder, C. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. *Environ. Exp. Bot.* **2002**, *48*, 75–92.

(21) Tattini, M.; Guidi, L.; Morassi-Bonzi, L.; Pinelli, P.; Remorini, D.; Degl'Innocenti, E.; Giordano, C.; Massai, R.; Agati, G. On the role of flavonoids in the integrated mechanisms of response of *Ligustrum vulgare* and *Phillyrea latifolia* to high solar radiation. *New Phytol.* **2005**, *167*, 457–470.

(22) Gattuso, G.; Caristi, C.; Gargiulli, C.; Bellocco, E.; Toscano, G.; Leuzzi, U. Flavonoid glycosides in bergamot juice (*Citrus bergamia* Risso). *J. Agric. Food. Chem.* **2006**, *54*, 3929–3935.

(23) 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.

(24) Hertog, M. G. L.; Hollman, P. C. H.; Venema, D. P. Optimization of a quantitative HPLC determination of potentially anticarcenogenic flavonoids in vegetables and fruits. *J. Agric. Food Chem.* **1992**, *40*, 1591–1598.

(25) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, 1970.

(26) Gil-Izquierdo, A.; Riquelme, T. M.; Porras, I.; Ferreres, F. Effect of the rootstock and interstock grafted in lemon tree (*Citrus limon* (L.) Burm) on the flavonoid content of lemon juice. *J. Agric. Food Chem.* **2004**, *52*, 324–331.

(27) Caristi, C.; Bellocco, E.; Panzera, V.; Toscazo, G.; Vadalá, R.; Leuzzi, U. Flavonoids detection by HPLC-DAD-MS-MS in lemon juices from Sicilian cultivars. *J. Agric. Food Chem.* **2003**, *51*, 3528–3534.

(28) Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F.; Tomás-Barberan, F. A. In vitro availability of flavonoids and other phenolics in orange juice. *J. Agric. Food Chem.* **2001**, *49*, 1035–1041.

(29) Vallejo, F.; Tomás-Barberán, F. A.; Ferreres, F. Characterisation of flavonols in broccoli (*Brassica oleracea* L. var. *italica*) by liquid chromatography-UV diode-array detection-electrospray ionization mass spectrometry. *J. Chromatogr. A* **2004**, *1054*, 181–193.

(30) Llorach, R.; Gil-Izquierdo, A.; Ferreres, F.; Thomás-Barberan, F. A. HPLC-DAD-ESI-MS/MS ESI charaterization of unusual highly glycosilated acylated flavonoids from cauliflower (*Brassica oleracea* L. var. *botrytis*) agroindustrial bydproducts. *J. Agric. Food Chem.* **2003**, *51*, 3895–3899.

(31) Ferreres, F.; Llorach, R.; Gil-Izquierdo, A. Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2004**, *39*, 312–321.

(32) Fabre, N.; Rustan, I.; De Hoffmann, E.; Quetin-Leclerq, J. Determination of flavone, flavonol and flavanones aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 707–715.

(33) Shi, P.; He, Q.; Song, Y.; Qu, H.; Cheng, Y. Characterization and identification of isomeric flavonoid *O*-diglycosides from *Citrus* in negative electrospray ionisation by ion trap mass spectrometry and time-of-flight mass spectrometry. *Anal. Chim. Acta* **2007**, *598*, 110–118.

(34) Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. HL-60 differentiating activity and flavonoid content of the readily extractable fraction prepared from *Citrus* juices. *J. Agric. Food Chem.* **1999**, *47*, 128–135.

(35) Dugo, P.; Presti, L. M.; Öhman, M.; Fazio, A.; Dugo, G.; Mondillo, L. Determination of flavonoids in citrus juice by micro-HPLC-ESI/MS. *J. Sep. Sci* **2005**, *28*, 1149–1156.

(36) Bratt, K.; Sunnerheim, K.; Bryngelsson, S.; Fagerlung, A.; Engman, L.; Andersson, R. E.; Dimberg, L. H. Avenanthramides in oats (*Avena sativa* L.) and structure-antioxidant activity relationships. *J. Agric. Food Chem.* **2003**, *51*, 594–600.

(37) Eun, J. C.; Won, J. L; Sung, H. C.; Sang, W. C. Proliferative effects of flavan-3-ols and propelargonidins from rhizomes of *Drynaria fortunei* on MCF-7 and osteoblastic cells. *Arch. Pharm. Res.* **2003**, *26*, 620–630.

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