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# Flavonoid Glycosides in Bergamot Juice (*Citrus bergamia* Risso)

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A comprehensive profile of flavonoids in bergamot juice was obtained by a single DAD-ESI-LC-MS-MS course. Eight flavonoids were found for the first time, five of these are *C*-glucosides (lucenin-2, stellarin-2, isovitexin, scoparin, and orientin 4′-methyl ether), and three are *O*-glycosides (rhoifolin 4′-*O*-glucoside, chrysoeriol 7-*O*-neohesperidoside-4′-*O*-glucoside, and chrysoeriol 7-*O*-neohesperidoside). A method is proposed to differentiate chrysoeriol and diosmetin derivatives, which are often indistinguishable by LC-MS-MS. In-depth knowledge of the flavonoid content is the starting point for bergamot juice exploitation in food industry applications.

**KEYWORDS:** ***Citrus bergamia*; flavonoids; *C*-glucosides; *O*-glycosides; HPLC-DAD-ESI-MS-MS**

## INTRODUCTION

Bergamot (*Citrus bergamia* Risso) is a plant hybrid of sour orange and lemon. It belongs to the family Rutaceae, genus *Citrus*. The plant is very sensitive to pedoclimatic conditions of soil, and to date, worldwide production of bergamot is concentrated almost exclusively in a narrow coastal strip in the Reggio Calabria (southern Italy) area. In the past, bergamot has been highly valued by the cosmetic and perfume industry since its essence is very rich in terpenes, esters, and alcohols possessing a very characteristic and intense fragrance. The development of synthetic essential oil production led to a drastic drop in commercial demand for bergamot. Nowadays, a growers' consortium in Reggio Calabria, "Consorzio del Bergamotto", promotes future applications for all parts of the bergamot fruit and further supports bergamot cultivation.

Flavonoids are phenolic derivatives with antioxidant ability. In particular, they can act as powerful free radical scavengers. In general, all citrus plants are rich sources of flavonoids, although they do not occur normally as aglycones (*1*−*5*) but rather as their more polar glycoside derivatives and specifically as their *O*-$\beta$-glycosides (*6*).

Flavonoid glycosides, found in citrus juice, flavedo, albedo, and leaf, present typical distribution patterns that are particular to each species. Lemon, for instance, is characterized by eriocitrin, hesperidin, and diosmin, whereas naringin, neohesperidin, and small amounts of neoeriocitrin are found in sour orange. Hesperidin, narirutin, and didymin are typical of sweet orange; naringin, narirutin, and to a lesser extent, hesperidin and neohesperidin, are found in grapefruit species (*7*−*13*). Bergamot seeds are an interesting source of glycosylated flavanones since they are rich in naringin and neohesperidin and contain also a fair amount of neoeriocitrin. There is almost no data on bergamot peels apart from their naringin content (*4*). The main components of bergamot juice, which have already been reported in the literature, have been identified as neoeriocitrin, naringin, and neohesperidin (*14*−*18*).

The aim of this work was to study the flavonoid components of bergamot juice, as a starting point for its commercial exploitation since it has so far been considered merely a byproduct of essential oil extraction.

## MATERIALS AND METHODS

**Materials.** The industrially processed bergamot juice was supplied by the "Ditta Visalli Diego Estrazione Essenze e Derivati", Melito di Porto Salvo (Reggio Calabria, Italy).

The investigation was carried out on 20 samples from the 2004−2005 fruit season. The juice was produced as a blend of three cultivars of *Citrus bergamia*: 'Fantastico', 'Femminello', and 'Castagnaro'.

**Reagents and Standard Solutions.** HPLC-grade acetonitrile and methanol were supplied by Sigma-Aldrich, and dimethylformamide (DMF) was from Carlo Erba (Milano, Italy). Diosmetin was supplied by Sigma-Aldrich (St. Louis, MO). Chrysoeriol, neodiosmin, eriocitrin, neoeriocitrin, naringin, and neohesperidin were from Extrasynthèse (Genay, France). They were used as standards. The calibration lines were obtained using DMF solutions of known concentration (10−100 mg/L).

**Sample Preparation.** DMF (10.0 mL) was added to the industrial bergamot juice (10.0 mL), and the mixture was centrifuged for 5 min at 3200 rpm. The supernatant liquid was filtered through an Iso-Disk P-34, 3 mm diameter PTFE membrane, 0.45 $\mu$m pore size (Supelco, Bellefonte, PA).

**Hydrolysis.** Acid hydrolysis was carried out on juice following the procedure of Hertog et al. (*19*). 6 M HCl (10 mL) in a methanol (25 mL)/water (10 mL) solution was added to 5 mL of bergamot juice to give a solution of 1.2 M HCl in 50% aqueous methanol. Ascorbic acid

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(50 mg) was added as an antioxidant. After refluxing at 90 °C for 20 h under stirring, the solution was allowed to cool at room temperature, vacuum-dried, and made up to 10 mL with water/DMF (1:1). The mixture was filtered through an Iso-Disk P-34 membrane and analyzed by HPLC.

**LC-MS-MS Analysis of Flavonoids.** LC-MS-MS analyses of samples were carried out with a ThermoQuest Model LCQ-Duo equipped with a diode array spectrophotometer and an ion trap mass spectrometer with an electrospray ionization source (ESI). Separation of flavonoids was performed on a 250 mm × 4.6 mm i.d., 5 μm Discovery C18 column, supplied by Supelco (Bellefonte, PA), equipped with a 20 mm × 4.0 mm guard column. 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 and 325 nm.

Operating parameters of the mass spectrometer were capillary temperature 250 °C; spray needle voltage set at 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 80−900 amu, in both positive and negative mode. The MS-MS spectra were obtained with 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.

