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Flavonoid Composition and Antioxidant Activity of Juices from Chinotto (*Citrus* × *myrtifolia* Raf.) Fruits at Different Ripening Stages

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The qualitative and quantitative compositions of chinotto juice in two different maturation periods were determined via chromatographic separation of extracted aliquots of juice of *Citrus* × *myrtifolia* Raf. by using reverse-phase LC-DAD-ESI-MS-MS. This provides a comprehensive chromatographic evaluation of 11 compounds (furanocoumarins and flavonoids *C*- and *O*-glycosides). Five flavonoids and two furanocoumarins were identified for the first time in chinotto juice: two *C*-glucosides (vicenin-2 and lucenin-2 4′-methyl ether), two *O*-glycosides (narirutin and rhoifolin), and a 3-hydroxy-3-methylglutaryl flavanone (brutieridin). Bergapten and epoxybergamottin were the primary furanocoumarins found. Overall, the juice from immature chinotto fruits is richer in bioactive compounds than that obtained from ripe fruits. The free radical and superoxide anion scavenging activities of juice from both green and ripe fruits were assessed, and results showed that the former is much more efficient in scavenging radical and superoxide species than the latter.

KEYWORDS: ***Citrus* × *myrtifolia* Raf.; HPLC-DAD-ESI-MS-MS; flavonoids; furocoumarins; antioxidant activity**

INTRODUCTION

Citrus × *myrtifolia* Raf. (sometimes referred to as *Citrus aurantium* L. var. *myrtifolia* Ker-Gawl) is a *Citrus* plant commonly known as myrtle-leaved orange or chinotto. Classified in 1961 by Tanaka (*1*), it is a small flowering tree growing to about 3 m tall, with small leaves closely reminiscent of myrtle and spherical fruits weighing up to 40−50 g, which is cultivated mostly in Italy (Liguria, Tuscany, Sicily, and Calabria regions), in southern France, and, to a lesser extent, in the citrus zones of the United States. Although in many countries it is grown only for ornamental uses, its sour-tasting fruits have a significant impact on the food industry. The smaller green ones (2−3 cm diameter) are used in the candy industry or in the preparation of jam, whereas the juice of ripe fruits is an essential flavor component of many bitters and aperitifs and, above all else, the primary ingredient of the 'Chinotto' Italian soft drink. In light of the growing distribution of this popular soft drink, which is also beginning to appear in several other countries (Americas, Australia), it is surprising that chinotto juice is among the least studied citrus juices, with only a few reports present in the literature (*2*, *3*).

Flavonoids are phenolic derivatives possessing remarkable health-promoting effects (*4*−*7*), a consequence of their marked antioxidant ability, and are generally found in vegetables, fruits, herbs, tea, and wine as secondary metabolites: *Citrus* juices are among the richest dietary sources of flavonoids (*8*). They are often found in *Citrus* juices as their glycoside derivatives and more specifically as their *O*-glycosides and *C*-glycosides, whereas aglycones occur less frequently in juices (*9*, *10*). Furthermore, flavonoid glycosides are present in distribution patterns that are unique to each species and can be regarded as chromatographic fingerprints for each species. Lemons, for instance, are characterized by an abundance of eriocitrin, hesperidin, and diosmin (*11*, *12*), whereas naringin, neohesperidin, and small amounts of neoeriocitrin are found in sour oranges (*13*). Hesperidin, narirutin, and didymin are typical of sweet oranges (*14*, *15*); naringin, narirutin, and, to a lesser extent, hesperidin and neohesperidin, are found in grapefruit species (*16*), and neoeriocitrin, naringin, and neohesperidin are the most abundant components of bergamot juice (*17*, *18*).

It is not entirely surprising that, similarly to what is reported for bergamot, chinotto juice is characterized by the predominance of the same flavanone 7-*O*-neohesperidoside components, that is, neoeriocitrin, naringin, and neohesperidin (even though reports differ on the presence in chinotto juice of eriocitrin (*2*) and didymin (*3*)). In fact, both *C. myrtifolia* and *C. bergamia* Risso are taxons closely related to the same parent species, *C. aurantium* L. (sour orange): chinotto is regarded as a mutation of sour orange, whereas bergamot is a cross between sour orange and citron (*C. medica* L.).

Taking into account the lack of information present in the literature and the relevance of chinotto juice in the soft drink industry, we hereby present the identification and quantification of nine flavonoids and two furocoumarins in crude juices from

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immature and mature fruits by HPLC-DAD separation coupled with MS-MS detection. Furthermore, we report the antioxidant activity of the juices toward DPPH$^{\bullet}$ radicals and superoxide anions.

