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Detailed Phytochemical Characterization of Bergamot Polyphenolic Fraction (BPF) by UPLC-DAD-MS and LC-NMR

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Supporting Information

ABSTRACT: Bergamot (*Citrus bergamia*) is cultivated in Southern Italy almost exclusively to produce the prized essential oil, a top note in several perfumes. The juice of bergamot, until recently poorly studied, is the object of a growing scientific interest due to its claimed activity to treat metabolic syndrome. The aim of this investigation was a detailed characterization of bergamot juice polyphenolic fraction (BPF) based on a UPLC-DAD-MS analysis complemented by preparative chromatographic separations, followed by NMR characterization of the isolated compounds. The combination of these techniques efficiently covered different classes of secondary metabolites, leading to the identification of 39 components, several of which had never been reported from bergamot. One of them, bergamjuicin (**35**), is a new flavanone glycoside, whose structure has been determined by MS and NMR techniques. The reported results could provide a guide for future routine analyses of BPF, a material of great nutraceutical and industrial interest.

KEYWORDS: *Citrus bergamia, bergamot polyphenolic fraction, NMR analysis, flavanones, limonoids, metabolic syndrome*

INTRODUCTION

Bergamot (*Citrus bergamia* Risso), a small tree belonging to the family Rutaceae and subfamily Esperidea, is defined as a natural hybrid between bitter orange (*Citrus aurantium* L.) and lemon [*Citrus limon* (L.) Burm. f.], or a mutation of the latter. The plant, presenting big leaves similar to those of lemon, blossoms during the winter, and gives white flowers and then round yellow fruits having the size of an orange. Bergamot has been known in the Mediterranean area for several centuries but, currently, the Italian bergamot production is limited to the Calabria region (Southern Italy) and, because the plant is very sensitive to the pedoclimatic conditions of the soil, it grows almost exclusively in the southern coastal area from Reggio Calabria to Locri. This cultivation area amounts to about 1500 ha and contributes over 95% of bergamot world production of essential oil,[1] obtained from rind of the bergamot fruit and widely used in the cosmetic industry. The scent of bergamot essential oil has been defined as a mixture of sweet light-orange oil and floral aroma, and this peculiarity made it a top note in prized perfumes, such as the Eau de Cologne composed by Farina at the beginning of the 18th century. In the last decades, the development of synthetic surrogates of bergamot essential oil has inevitably contributed, together with other reasons, to a significant decrease in the commercial demand for bergamot oil.

Until recently, the other parts of the bergamot plant had been almost completely neglected and considered only as waste products of the essential oil industry, representing an environmental and economic problem more than a resource. Not surprisingly, among the about 1000 papers/patents present in the scientific literature on *Citrus bergamia*,[2] the vast majority investigated the phytochemical composition (dominated by limonene, linalool, and linalyl acetate),[3] the antioxidant potential, improvement of cognitive functions, and anti-inflammatory and antiproliferative activities exerted by its essential oil.[4] Probably due to the scarce commercial attention shown by the fruit industry, the phytochemical profile of bergamot fruit in the form of juice and derived extracts is probably the least studied among all the citrus species. However, in recent years, the situation is rapidly changing and the scientific community has been evidencing the marked antioxidant activity and the interesting nutraceutical potential of bergamot juice. Hypolipemic, hypoglycemic, and anti-inflammatory activities and, more generally, effectiveness in the treatment of metabolic syndrome symptoms have been claimed.[5−7] In particular, a bergamot enriched polyphenolic fraction (BPF), obtained from the peeled fruit, has been documented in several trials for its lipid-lowering effects associated with significant reduction of cardiovascular damage.[8,9] Recently, a lecithin food-grade delivery system (Phytosome) has been applied to BPF, achieving higher bioavailability and better absorption of the dispersed state of the phytocomplex (Vazguard).

These encouraging premises have stimulated a number of phytochemical characterizations of bergamot juice, resulting in the classification of its main components into three structural classes: (i) flavanone glycosides, (ii) flavone *C*- and *O*-

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glycosides, and (iii) polymethoxyflavones.[10] Compared to other citrus fruits, bergamot fruit juice is characterized by relatively higher amounts of metabolites belonging to the first class: along with neoeriocitrin (**1**), naringin (**2**), and neohesperidin (**3**), a couple of flavanone glycosides showing an ester linkage between a sugar unit and 3-hydroxy-3-methylglutaric acid (HMGA), named melitidin (**4**) and brutieridin (**5**),[11] are among the major components of the juice.