**Quantitative Evaluation of Flavonoid Content.** Quantitative analysis was carried out by integration of the areas of the peaks from the chromatogram at 325 nm, by using the Genesis peak detection algorithm integrated in the ThermoQuest software.

**3-D Structures Computer Simulation.** Structure refinement was performed by semiempirical calculations on ground-state conformers and conformer distribution, by applying the AM1, PM3, and MNDO force fields included in the Spartan-04 package (Wavefunction, Inc.) on a SUN workstation.

**Spectroscopic Data. Compound 1.** RT, 13.8 min; UV, 258 (sh), 270, 349 nm; MS, 611 $[M + H]^+$, 609 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 611 $[M + H]^+$ (2), 593 $[(M + H) - 18]^+$ (100), 575 $[(M + H) - 36]^+$ (9), 545 $[(M + H) - 66]^+$ (8), 539 $[(M + H) - 72]^+$ (3), 527 $[(M + H) - 84]^+$ (2), 491 $[(M + H) - 120]^+$ (6), 473 $[(M + H) - 138]^+$ (14); MS-MS focused on $[M - H]^-$, 609 $[M - H]^-$ (2), 591 $[(M - H) - 18]^-$ (8), 519 $[(M - H) - 90]^-$ (28), 489 $[(M - H) - 120]^-$ (100), 399 $[(M - H) - 210]^-$ (A + 113) (2), 369 $[(M - H) - 240]^-$ (A + 83) (3).

**Compound 2.** RT, 15.3 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 3.** RT, 15.9 min; UV, 256 (sh), 271, 347 nm; MS, 625 $[M + H]^+$, 623 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 625 $[M + H]^+$ (2), 607 $[(M + H) - 18]^+$ (100), 589 $[(M + H) - 36]^+$ (8), 559 $[(M + H) - 66]^+$ (2), 505 $[(M + H) - 120]^+$ (4), 487 $[(M + H) - 138]^+$ (6); MS-MS focused on $[M - H]^-$, 623 $[M - H]^-$ (25), 605 $[(M - H) - 18]^-$ (2), 533 $[(M - H) - 90]^-$ (15), 503 $[(M - H) - 120]^-$ (100), 413 $[(M - H) - 210]^-$ (A + 113) (4), 383 $[(M - H) - 240]^-$ (A + 83) (14).

**Compound 4.** RT, 16.5 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 5.** RT, 16.8 min; UV, 268, 324 nm; MS, 741 $[M + H]^+$, 739 $[M - H]^-$; sidMS, 739 $[M - H]^-$ (48), 593 $[(M - H) - 146]^-$ (42), 577 $[(M - H) - 162]^-$ (100), 431 $[(M - H) - 308]^-$ (3), 269 $[(M - H) - 308 - 162]^-$ (A) (3); MS-MS focused on $[M + H]^+$, 741 $[M + H]^+$ (10), 595 $[(M + H) - 146]^+$ (14), 579 $[(M + H) - 162]^+$ (<1), 433 $[(M + H) - 308]^+$ (100), 271 $[(M + H) - 308 - 162]^+$ (A) (4).

**Compound 6.** RT, 17.5 min; UV, 249 (sh), 268, 337 nm; MS, 771 $[M + H]^+$, 769 $[M - H]^-$; sidMS, 771 $[M + H]^+$ (100), 463 $[(M + H) - 308]^+$ (9), 301 $[(M + H) - 308 - 162]^+$(9); 769 $[M - H]^-$ (4), 607 $[(M - H) - 162]^-$ (100), 299 $[(M - H) - 308 - 162]^-$ (A) (2); MS-MS focused on $[M + H]^+$, 771 $[M + H]^+$ (34), 625 $[(M + H) - 146]^+$ (18), 609 $[(M + H) - 162]^+$ (13), 463 $[(M + H) - 308]^+$ (100), 301 $[(M + H) - 308 - 162]^+$ (A) (23); sidMS-MS focused on the aglycone $[(M - H) - 308 - 162]^-$, 299 $[(M - H) - 308 - 162]^-$ (18), 284 $[(M - H) - 308 - 162 - 15]^-$ (A − 15) (100).

**Compound 7.** RT, 19.6 min; UV, 283 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 8.** RT, 19.7 min; UV, 269, 336 nm; MS, 433 $[M + H]^+$, 431 $[M - H]^-$; MS-MS focused on $[M - H]^-$, 431 $[M - H]^-$ (1), 413 $[(M - H) - 18]^-$ (6), 341 $[(M - H) - 90]^-$ (24), 311 $[(M - H) - 120]^-$ (100).

**Compound 9.** RT, 20.4 min; UV, 283 nm; MS, 597 $[M + H]^+$, 595 $[M - H]^-$; MS-MS focused on $[M + H]^+$, 597 $[M + H]^+$ (3), 579 $[(M + H) - 18]^+$ (22), 561 $[(M + H)\text{-}36]^+$ (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 10.** RT, 20.7 min; UV, 256, 268, 347 nm; MS, 463 $[M + H]^+$, 461 $[M - H]^-$, MS-MS focused on $[M + H]^+$, 463 $[M + H]^+$ (1), 445 $[(M + H) - 18]^+$ (100), 427 $[(M + H) - 36]^+$ (18), 409 $[(M + H) - 54]^+$ (2), 397 $[(M + H) - 66]^+$ (17), 373 $[(M + H) - 90]^+$ (1), 367 $[(M + H) - 96]^+$ (3), 343 $[(M + H) - 120]^+$ (9), 325 $[(M + H) - 138]^+$ (1), 313 $[(M + H) - 150]^+$ (1); MS-MS focused on $[M - H]^-$, 461 $[M - H]^-$ (4), 371 $[(M - H) - 90]^-$ (8), 341 $[(M - H) - 120]^-$ (100).