EXPERIMENTAL PROCEDURES

Material and Reagents. *Citrus* × *myrtifolia* Raf. (chinotto) fruits were supplied by Azienda Agricola Vivaistica Formica (Milazzo, ME, Italy). They were collected in two different stages of ripening, immature (harvested in June about 3 months after the flowering period, which takes place in March) and in November, at the beginning of the harvesting period, which goes from October to late January. Immature fruits were green oblate spheroids with a diameter of about 2−3 cm, whereas ripe fruits were orange oblate spheroids with a diameter of about 5−6 cm. Juice samples from immature and mature chinotto fruits were prepared by hand squeezing the fresh fruit, from which the peel had previously been removed. The juices were stored at −20 °C until needed for the study. The investigation was carried out on 10 samples for each cultivar from the 2007−2008 fruit season.

Reagents and Standard Solutions. HPLC-grade acetonitrile and methanol and bergapten were supplied by Sigma-Aldrich (St. Louis, MO), and dimethylformamide (DMF) was supplied by Carlo Erba (Milano, Italy). Eriocitrin, neoeriocitrin, naringin, and neohesperidin were obtained from Extrasynthèse (Genay, France). Vicenin-2 and lucenin-2 4′-methyl ether were separated from orange and citron juices, respectively (*19*). All of these compounds were used as standards. The calibration lines were obtained using DMF solutions of known concentration (1−100 mg/L). Quantitative analysis was carried out by integration of the areas of the peaks from the chromatogram at 280, 325, and 310 nm for flavonoids and furocoumarins, respectively, by using the Genesis peak detection algorithm integrated in the ThermoQuest software. Vicenin-2 and lucenin-2 4′-methyl ether were expressed as equivalent of the latter.

Preparative HPLC. Preparative HPLC was carried out using a Shimadzu model LC 8 A HPLC instrument (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-10 A vp UV−vis detector. The column used was a 250 mm × 21.2 mm i.d., 5 μm, Discovery C18 (Supelco, Bellefonte, PA). The injection loop was 2.0 mL, and the flow rate was 10.0 mL/min at room temperature (20 °C). Analysis was carried out at 310 nm. The mobile phase consisted of an isocratic mobile phase of acetonitrile and H_2O (40:60 v/v) for 50 min. The first component was eluted at t_R of 36.0 min and the second at t_R of 41.24 min.

Sample Preparation. DMF (10.0 mL) was added to the juice (10.0 mL), and the mixture was centrifuged for 5 min at 3200 rpm. The supernatant liquid was filtered through an Iso-Disc P-34, 3 mm diameter, PTFE membrane, 0.45 μm pore size (Supelco).

LC-MS-MS Analyses of Flavonoids and Furocoumarins. LC-MS-MS analyses of samples were carried out with a ThermoQuest Model LCQ-Duo (Thermo Fisher Scientific Inc., Waltham, MA) equipped with a diode array spectrophotometer and an ion trap mass spectrometer with an electrospray ionization source (ESI). The column used for the separation of each compound was a 250 mm × 4.6 mm i.d., 5 μm, Discovery C18, with a 20 mm × 4 mm guard column of the same material (Supelco). The Discovery C18 column was placed in a column oven set at 30 °C. The injection loop was 20 μL, and the flow rate was 1.0 mL/min. The mobile phase consisted of a linear gradient of acetonitrile in H_2O as follows: 5−20% (0−15 min), 20−30% (15−20 min), 30−100% (20−35 min), 100% (35−40 min), 100−5% (40−45 min), and 5% (45−55 min). UV spectra were recorded between 200 and 450 nm, and simultaneous detection by diode array was performed at 278, 310, and 325 nm. Operating parameters of the mass spectrometer were capillary temperature, 250 °C; spray needle voltage, 4.50 kV; ES capillary voltage, +3 and −47 V for positive and negative polarity, respectively; and tube lens offset, 0 and −25 V for positive and negative polarity, respectively. Nitrogen was used as a sheath gas with a flow of 50 arbitrary units. Mass analysis was carried out in full-scan mode from 80 to 900 amu, in both positive and negative mode. The MS-MS spectra were obtained using an applied collision energy of 20−30% of instrument maximum. A source fragmentation of 20 V as a collision energy was used in MS and MS-MS analysis when required. Each sample was tested three times and gave superimposable chromatograms.

Identification of Compounds. Compounds **1**−**11** were identified by their retention time, UV spectra, and MS and MS-MS data and by comparison with a standard sample, when available.

*Compound **1**:* t_R, 15.05 min; UV, 271, 335 nm; MS, 595 $[(M + H)]^+$, 593 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 595 $[M + H]^+$ (1), 577 $[M + H - 18]^+$ (100), 559 $[M + H - 36]^+$ (14), 529 $[M + H - 66]^+$ (9), 523 $[M + H - 72]^+$ (2), 511 $[M + H - 84]^+$ (4), 475 $[M + H - 120]^+$ (7), 457 $[M + H - 138]^+$ (19); MS-MS focused on $[M - H]^-$, 593 $[M - H]^-$ (6), 575 $[M - H - 18]^-$ (11), 503 $[M - H - 90]^-$ (30), 473 $[M - H - 120]^-$ (100), 383 $[M - H - 210]^-$ (A + 113) (5), 353 $[M - H - 240]^-$ (A + 83) (11).