The majority of the analytical profiles of bergamot fruit preparations available to date have been outlined by means of HPLC/mass spectrometry (MS) techniques employing RP18 columns packed with conventional particles. This approach, while allowing highly sensitive and rapid analyses, often fails to identify some metabolites contained in the vegetal matrix even in abundant amounts. On the other hand, preparative chromatographic purification followed by NMR identification has been rarely applied and only to selected classes of compounds such as furanocoumarins[12] and limonoids.[13]

Although preliminary profile of the polyphenolic fraction of BPF has been recently reported by Salerno et al.,[14] a detailed characterization of bergamot fruit preparations is still lacking. This constituted the rationale for the present investigation, in which a metabolic profile of BPF has been obtained by UPLC-DAD-MS combined to a preparative chromatography using two complementary stationary phases (semipreparative RP-18 column and Sephadex LH-20), followed by NMR-based identification of the isolated metabolites. This combined approach provided the most comprehensive description of bergamot juice/BPF available to date, disclosing the presence of several compounds never reported before for bergamot, one of which, named bergamjuicin (**35**), was unprecedented in the scientific literature. The structural elucidation of this compound will be also described here.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations ($CHCl_3$) were measured at 589 nm on a P2000 Jasco (Dunmow, UK) polarimeter. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Varian INOVA spectrometer. Column chromatography was performed on Sephadex LH-20 column (Pharmacia, Uppsala, Sweden). RP-HPLC-UV−vis separations were performed on an Agilent instrument, using a 1260 Quat Pump VL system, equipped with a 1260 VWD VL UV−vis detector, a Supelco Ascentis C18, 5 μ 10 mm × 250 mm column, and a Rheodyne injector. HPLC-RI separations were performed on a Knauer (Berlin, Germany) 1800 apparatus equipped with a refractive index detector. LUNA (normal phase, SI60, or reverse-phase RP18, 250 mm × 4 mm) (Phenomenex) columns were used with 0.8 mL/min as flow rate and isocratic elution with methanol/water mixtures at room temperature. Thin-layer chromatography (TLC) was performed on plates coated with silica gel 60 F254 Merck, 0.25 mm

Sample Preparation. Bergamot juice (BJ) was obtained from both hand-squeezed fresh fruits and from peeled-off fruits by industrial pressing and squeezing; samples were analyzed after filtration at 0.45 μm with a disposable PVDF filter. Bergamot polyphenolic fraction (BPF) (H&AD, Polistena, RC, Italy) was obtained from bergamot (peeled-off fruit) industrial juice as already described.[6] Briefly, bergamot juice was clarified by ultrafiltration, loaded on a polystyrene resin column (Mitsubishi), eluted by a mild KOH solution (pH = 8.5), and neutralized by filtration on cationic resin at acidic pH. Finally, it was vacuum-dried and minced to the desired particle size to obtain BPF powder. Methanol solutions (2.5 and 10 mg/mL) of BPF were prepared for UPLC-DAD-UV and UPLC-DAD-MS analysis, respectively. Both solutions were filtered at 0.45 μm with disposable PTFE filters before injection.

UPLC-DAD-MS Analysis. UPLC-DAD-UV were performed on a Waters (Milford, MA, USA) Acquity H-class system equipped with a PDA detector, thermostated column compartment and Empower software; column Waters BEH C18 (100 mm × 2.1 mm, 1.7 μm particle size). Analysis conditions: flow rate 0.5 mL/min, injection volume 0.5 μL, column oven temperature 35 °C. UV detection at 285 nm. PDA spectra in the 190−600 nm range. The mobile phase consisted of a ternary system including (A) 0.2% V/V of HCOOH in H_2O, (B) CH_3CN, and (C) CH_3OH with the following gradient elution: starting conditions, A 83%, B 5% C 12%; 6.5 min, A 72% B 16% C12%; 7.5 min, A 72% B 16% C12%; 12 min, B 50% C 50%; 13 min, B 50% C 50%, 15 min, initial conditions. The total run time, including column wash and equilibration, was 16 min. For UPLC-MS^n experiments: Accela 1250 pump and Open Accela AS, LTQ-XL ion trap mass spectrometer (Thermo-Scientific, Waltham, MA, USA) equipped with an ESI interface and Excalibur data system. MS analysis was carried out in full scan mode, recording spectra both in positive and negative ions mode. Precursor ions $[M + H]^+$ and/or $[M - H]^-$ were isolated with an isolation width of 2.0 m/z units and fragmented using an activation amplitude of 35%. Operating parameters of the mass spectrometer were capillary temperature 350 °C; spray needle voltage set at 4.50 kV (positive) and 4.00 kV (negative); ES capillary voltage +47 and −49 V for positive and negative polarity, respectively. For HR-ESIMS experiments LTQ-Orbitrap mass spectrometer equipped with an ESI interface and Excalibur data system was used. HR-MS analysis was carried out in full scan mode operation at a resolution of 30000 recording MS spectra either in negative or positive mode. The instrument was calibrated by Thermo calibration solutions prior to beginning the analysis.

Chromatographic Purification. BPF was chromatographed on two different stationary phases (Sephadex LH-20 and RP-18) followed by HPLC purifications. All the pure compounds obtained with this procedure were analyzed by ^1H NMR.