**Compound 11.** RT, 20.9 min; UV, 253 (sh), 270, 348 nm; MS, 463 $[M + H]^+$, 461 $[M - H]^-$, MS-MS focused on $[M + H]^+$, 463 $[M + H]^+$ (1), 445 $[(M + H) - 18]^+$ (100), 427 $[(M + H) - 36]^+$ (23), 409 $[(M + H) - 54]^+$ (11), 397 $[(M + H) - 66]^+$ (54), 367 $[(M + H) - 96]^+$ (23), 343 $[(M + H) - 120]^+$ (21), 313 $[(M + H) - 150]^+$ (1); MS-MS focused on $[M - H]^-$, 461 $[M - H]^-$ (29), 371 $[(M - H) - 90]^-$ (13), 341 $[(M - H) - 120]^-$ (100).

**Compound 12.** RT, 22.1 min; UV, 267, 337 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 13.** RT, 22.3 min; UV, 281 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 $[(M - H) - 266]^-$ (16), 271 $[(M - H) - 308]^-$ (A) (25).

**Compound 14.** RT, 22.5 min; UV, 252, 268, 347 nm; MS, 609 $[M + H]^+$, 607 $[M - H]^-$; sidMS, 609 $[M + H]^+$ (100), 463 $[(M + H) - 146]^+$ (3), 301 $[(M + H) - 308]^+$ (A) (39), 607 $[M - H]^-$ (100), 299 $[(M - H) - 308]^-$ (A) (38); MS-MS focused on $[M + H]^+$, 609 $[M + H]^+$ (12), 463 $[(M + H) - 146]^+$ (21), 301 $[(M + H) - 308]^+$ (A) (100); MS-MS focused on $[M - H]^-$, 607 $[M - H]^-$ (75), 487 $[(M - H) - 120]^-$ (<1), 461 $[(M - H) - 146]^-$ (1), 341 $[(M - H) - 266]^-$ (1), 299 $[(M - H) - 308]^-$ (A) (100), 284 $[(M - H) - 308 - 15]^-$ (A − 15) (12); sidMS-MS focused on $[(M - H) - 308]^-$, 299 $[(M - H) - 308]^-$ (37), 284 $[(M - H) - 308 - 15]^-$ (100).

**Compound 15.** RT, 22.7 min; UV, 253, 268, 345 nm; MS, 609 $[M + H]^+$, 607 $[M - H]^-$; sidMS, 609 $[M + H]^+$ (100), 463 $[(M + H) -$

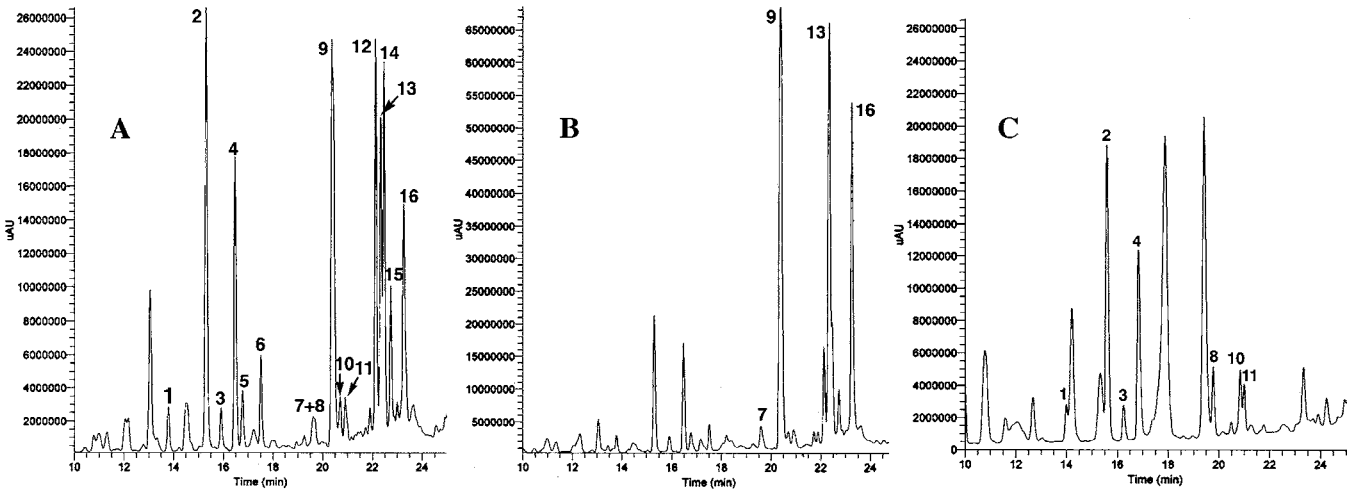
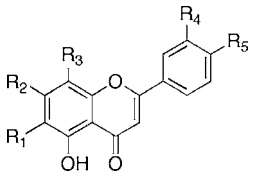



**Figure 1.** Typical chromatograms of bergamot juice: (**A**) 325 nm and (**B**) 278 nm. Components 1–16 were identified as follows: 1, lucenin-2; 2, vicenin-2; 3, stellarin-2; 4, lucenin-2 4′-methyl ether; 5, rhoifolin 4′-glucoside; 6, chrysoeriol 7-*O*-neohesperidoside-4′-glucoside; 7, eriocitrin; 8, isovitexin; 9, neoeriocitrin; 10, scoparin; 11, orientin 4′-methyl ether; 12, rhoifolin; 13, naringin; 14, chrysoeriol 7-*O*-neohesperidoside; 15, neodiosmin; and 16, neohesperidin. (**C**) DAD chromatogram at 325 nm of hydrolyzed bergamot juice. Acid hydrolysis resistant components: 1, lucenin-2; 2, vicenin-2; 3, stellarin-2; 4, lucenin-2 4′-methyl ether; 8, isovitexin; 10, scoparin; and 11, orientin 4′-methyl ether.