*Compound **2**:* t_R, 16.29 min; UV, 257 (sh), 271, 348 nm; MS, 625 $[M + H]^+$, 623 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 625 $[M + H]^+$ (2), 607 $[M + H - 18]^+$ (100), 589 $[M + H - 36]^+$ (11), 559 $[M + H - 66]^+$ (6), 541 $[M + H - 84]^+$ (1), 505 $[M + H - 120]^+$ (6), 487 $[M + H - 138]^+$ (11); MS-MS focused on $[M - H]^-$, 623 $[M - H]^-$ (44), 605 $[M - H - 18]^-$ (4), 533 $[M - H - 90]^-$ (12), 503 $[M - H - 120]^-$ (100), 413 $[M - H - 210]^-$ (A + 113) (3), 383 $[M - H - 240]^-$ (A + 83) (10).

*Compound **3**:* t_R, 19.5 min; UV, 284, 319 nm; MS, 597 $[M + H]^+$, 595 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 597 $[M + H]^+$ (1), 579 $[M + H - 18]^+$ (18), 561 $[M + H - 36]^+$ (21), 543 $[M + H - 54]^+$ (6), 477 $[M + H - 120]^+$ (7), 451 $[M + H - 146]^+$ (50), 435 $[M + H - 162]^+$ (100), 331 $[M + H - 266]^+$ (14), 289 $[M + H - 308]^+$ (A) (13); MS-MS focused on $[M - H]^-$, 595 $[M - H]^-$ (8), 287 $[M - H - 308]^-$ (A) (100).

*Compound **4**:* t_R, 20.29 min; UV, 283, 329 nm; MS, 597 $[M + H]^+$, 595 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 597 $[M + H]^+$ (3), 579 $[M + H - 18]^+$ (22), 561 $[M + H - 36]^+$ (11), 543 $[M + H - 54]^+$ (5), 451 $[M + H - 146]^+$ (72), 435 $[M + H - 162]^+$ (100), 331 $[M + H - 266]^+$ (13), 289 $[M + H - 308]^+$ (A) (15); MS-MS focused on $[M - H]^-$, 595 $[M - H]^-$ (25), 475 $[M - H - 120]^-$ (2), 459 $[M - H - 136]^-$ (100), 287 $[M - H - 308]^-$ (A) (8).

*Compound **5**:* t_R, 21.6 min; UV, 283, 323 nm; MS, 581 $[M + H]^+$, 579 $[M - H]^-$.

*Compound **6**:* t_R, 22.05 min; UV, 268, 333 nm; MS, 579 $[M + H]^+$, 577 $[M - H]^-$; MS-MS focused on $[M - H]^-$, 577 $[M - H]^-$ (79), 457 $[M - H - 120]^-$ (6), 431 $[M - H - 146]^-$ (5), 311 $[M - H - 266]^-$ (6), 269 $[M - H - 308]^-$ (A) (100).

*Compound **7**:* t_R, 22.4 min; UV, 282, 326 nm; MS, 581 $[M + H]^+$, 579 $[M - H]^-$; MS-MS focused on $[M - H]^-$, 579 $[M - H]^-$ (34), 459 $[M - H - 120]^-$ (100), 417 $[M - H - 162]^-$ (2), 313 $[M - H - 66]^-$ (16), 271 $[M - H - 308]^-$ (A) (25).

*Compound **8**:* t_R, 23.22 min; UV, 282, 324 nm; MS, 611 $[M + H]^+$, 609 $[M - H]^-$; MS-MS focused on $[M - H]^-$, 609 $[M - H]^-$ (82), 489 $[M - H - 120]^-$ (15), 447 $[M - H - 162]^-$ (4), 343 $[M - H - 266]^-$ (25), 301 $[M - H - 308]^-$ (A) (100).

*Compound **9**:* t_R, 24.66 min; UV, 284, 325 nm; MS, 755 $[M + H]^+$, 753 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 755 $[M + H]^+$ (15), 753 $[M + H - 102]^+$ (62), 611 $[M + H - 42]^+$ (32), 303 $[M + H - 308]^+$ (A) (7); MS-MS focused on $[M - H]^-$, 753 $[M - H]^-$ (28), 651 $[M - H - 102]^-$ (65), 609 $[M - H - 144]^-$ (4), 301 $[M - H - 308]^-$ (A) (15).