**Table 1.** Flavone *C*-Glucosides (**1**–**4**, **8**, **10**, and **11**) and Flavone *O*-Glycosides (**5**, **6**, **12**, **14**, and **15**)



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | structure assignment |
|---|---|---|---|---|---|---|
| **1** | Glu | OH | Glu | OH | OH | luteolin 6,8-di-*C*-glucoside (lucenin-2) |
| **2** | Glu | OH | Glu | H | OH | apigenin 6,8-di-*C*-glucoside (vicenin-2) |
| **3** | Glu | OH | Glu | OMe | OH | chrysoeriol 6,8-di-*C*-glucoside (stellarin-2) |
| **4** | Glu | OH | Glu | OH | OMe | diosmetin 6,8-di-*C*-glucoside (lucenin-2 4′-methyl ether) |
| **5** | H | *O*-NH[a] | H | H | *O*-Glu | apigenin 7-*O*-neohesperidoside-4′-glucoside (rhoifolin 4′-glucoside) |
| **6** | H | *O*-NH[a] | H | OMe | *O*-Glu | chrysoeriol 7-*O*-neohesperidoside-4′-glucoside |
| **8** | Glu | OH | H | H | OH | apigenin 6-*C*-glucoside (isovitexin) |
| **10** | H | OH | Glu | OMe | OH | chrysoeriol 8-*C*-glucoside (scoparin) |
| **11** | H | OH | Glu | OH | OMe | diosmetin 8-*C*-glucoside (orientin 4′-methyl ether) |
| **12** | H | *O*-NH[a] | H | H | OH | apigenin 7-*O*-neohesperidoside (rhoifolin) |
| **14** | H | *O*-NH[a] | H | OMe | OH | chrysoeriol 7-*O*-neohesperidoside |
| **15** | H | *O*-NH[a] | H | OH | OMe | diosmetin 7-*O*-neohesperidoside (neodiosmin) |

[a] *O*-Neohesperidose.

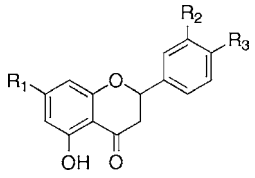
146]$^+$ (2), 301 [(M + H) − 308]$^+$ (A) (39); 607 [M − H]$^-$ (100), 461 [(M − H) − 146]$^-$ (1), 299 [(M − H) − 308]$^-$ (A) (77); MS-MS focused on [M + H]$^+$, 609 [M + H]$^+$ (6), 463 [(M + H) − 146]$^+$ (20), 301 [(M + H) − 308]$^+$ (A) (100); MS-MS focused on [M − H]$^-$, 607 [M − H]$^-$ (4), 487 [(M − H) − 120]$^-$ (2), 341 [(M − H) − 266]$^-$ (3), 299 [(M − H) − 308]$^-$ (A) (100), 284 [(M − H) − 308 − 15]$^-$ (A − 15) (10); sidMS-MS focused on [(M − H) − 308]$^-$, 299 [(M − H) − 308]$^-$ (3), 284 [(M − H) − 308 − 15]$^-$ (100).

**Compound 16.** RT, 23.2 min; UV, 283 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).

## RESULTS AND DISCUSSION

A DAD chromatogram at 325 nm of a sample of industrially processed bergamot juice is reported in **Figure 1A**. Peaks 1−16 correspond to identified flavonoid glycosides (**Tables 1** and **2**). **Figure 1B** reports the simultaneous chromatogram at 278 nm,

**Table 2.** Flavanone *O*-Glycosides (**7**, **9**, **13**, and **16**)



| | $R_1$ | $R_2$ | $R_3$ | structure assignment |
|---|---|---|---|---|
| **7** | *O*-Ru[a] | OH | OH | eriodictyol 7-*O*-rutinoside (eriocitrin) |
| **9** | *O*-NH[b] | OH | OH | eriodictyol 7-*O*-neohesperidoside (neoeriocitrin) |
| **13** | *O*-NH[b] | H | OH | naringenin 7-*O*-neohesperidoside (naringin) |
| **16** | *O*-NH[b] | OH | OMe | hesperetin 7-*O*-neohesperidoside (neohesperidin) |

[a] *O*-Rutinose. [b] *O*-Neohesperidose.

which allows the identification of compounds **7**, **9**, **13**, and **16** as flavanones from the observed increase in relative absorptions. Analysis of first-order MS spectra recorded for each peak together with MS-MS experiments, UV comparison, and retention time (RT) led to the following structure assignments.

**Flavone *C*-Glucosides.** Flavone *C*-glucosides are resistant to acid hydrolysis. Comparing the DAD chromatogram at 325 nm of juice (**Figure 1A**) with the corresponding one of hydrolyzed juice (**Figure 1C**), it is possible to observe that the peaks for compounds **1**−**4**, **8**, **10**, and **11** appear unchanged. This behavior, along with the absence of the aglycone ion in the first-order MS, suggests that these compounds are flavone *C*-glycosides.