*Compound **10**:* t_R, 29.24 min; UV, 221, 249, 260, 268, 311 nm; MS, 217 $[M + H]^+$; MS-MS focused on $[M + H]^+$, 202 $[M + H - 15]^+$ (100).

*Compound **11**:* t_R, 29.61 min; UV, 221, 250, 258 (sh), 311 nm; MS, 355 $[M + H]^+$; MS-MS focused on $[M + H]^+$, 355 $[M + H]^+$ (25), 337 $[M + H - 18]^+$ (70), 203 $[M + H - 134]^+$ (8).

Hydrolysis. Acid hydrolysis was carried out on juices following the procedure reported by Hertog et al. (*20*). HCl (10 mL, 6 M) in a methanol (25 mL)/water (10 mL) solution was added to 5 mL of chinotto juice to give a solution of 1.2 M HCl in 50% aqueous methanol. Ascorbic acid (50 mg) was added as antioxidant. After refluxing at 90 °C for 20 h under stirring, the solution was allowed to cool at room temperature, vacuum-dried, and brought to 10 mL with water/DMF (1:1). The mixture was filtered through an Iso-Disc P-34 membrane and analyzed by HPLC.

DPPH$^{\bullet}$ Assay. The antioxidant ability of crude juice obtained by immature or mature fruits of *Citrus myrtifolia* was assessed using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$). The assay was carried out according to the literature procedure (*21*) with minor modifications. The chinotto juice was centrifuged at 4000 rpm for 10 min, and the supernatant was collected for analysis. Fifty microliters of supernatant was mixed with 63 μM DPPH$^{\bullet}$ in methanol, to a final volume of 4.0 mL. Control contains all of the components except juice supernatant. The change in absorbance at 517 nm was monitored over 50 min. The inhibition percentage of radical

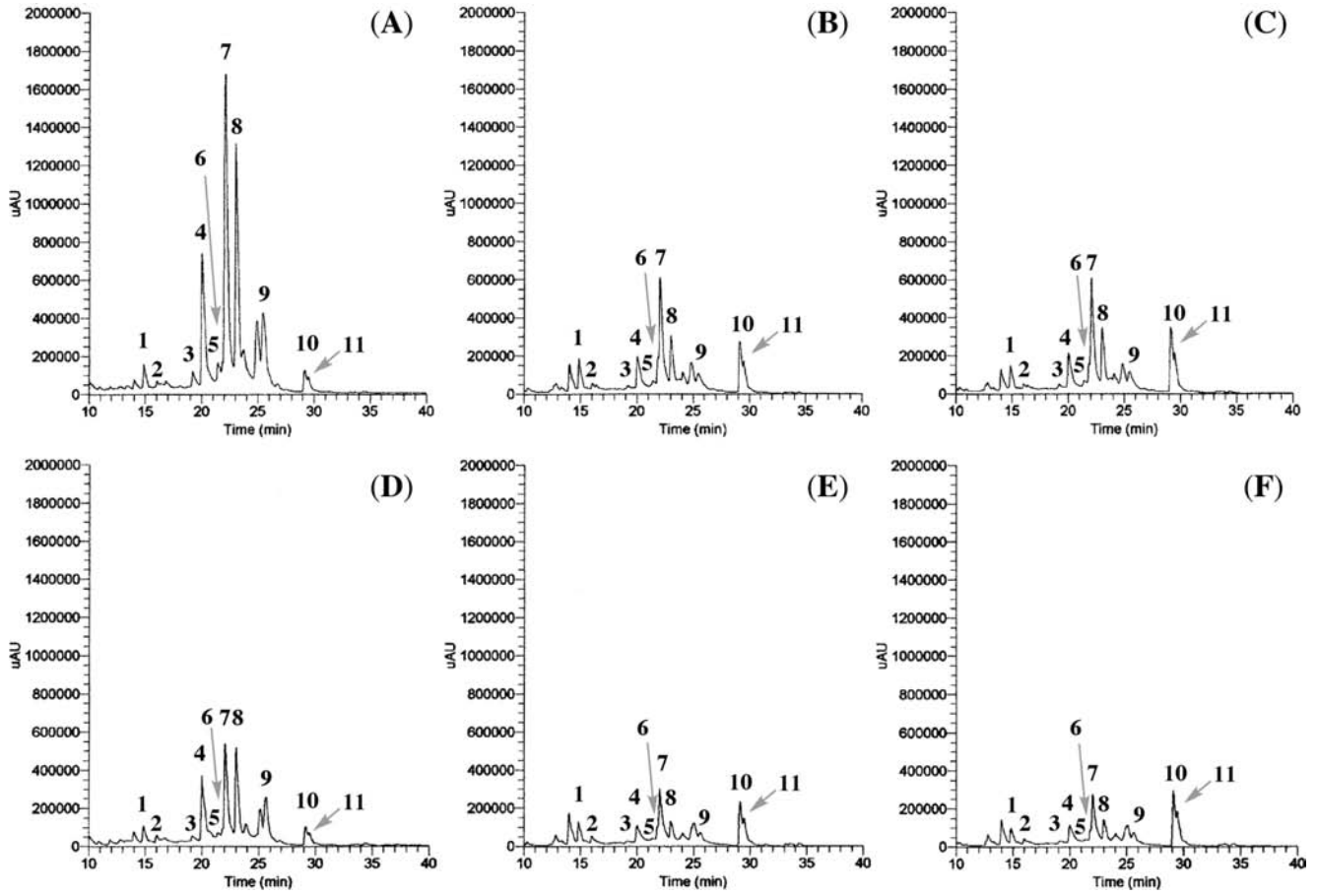


Figure 1. Typical DAD chromatograms of *C. myrtifolia* juices from immature (top) and ripe (bottom) fruits recorded at 278 (**A**, **D**), 325 (**B**, **E**), and 310 nm (**C**, **F**). Compounds **1**−**11** were identified as follows: **1**, vicenin-2; **2**, lucenin-2 4′-methyl ether; **3**, eriocitrin; **4**, neoeriocitrin; **5**, narirutin; **6**, rhoifolin; **7**, naringin; **8**, neohesperidin; **9**, brutieridin; **10**, bergapten; **11**, epoxybergamottin.

scavenging activity was calculated by the equation

$$\text{inhibition } (\%) = \frac{A_0 - A_S}{A_0} \times 100 \quad (1)$$

where A_0 is the absorbance of the control and A_S is the absorbance of the sample after 50 min of incubation. All tests were run in triplicate, and the results are expressed as mean ± standard deviation (SD). Trolox (1−100 μM) was chosen as a standard antioxidant, and juice activity is expressed in micromolar Trolox equivalents (μM TE).

Superoxide Anion Scavenging Assay. Superoxide anion scavenging activity of chinotto juice was performed according to a minor modification of the method described by Nishimiki et al. (*22*). The reaction mix in a final volume of 1.0 mL was composed of 80 mM phosphate buffer (pH 7.4), 499 μM nitroblue tetrazolium (NBT), 187 μM nicotinamide adenine dinucleotide reduced form (NADH), and 6 μM phenazine methosulfate (PMS) with or without 50 μL of tested juices. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion scavenging activity was calculated according to eq **1**. Trolox (1−100 μM) was chosen as a standard antioxidant, and juice activity is expressed in micromolar Trolox equivalents (μM TE).

Statistical Analysis. The data were analyzed by one-way analysis of variance (ANOVA). The significance of the difference between the samples for each experimental test condition was assayed by using Student's *t* test for each paired experiment. A $P < 0.05$ was regarded as indicating a significant difference.

RESULTS AND DISCUSSION

Over the past few years, we have laid out a procedure for the direct analysis of crude *Citrus* juices (*17*−*19*). The same protocol was applied to *C. myrtifolia* juice. Hand-squeezed chinotto juice was mixed with DMF, centrifuged, filtered, and finally analyzed by reversed-phase HPLC-DAD-MS-MS. *Citrus* juice is a complex food matrix, but the DAD chromatograms at 278, 325, and 310 nm of juices from immature or ripe chinotto fruits (**Figure 1**) and the UV spectra recorded in correspondence of each chromatographic peak showed that it contains different classes of compounds such as flavonoids and furanocoumarins. Comparison of the relative absorbances at 278 and 325 nm allowed for the discrimination of flavanone and flavone derivatives, whereas the DAD chromatogram at 310 nm could be used to efficiently detect furocoumarins.

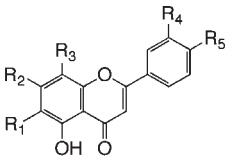
Observation of traces **A** and **B** in **Figure 1**, corresponding to juice samples from unripe fruits, provided initial evidence on the assignment of a flavone skeleton for compounds **1**, **2**, and **6** and a flavanone one for derivatives **3**−**5** and **7**−**9**. Besides, inspection of the spectrum after acidic hydrolysis showed that compounds **1** and **2** were unchanged, indicating that they are flavone-*C*-glycosides rather than -*O*-glycosides (*17*). Drawing on the reported data of the closely related bergamot juice (*18*), it was possible to obtain an unambiguous structural assignment for compounds **1**−**8**, owing to the excellent agreement of their first-order ESI-MS spectra recorded for each peak, MS-MS data, UV data, and retention time (t_R) (**Figures 2** and **3**).