The MS-MS spectra, obtained by focusing on each [M − H]$^-$ ion of compounds **1**−**4**, exhibited the same pattern of fragmentation ([(M − H) − 18]$^-$, [(M − H) − 90]$^-$, [(M − H) − 120]$^-$, [A + 113]$^-$, and [A + 83]$^-$), typical of di-*C*-glycosylflavones. Positions 6 and 8 appeared to be substituted in each case since the maximum of band II was located at 270 nm or higher. The absence of the [(M − H) − 60]$^-$ fragment in these MS-MS spectra, which is usually generated by the fragmentation of pentose derivatives, along with the presence of the [(M − H) − 120]$^-$ (base peak) and [(M − H) − 90]$^-$ peaks, suggested that the sugar substituents were hexoses (*19*). The ions *m*/*z* [A + 113]$^-$ and [A + 83]$^-$ correspond to the

aglycone bearing sugar fragments, and they are particularly important for aglycone identification.

Full MS and MS-MS spectra of compounds **8**, **10**, and **11** did not provide any evidence regarding the nature of the aglycones. However, the presence of $[(M - H) - 90]^-$ and $[(M - H) - 120]^-$ confirms that these compounds are mono-*C*-hexosylated. The position of the sugar residue can be assigned by observation of the peak for the $[(M - H) - 18]^-$ fragment. When this is present, as in compound **8**, the sugar substituent is located in position 6. On the other hand, its absence indicates that the sugar lies on the 8 position, as for compounds **10** and **11**.

**Compound 1.** The MS-MS spectrum in positive mode focused on *m*/*z* 611 ($[M + H]^+$) was not particularly useful for structural identification; only peaks derived from the fragmentation of the sugar units were observed. The presence of $[A + 113]^-$ and $[A + 83]^-$ peaks in the MS-MS spectrum focused on *m*/*z* 609 ($[M - H]^-$) indicated that the aglycone has a molecular weight of 286.

The UV spectrum showed typical absorptions for a disubstituted tetrahydroxyflavone. The loss of 90 and 120 mass units from the pseudomolecular ion $[M - H]^-$ (as discussed previously), along with the shift observed for band II in the UV spectrum, confirmed the sugar substituents at positions 6 and 8 to be hexoses.

Retention time, UV, and negative mode MS-MS spectra led to the identification of compound **1** as luteolin 6,8-di-*C*-glucoside (lucenin-2). This assignment agrees with the observation that data reported above are superimposable with those previously reported in the literature for lucenin-2 (*20*). This is the first time that lucenin-2 has been found in bergamot juice.

**Compounds 2 and 4.** Compounds **2** and **4** were identified as apigenin 6,8-di-*C*-glucoside (vicenin-2) and diosmetin 6,8-di-*C*-glucoside (lucenin-2 4′-methyl ether), respectively, by comparison with previously reported RTs and spectroscopic data (*13*, *21*).

**Compound 3.** The aglycone ($M_w = 300$, as observed in the MS-MS spectrum in negative mode) could be either diosmetin or chrysoeriol. The fragmentation shows that the linked sugars are hexoses. Band II value in UV spectra confirmed the presence of the sugars at the 6 and 8 positions. Compound **3** presented spectroscopic data and RT consistent with those of stellarin-2, which has been already identified as a component of lemon juice (*22*). It was therefore identified as chrysoeriol 6,8-di-*C*-glucoside (stellarin-2). This is the first time that stellarin-2 has been found in bergamot juice.

Compounds **3** and **4** are isomers with a hydroxyl group in the 4′ and 3′ positions, respectively. It has been suggested (*22*) that the shorter RT of chrysoeriol derivative **3** may be a consequence of the higher acidity of the 4′-OH group, with respect to the 3′-OH group of diosmetin derivative **4**. However, when standard chrysoeriol and diosmetin were subjected to HPLC analysis, in a control experiment carried out under the same experimental conditions employed for the bergamot juice analysis, it was observed that both flavones have the same RT, demonstrating that a difference in acidity is unlikely to be responsible for the difference in RT displayed by the C-glycosylated derivatives **3** and **4**.

Computer simulations carried out on compounds **3** and **4** showed that the glycosyl moiety in position 8 of the diosmetin derivative **4** can form a hydrogen bond with the 3′-OH group (**Figure 2**), whereas the chrysoeriol derivative cannot form intramolecular hydrogen bonds with the 4′-OH group. This hydrogen bond may be responsible for a lower overall polarity, and hence higher RT, of diosmetin derivative **4** with respect to chrysoeriol derivative **3**.

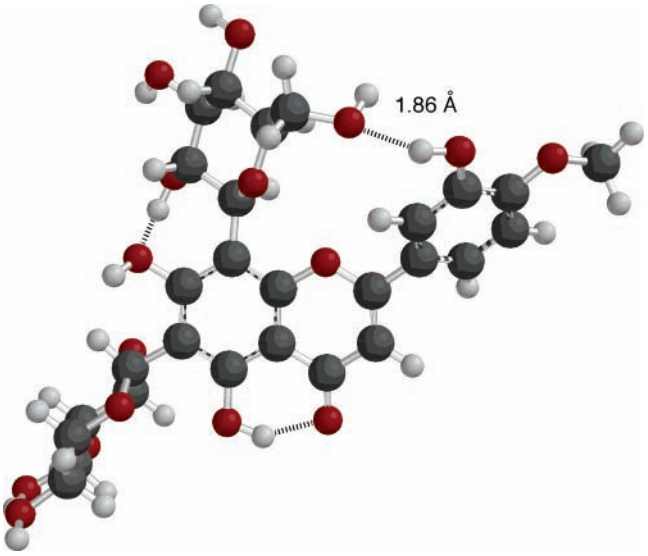



**Figure 2.** Computer model of 6,8-di-*C*-glucosyl diosmetin (component **4**). Hydrogen bonds are shown as dashed lines. Colors are follows: carbon, black; oxygen, red; and hydrogen, white.