The UV spectrum of compound **9** indicated the flavanone nature of the aglycone, and it is in agreement with the one reported for brutieridin (*23*). Fragmentation in the negative mode MS-MS spectrum focused on the pseudomolecular ion at 753 showed the appearance of peaks corresponding to the loss of

$C_4H_6O_3$ (*m*/*z* 651, $[M - H - 102]^-$) and $C_6H_8O_4$ (*m*/*z* 609, $[M - H - 144]^-$), both compatible with the presence of a 3-hydroxy-3-methylglutaryl substituent on one of the saccharide residues. Compound **9** was therefore identified as hesperetin 7-*O*-[2″-α-L-rhamnosyl-6″-(3‴′-hydroxy-3‴′-methylglutaryl)-β-D-glucoside] (brutieridin) in accordance with the spectroscopic data and the fragmentation pattern recently reported (*23*) (**Figure 4**).

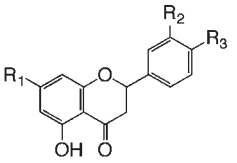
Compounds **10** and **11** both showed DAD-UV absorption maxima at 221 and 311, indicating the probable presence of coumarin nuclei (*24*). On the basis of its t_R, UV, MS, and MS-MS data ($[M + H]^+$ *m*/*z* 217; $[M + H - CH_3]^+$ *m*/*z* 202), compound **10** was identified as bergapten (**Figure 4**), which was also previously found in bergamot juice (*17*, *18*).

As for compound **11**, t_R 29.61 min, the ESI-MS spectrum in positive mode showed the presence of a pseudomolecular ion (*m*/*z* 355, $[M + H]^+$), but the DAD spectrum was not conclusive, as a result of a partial overlapping with the peak for compound **10**. We therefore set up a preparative HPLC procedure to permit its separate collection. The isocratic separation was carried out on a C18 column, using water/acetonitrile (40:60 v/v) as the mobile phase (**Figure 5**). The collected furocoumarin was then analyzed, and the APCI-MS-MS spectrum in positive mode (focused on *m*/*z* 355) showed the appearance of two fragments with *m*/*z* 337 ($[(M + H - H_2O]^+$) and *m*/*z* 203 ($[M + H - C_{10}H_{17}O]^+$). This fragmentation pattern, along with UV absorbances at 221, 250, and 258, led us to assign compound **11** as epoxybergamottin (**Figure 4**) as a result of the excellent agreement with literature data (*25*).



	R_1	R_2	R_3	R_4	R_5	Structure assignment
1	Glu	OH	Glu	H	OH	Apigenin 6,8-di-*C*-glucoside (Vicenin-2)
2	Glu	OH	Glu	OH	OMe	Diosmetin 6,8-di-*C*-glucoside (Lucenin-2 4′-methyl ether)
6	H	*O*-Nh†	H	H	OH	Apigenin 7-*O*-neohesperidoside (Rhoifolin)

Figure 2. Flavone-*C*-glucosides (**1**, **2**) and flavone-*O*-glycosides (**6**). †*O*-Neohesperidose.



	R_1	R_2	R_3	Structure assignment
3	*O*-Ru†	OH	OH	Eriodictyol 7-*O*-rutinoside (Eriocitrin)
4	*O*-Nh‡	OH	OH	Eriodictyol 7-*O*-neohesperidoside (Neoeriocitrin)
5	*O*-Ru†	H	OH	Naringenin 7-*O*-rutinoside (Narirutin)
7	*O*-Nh‡	H	OH	Naringenin 7-*O*-neohesperidoside (Naringin)
8	*O*-Nh‡	OH	OMe	Hesperetin 7-*O*-neohesperidoside (Neohesperidin)

Figure 3. Flavanone-*O*-glycosides (**3**−**5**, **7**, **8**). †*O*-Rutinose; ‡*O*-neohesperidose.

Quantitative Evaluation. Neoeriocitrin (**4**), naringin (**7**), neohesperidin (**8**), and brutieridin (**9**) are the flavanone-*O*-glycosides found in the highest amounts in juices from both immature fruits (7.15 ± 0.27, 19.95 ± 0.31, 18.24 ± 0.23, and 8.33 ± 0.42 mg/L, respectively) and mature fruits (3.08 ± 0.16, 6.08 ± 0.23, 5.76 ± 0.24, and 2.19 ± 0.26 mg/L, respectively) (**Table 1**). Small amounts of eriocitrin (**3**), narirutin (**5**), and rhoifolin (**6**) were also found, again in juice from both immature fruits (0.76 ± 0.042, 0.75 ± 0.021, and 0.93 ± 0.022 mg/L, respectively) and mature fruits (0.26 ± 0.028, 0.08 ± 0.032, and 0.11 ± 0.19 mg/L, respectively). The flavone-*C*-glucosides vicenin-2 (**1**) and lucenin-2 4′-methyl ether (**2**) were found to be present in significant amounts both in immature chinotto juice (0.92 ± 0.021 and 0.35 ± 0.028 mg/L, respectively) and in the mature one (0.58 ± 0.049 and 0.20 ± 0.021 mg/L, respectively). These data confirm once again the similarity between bergamot and chinotto juices, because vicenin-2 and lucenin-2 4′-methyl ether are the most abundant flavone-*C*-glucosides in bergamot juice (*17*, *18*). Furthermore, the furocoumarins, bergapten (**10**) and epoxybergamottin (**11**), were quantified as 0.91 ± 0.03 and 0.75 ± 0.05 mg/L in the juice from immature fruits and as 0.67 ± 0.02 and 0.24 ± 0.03 mg/L in the juice of mature ones.

Interestingly, a progressive decrease in the flavonoid content over ripening is evident from the data described. Green chinotto fruits are relatively richer in flavonoids and furocoumarins than ripe fruits, going from a total flavonoid content of 57.4 mg/L for the former to 18.3 mg/L for the latter. However, both amounts are significantly lower than what has been observed for bergamot (*18*) (372−512 mg/L, depending on the cultivar).

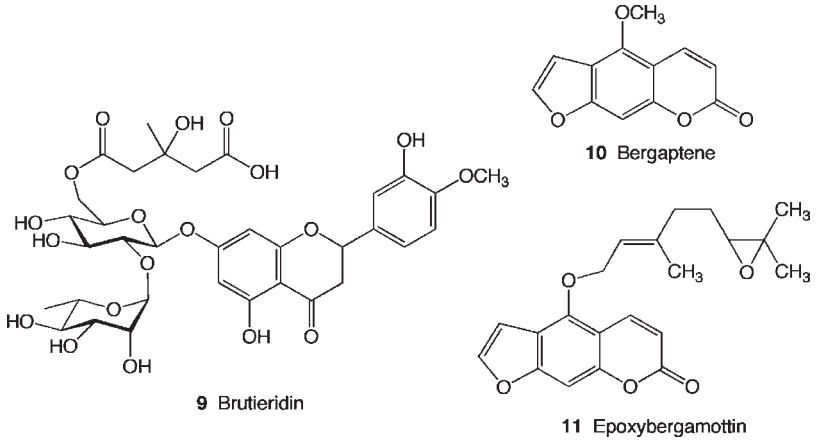


Figure 4. Structures for compounds **9**−**11**.

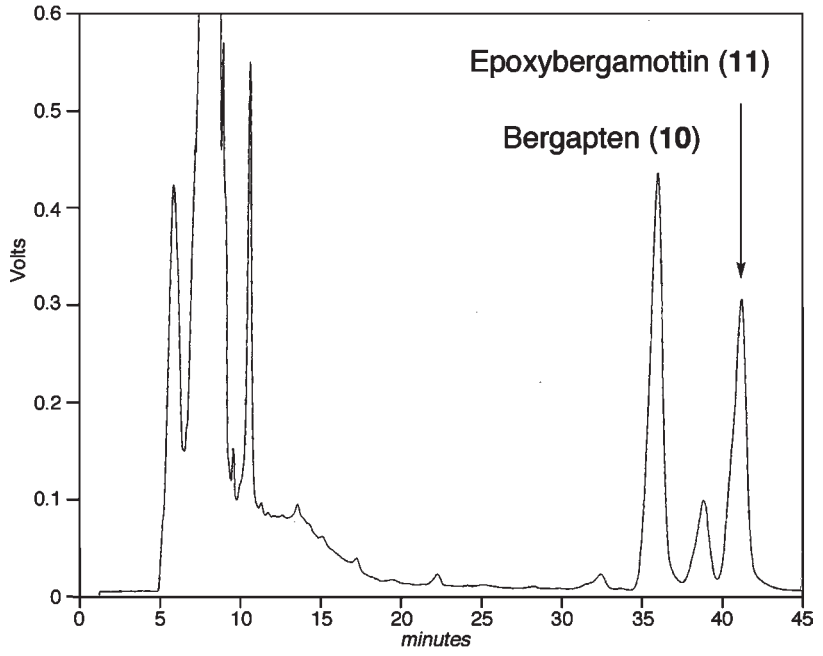


Figure 5. Preparative HPLC chromatogram for the collection of furocoumarins **10** and **11** from *C. myrtifolia* juice.