**Compound 8.** UV absorptions of compound **8** were very similar to those of both apigenin 8-*C*-glucoside (vitexin) and apigenin 6-*C*-glucoside (isovitexin). The loss of 90 and 120 (base peak) amu from the pseudomolecular ion $[M - H]^-$ indicated the presence of a hexose as substituent in the apigenin skeleton. The fragmentation in the MS-MS spectrum in negative mode of component **8** gave additional information to differentiate between these two possible structures: the abundance of the ion at *m*/*z* 341 ($[(M - H) - 90]^-$ (*24*)) was compatible with that expected for isovitexin, rather than vitexin (*20*, *23*). Furthermore, the presence of the ion $[(M - H) - 18]^-$ sustained the attribution since the loss of 18 amu is not observed in the MS-MS spectrum of vitexin. We report here for the first time the presence of isovitexin in bergamot juice.

**Compounds 10 and 11.** The first-order MS spectra of compounds **10** and **11** showed the same pseudomolecular ion $[M - H]^-$ *m*/*z* 461. In both MS-MS spectra, the ions $[(M - H) - 90]^-$ and $[(M - H) - 120]^-$ suggested the presence of a single hexose as substituent at the 6, 8, or 3 position. UV spectra of both **10** and **11** indicated the presence, for the aglycone, of chrysoeriol or diosmetin.

The absence of the $[(M - H) - 18]^-$ fragment in the $[M - H]^-$ MS-MS spectra for compounds **10** and **11** suggests that the hexose substituent is located at the 8 or 3 position rather than at the 6 position. Since substitution at the 3 position in mono glucosyl-flavones has never been observed in *Citrus* species, it is likely that compounds **10** and **11** bear the hexose moiety at the 8 position.

The peak with a lower RT can be attributed to a chrysoeriol derivative, as a result of the difference of polarity with respect to analogous diosmetin derivatives for the reasons discussed previously. Therefore, compound **10** can be identified as chrysoeriol 8-*C*-glucoside (scoparin), which has previously been reported as present in *Citrus* species (*24*). Compound **11**, considering the higher RT, can be identified as diosmetin 8-*C*-glucoside (orientin 4′-methyl ether). This assignment is in agreement with the reported literature data on its presence in *Citrus* species (*25*). Scoparin and orientin 4′-methyl ether are now, for the first time, reported in bergamot juice.

**Flavonoid *O*-Glycosides.** Flavonoid *O*-glycosides are easily hydrolyzed in an acidic medium. Comparison of the DAD chromatograms at 325 nm before and after hydrolysis (**Figure 1A**,**C**) suggests that compounds **5**−**7**, **9**, and **12**−**16** are *O*-glycosides. Compounds **5**, **6**, **12**, **14**, and **15** show UV absorptions typical of flavone derivatives, whereas compounds **7**, **9**, **13**, and **16** present spectra compatible with flavanone structures. Positive and negative ion MS-MS analysis allows the identification of both the aglycones and the saccharide substituents. In *Citrus* species, the carbohydrate components are usually either monosaccharides (glucose, for instance) or disaccharides (rutinose or neohesperidose), and they are generally attached to the aglycone in positions 7 and 4′. The position of the interglycosidic bond (1 → 2 or 1 → 6) can be determined by a previously reported procedure that also provides a means to discriminate between flavanones and flavones derivatives (*26*).

**Compound 5.** The UV spectrum of compound **5** showed a hypsochromic shift on band I compatible with a 4′ substitution on an apigenin skeleton (*27*). The MS-MS focused on $[M + H]^+$ (*m*/*z* 741) showed fragments at *m*/*z* 595, 433, and 271 (aglycone) resulting from the successive loss of a rhamnose and two glucose units, typical of a tri-*O*-glycosyl flavonoid. To determine the interglycosidic bond position, the procedure described in the guidelines cited previously was applied (*26*). The higher relative abundance of the *m*/*z* 433 (100) fragment, with respect to the *m*/ *z* 579 (unobserved) and *m*/*z* 595 (14) ones, suggested a *O*-neohesperidoside (L-rha-(1 → 2)-D-glu) as the disaccharide component and demonstrated also that the aglycone possesses a flavone skeleton. Flavone tri-*O*-glycosides in the *Citrus* species generally occur as glucosyl-neohesperidosyl or glucosyl-rutinosyl derivatives, with the disaccharide linked to the 7 position. Therefore, compound **5** was identified as apigenin 7-*O*-neohesperidoside-4′-*O*-glucoside, which was found for the first time in bergamot juice.

**Compound 6.** UV spectroscopic data for compound **6** are consistent with the presence of a chrysoeriol/diosmetin aglycone with a hypsochromic shift of band I (ca. 10 nm) caused by a substituent in position 4′. According to the previous guidelines (*26*), evaluation of the relative intensities of peaks at *m*/*z* 463 (100), 609 (13), and 625 (18) in the positive mode MS-MS spectrum, obtained by focusing on the pseudomolecular ion *m*/*z* 771, showed the presence of a glucose unit along with a disaccharide (neohesperidoside) on a flavone skeleton. In line with literature on the *Citrus* species, the disaccharide substitution was assigned to position 7 and the glucose substitution at position 4′.

Negative mode MS-MS focused on *m*/*z* 299 showed the loss of a methyl group (*m*/*z* 284 (100)) from the pseudomolecular ion *m*/*z* 299 (18) of the aglycone. MS-MS spectrum of a chrysoeriol standard in the same experimental conditions presented a similar fragmentation pattern (*m*/*z* 284 (100), *m*/*z* 299 (20), whereas a diosmetin standard showed a $[M - H]^-$ peak with a relative abundance of only 1%. This behavior can be explained by considering that a methoxyl group in the 4′ position (as in diosmetin) is more prone to loss of the methyl moiety with respect to the isomeric derivative bearing the -OMe substituent in the 3′ position since the resulting radical is more stabilized in the former case. On the basis of this evidence, compound **6** can be identified as chrysoeriol 7-*O*-neohesperidoside-4′-*O*-glucoside, undiscovered up to now in bergamot juice.

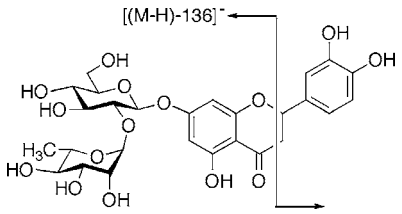



**Figure 3.** Retrocyclization involving the aglycone of neoeriocitrin (compound **9**).

As a general rule, we suggest comparing the relative abundance of the pseudomolecular $[A - H]^-$ ion with respect to the $[A - H - CH_3]^{\bullet -}$ peak, in MS-MS spectra focused on the $[A - H]^-$ fragment, as a convenient method to distinguish between isomeric chrysoeriol/diosmetin compounds.

**Compounds 7 and 9.** The DAD UV spectrum of compound **7** showed the flavanone nature of the aglycone. MS-MS in positive mode focused on *m*/*z* 597 $[M + H]^+$ presented several fragments derived from the subsequent loss of water, as already reported in the literature (*26*). The presence of a disaccharide substituent was highlighted by the presence of $[(M + H) - 146]^+$, $[(M + H) - 162]^+$, and $[(M + H) - 308]^+$ fragments. According to the guidelines (*28*), given that the base peak is the $[(M + H) - 162]^+$ fragment, it was possible to assign a flavanone skeleton to the aglycone of compound **7**. Negative mode MS-MS focused on *m*/*z* 595 ($[M - H]^-$) allowed the identification of the 1 → 6 interglycosidic bond position: the most abundant peak was *m*/*z* 287, which confirmed the loss of a disaccharide from the pseudomolecular ion, and the absence of a $[(M - H) - 120]^-$ peak demonstrated that the disaccharide is rutinose. By comparison of the spectroscopic data of this compound with those for standard eriocitrin (which is coeluted with **7**), compound **7** is identified as eriocitrin, in agreement with literature data (*15*).

The MS-MS spectrum in positive mode, focused on *m*/*z* 597 ($[M + H]^+$) for compound **9**, was almost identical to the one for the isomeric compound **7** thus confirming the flavanone nature of **9**, which was also evident from the DAD UV spectrum. Negative mode MS-MS spectrum, focused on *m*/*z* 595, $[M - H]^-$, presented an unexpected $[(M - H) - 136]^-$ fragment.

Inspection of the pseudomolecular ion $[M - H]^-$ of component **9** by selected reaction monitoring (SRM) showed, along with a fragmentation that produced the peak $[(M - H) - 136]^-$ (100), an alternative pathway that led to the loss of the entire disaccharide (*m*/*z* 287 $[(M - H) - 308]^-$). The resulting peak, however, had a much lower intensity (8). Compound **9** coelutes with standard neoeriocitrin, and the spectroscopic data coincide. Therefore, **9** is identified as neoeriocitrin, in agreement with literature data that indicate neoeriocitrin as one of the three major flavanone glycosides found in bergamot *Citrus* along with naringin and neohesperidin (*14*, *15*).

The appearance of the $[(M - H) - 136]^-$ fragment, which was observed in compound **9** (a neohesperidoside) but not in compound **7** (a rutinoside), could be explained with a fragmentation pathway alternative to the sugar fragmentation, which would have led to the expected $[(M - H) - 120]^-$, a fragment typical of 1 → 2-linked disaccharides (*26*). We suggest that the loss of 136 mass units is the consequence of a retrocyclization involving the aglycone as shown in **Figure 3**.

**Compound 12.** The UV spectrum of compound **12** was compatible with an apigenin aglycone. The absence of shift for the $\lambda_{max}$ (337 nm, band I) indicated a free hydroxyl group in position 4′. Negative mode MS-MS focused on the *m*/*z* 577 ion showed the presence of a disaccharide with $M_w$ 308 on an

apigenin skeleton, which is composed of a rhamnose unit ([(M − H) − 146]$^-$) connected to a glucose unit ([(M − H) − 162]$^-$) via a 1 → 2 glycosidic bond (confirmed by the typical presence of a [(M − H) − 120]$^-$ fragment from neohesperidosides). Since the disaccharide can be considered linked to position 7, compound **12** has been identified as apigenin 7-*O*-neohesperidoside (rhoifolin), which has already been found in edible parts of bergamot (*15*).

**Compound 13.** The UV spectrum showed the flavanonic nature of compound **13**. Negative ion MS-MS focused on the [M − H]$^-$ ion highlights the presence of a neohesperidose disaccharide (1 → 2 linkage). Standard naringin coeluted with **13** and possesses spectroscopic data highly consistent with compound **13**, which can thus be identified as naringin (*14*, *15*).

**Compounds 14 and 15.** The UV spectra of isomeric compounds **14** and **15** were both compatible with a chrysoeriol/diosmetin aglycone. For both these compounds, MS-MS in positive mode demonstrated the presence of a disaccharide composed of rhamnose and glucose because of the consecutive loss of 146 and 162 mass units. The nature of (1 → 2) interglycosidic linkage can be suggested by the evidence that the [(M + H) − 308]$^+$ fragment is much more abundant than the [(M + H) − 146]$^+$ fragment in the MS-MS spectra of compounds **14** and **15** (*26*).