Table 1. Flavone-*C*-glucoside (**1** and **2**), Flavone-*O*-glycoside (**6**), Flavanone-*O*-glycoside (**3**−**5**, **7**−**9**), and Furocoumarin (**10** and **11**) Contents in *C. myrtifolia* Juice

		juice of immature chinotto		juice of mature chinotto	
		mean (mg/L)	range (mg/L)	mean (mg/L)	range (mg/L)
flavonoids					
1	vicenin-2	0.92	0.90−0.93	0.58	0.55−0.62
2	lucenin-2 4′-OMe	0.35	0.33−0.37	0.20	0.18−0.21
3	eriocitrin	0.76	0.71−0.77	0.26	0.23−0.27
4	neoeriocitrin	7.15	6.87−7.25	3.08	3.00−3.23
5	narirutin	0.75	0.73−0.76	0.08	0.07−0.13
6	rhoifolin	0.93	0.91−0.94	0.11	0.09−0.12
7	naringin	19.95	19.69−20.13	6.08	5.92−6.25
8	neohesperidin	18.24	18.11−18.43	5.76	5.57−5.91
9	brutieridin	8.33	8.03−8.62	2.19	1.97−2.33
furocoumarins					
10	bergapten	0.91	0.88−0.92	0.67	0.64−0.69
11	epoxybergamottin	0.75	0.73−0.77	0.24	0.21−0.25

Antioxidant Assays. To further demonstrate the potential health-promoting properties of *C. myrtifolia*, we carried out a screening on the antioxidant abilities of the juices from immature and mature *C. myrtifolia* fruits. In vitro free radical scavenging activity was determined with the DPPH$^{\bullet}$ assay, which measures, in our case, the ability of a crude *Citrus* juice as such to bleach a 2,2-diphenyl-1-picrylhydrazyl solution, and the results are expressed as Trolox equivalents (TE). Both juices reacted and quenched DPPH$^{\bullet}$ radicals, showing significant antioxidant power. As reported in **Figure 6** (left), juice from immature chinotto showed the highest capacity of bleaching DPPH$^{\bullet}$ free radicals, reaching 13.1 μM TE. However, a good albeit lower antioxidant activity was determined for ripe chinotto juice (8.9 μM TE). The capability of crude juices to neutralize superoxide anion formed by the reduction of NBT with NADH mediated by PMS under aerobic conditions follows the same trend, and it is reported in **Figure 6** (right). Both chinotto juices induce a significant decrease of the absorbance value at 560 nm, reaching values of 184 μM TE for green chinotto juice and 144 μM TE for

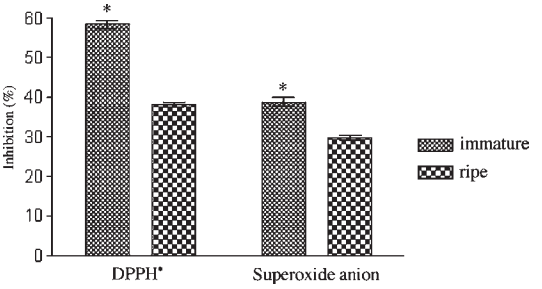


Figure 6. DPPH$^{\bullet}$ (left) and anion superoxide (right) scavenging activityies of juices from immature and mature *C. myrtifolia* fruits. Each value represents the mean ± SD (n = 3). Asterisks indicate a significant difference between the samples at $P < 0.05$.

the one obtained from ripe chinotto. Both DPPH$^{\bullet}$ radical and superoxide scavenging differences were found to be statistically relevant at a $P < 0.05$.

The values obtained for the antioxidant activity are quite interesting, even if they do not appear to be correlated to the total flavonoid content (for instance, the richer crude bergamot juice was found to have lower antioxidant activity than chinotto (*18*)). Neoeriocitrin **4** and eriocitrin **3** are probably the flavonoids that contribute the most to the total antioxidant activity, because the presence of a catecholic B-ring confers greater stability to aroxyl radicals formed upon DPPH$^{\bullet}$ quenching (*26*). On the other hand **3** and **4** are not overly abundant in chinotto juice, and therefore they cannot be held responsible for such a high activity, which could possibly be ascribed to the ascorbic acid content of the juice (895 mg/L (*2*)). However, these findings are in line with reported data on chinotto-based soft drinks, which outclass other beverages such as cola soft drinks in terms of antioxidant ability (*27*).

The total content of flavonoids and furocoumarins in *C. myrtifolia* juice obtained from fruits at two different stages of ripening has been determined by applying an HPLC-DAD-ESI-MS-MS protocol that we have optimized over the past few years for the direct analysis of crude *Citrus* juices. Chinotto juice contains a number of bioactive components, including significant amounts

of the statin-like derivative brutieridin, which has been found in *C. myrtifolia* for the first time. The antioxidant activity of juices from green and ripe chinotto was tested by DPPH$^{\bullet}$ radical bleaching and superoxide anion scavenging, and it was shown that immature chinotto fruits, in particular, yield a juice with a remarkable antioxidant power. In light of the great commercial interest in research on dietary products that can extend the antioxidant pool available for humans, and considering the growing diffusion of chinotto-based soft drinks, the results reported in this study may confer further impulse to the industrial application of such an interesting and little-known fruit.

ABBREVIATIONS USED

DPPH$^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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