The different nature of the aglycones can be confirmed by negative mode MS-MS focused on the *m*/*z* 299 [A − H]$^-$ ion: in the case of compound **14**, as opposed to **15**, it was possible to observe a greater abundance of the [A − H]$^-$ fragment with respect to [(A − H) − 15]$^{\bullet -}$ fragment. This behavior was also observed for standard chrysoeriol and diosmetin, as discussed previously. Moreover, standard neodiosmin coeluted with **15**, and their spectral data are highly consistent. Therefore, compound **14** is identified as chrysoeriol 7-*O*-neohesperidoside, whereas compound **15** was identified as diosmetin 7-*O*-neohesperidoside (neodiosmin). Compound **14** was identified in bergamot juice for the first time, whereas the presence of neodiosmin (compound **15**) in the edible part of bergamot has been reported previously (*15*).

**Compound 16.** The UV spectrum of compound **16** indicated the flavanone nature of the aglycone. Fragmentation in the negative mode MS-MS spectrum (focused on *m*/*z* 609) showed the typical pattern for a 1 → 2-linked rhamnose-glucose disaccharide. Standard neohesperidin presents very similar spectroscopic data and coelutes with compound **16**. Compound **16** was therefore identified as neohesperidin in accordance with the literature data (*14*, *15*).

**Quantitative Evaluation.** It is well-known that neoeriocitrin, naringin, and neohesperidin are the flavanone-*O*-glycosides to be found in the highest amounts in bergamot juice (257.0−295.8, 248.1−274.6, and 206.6−235.7 mg/L, respectively) (**Table 3**). The concentration of compounds determined in this paper agrees well with literature values (*14*, *15*). Eriocitrin was also found (13.4−15.6 mg/L), as expected, and in accordance with previous reports (*15*) dealing with the edible parts of bergamot.

Rhoifolin (53.2−68.1 mg/L) and neodiosmin (19.0−27.1 mg/L) were found, and again, this confirms previous determinations for the edible parts of bergamot (*15*). Two chrysoeriol derivatives, chrysoeriol 7-*O*-neohesperidoside-4′-*O*-glucoside and chrysoeriol 7-*O*-neohesperidoside, were found in considerable amounts (11.6−13.2 and 42.1−55.7 mg/L, respectively). Rhoifolin 4′-*O*-glucoside, an apigenin derivative, was identified for the first time. A small amount (7.3−8.9 mg/L) was found to be present. It should be mentioned that, in the absence of flavone-*O*-glycoside standards, these amounts were determined by employing a neodiosmin calibration curve.

**Table 3.** Flavone *C*-Glucosides (**1**–**4**, **8**, **10**, and **11**), Flavone *O*-Glycosides (**5**, **6**, **12**, **14**, and **15**), and Flavanone *O*-Glycosides (**7**, **9**, **13**, and **16**)

| compound | structure assignment | mg/L[a] |
|---|---|---|
| **1** | lucenin-2 | 6.4–7.5 |
| **2** | vicenin-2 | 58.3–66.2 |
| **3** | stellarin-2 | 5.8–7.3 |
| **4** | lucenin-2 4′-methyl ether | 37.9–49.7 |
| **5** | rhoifolin 4′-glucoside | 7.3–8.9 |
| **6** | chrysoeriol 7-*O*-neohesperidoside-4′-glucoside | 11.6–13.2 |
| **7** | eriocitrin | 13.4–15.6 |
| **8** | isovitexin | 4.5–5.3 |
| **9** | neoeriocitrin | 257.0–295.8 |
| **10** | scoparin | 7.2–7.9 |
| **11** | orientin 4′-methyl ether | 7.6–8.8 |
| **12** | rhoifolin | 53.2–68.1 |
| **13** | naringin | 248.1–274.6 |
| **14** | chrysoeriol 7-*O*-neohesperidoside | 42.1–55.7 |
| **15** | neodiosmin | 19.0–27.1 |
| **16** | neohesperidin | 206.6–235.7 |

[a] Contents (mg/L) as range values of bergamot juice.

The presence of vicenin-2 (58.3−66.2 mg/L) and lucenin-2 4′-methyl ether (37.9−49.7 mg/L) is consistent with the nature of bergamot, which is a hybrid of orange and lemon since 6,8-di-*C*-glucosyl apigenin (vicenin-2) is quite abundant in orange juice, while diosmetin 6,8-di-*C*-glucoside (lucenin-2 4′methyl ether) is characteristic of lemon juice (*21*). Concentrations of lucenin-2, stellarin-2, isovitexin, scoparin, and orientin 4′-methyl ether, found for the first time in bergamot juice, are all below 10 mg/L. For the determination of flavone-*C*-glycosides, the calibration curve was obtained from diosmetin-6,8-di-*C*-glucoside that we had previously extracted from lemon juice (*13*).

The LC-MS-MS method is highly selective and sensitive for the identification of phenolic components in *C. bergamia* juice, even at very low concentrations. The coupled UV MS (negative and positive) analysis on the crude juice provides a complete investigation for all of the peaks of the chromatogram. MS-MS experiments allow a complete structural elucidation for all the investigated species. By employing this methodology, it has been possible to identify for the first time in bergamot juice five *C*-glucosides (lucenin-2, stellarin-2, isovitexin, scoparin, and orientin 4′-methyl ether) along with three *O*-glycosides (rhoifolin 4′-*O*-glucoside, chrysoeriol 7-*O*-neohesperidoside-4′-*O*-glucoside, and chrysoeriol-7-*O*-neohesperidoside).

This detailed description of the profile of flavonoid components in bergamot juice, which includes derivatives present in very low concentrations, demonstrates the presence of an interesting richness of compounds possessing efficient antioxidant ability. Furthermore, the abundance of flavonoid constituents could lead to a possible increase in the use of this juice in the food industry since bergamot juice is even richer than lemon or orange juice (*12*, *13*).

## ABBREVIATIONS USED

A, aglycone; SRM, selected reaction monitoring.

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