Sephadex LH-20. A methanolic solution of an aliquot of BPF (6 g) was chromatographed in 2 g lots on a Sephadex LH-20 (Pharmacia) column, eluting isocratically with CH_3OH. Fractions of 20 mL were collected and analyzed by TLC using $BuOH/CH_3COOH/H_2O$ (60:15:25, v/v/v) as eluent. A total of 10 fractions (A−J) were thus obtained, and selected fractions were further purified by reverse-phase HPLC. Fraction D (157.2 mg) was chromatographed by HPLC (CH_3OH/H_2O, 50:50, flow rate 2.5 mL/min) to afford bergamjuicin (**35**, 6.7 mg, R_t 7.0 min) and nomilin acid glucoside (**39**, 5.7 mg, R_t 12 min). Fraction G (105 mg) was separated by semipreparative HPLC (CH_3OH/H_2O, 45:55, flow rate 2.5 mL/min) to afford pure eriocitrin (**7**, 18.8 mg, R_t 14 min), naringenin-7-*O*-β-glucoside (**9**, 9.8 mg, R_t 19 min), hesperetin-7-*O*-β-glucoside (**34**, 8.3 mg, R_t 22 min), demethoxycentaureidin-7-*O*-β-glucoside (**22**, 1.1 mg, R_t 23 min), and two subfractions (subfractions G13 and G14), whose further purification by analytical RP-HPLC ($MeOH/H_2O$ 50:50, flow rate 0.8 mL/min) yielded apigenin-6-*C*-glucoside (**16**, 1.0 mg, R_t 6.1 min), diosmetin-7-*O*-β-glucoside (**21**, 1.9 mg, R_t 7.6 min), and diosmetin-8-C-glucoside (**19**, 3.9 mg, R_t 8.6 min). Fraction H (65 mg), was separated by HPLC (CH_3OH/H_2O, 55:45, flow rate 1.0 mL/min) to afford pure eriodictyol (**31**, 12.4 mg, R_t 8.9 min), naringenin (**30**, 12.2 mg, R_t 14.1 min), and hesperetin (**32**, 10.3 mg, R_t 15.3 min).

RP-18 HPLC. Another sample of BPF (500 mg) was separated by semipreparative RP-18 HPLC performed on an Agilent instrument, using Supelco Ascentis C18, 5 μm 250 mm × 10 mm column. The mobile phase was a mixture of A, water−formic acid (99.8:0.2, v/v), B, acetonitrile, and C, methanol with a gradient programmed as follows: starting conditions, A 75% B 15% C 10%; 20 min, A 50% B 50%; 30 min, A 75% B 15% C 10%. The injected volume was 500 μL, and flow rate was 2.5 mL/min. The UV detection wavelength was set at 325 nm. This separation afforded 12 fractions (A−L). Fraction A was identified as apigenin-6,8-di-C-glucoside (**12**, 8.5 mg, R_t 7.6 min), fraction B was identified as neoeriocitrin (**1**, 31 mg, R_t 11.3 min), fraction C was identified as naringin (**2**, 49.8 mg, R_t 12.3 min), fraction E was identified as neohesperidin (**3**, 30.1 mg, R_t 13.6 min), fraction G was identified as brutieridin (**5**, 26 mg, R_t 18.7 min), fraction H was identified as eriodictyol-7-*O*-β-glucoside (**33**, 3.9 mg,

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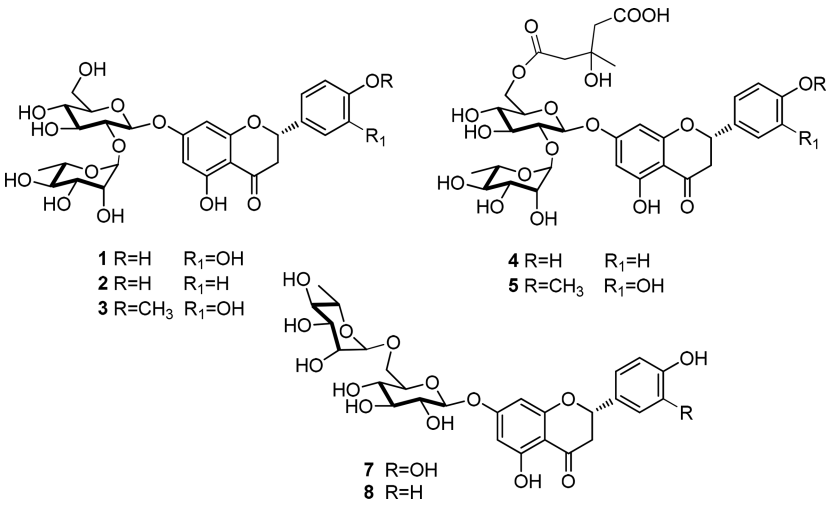

Figure 1. Chemical structure of the main flavanones of BPF.

R_t 19.7 min), fraction J was identified as naringenin (**30**, 3.8 mg, R_t 23.2 min), and fraction L was identified as nomilinic acid (**38**, 4.3 mg, R_t 29.2 min). A series of fractions needed a further purification on analytical HPLC. Fraction D was rechromatographed by RP-18 HPLC-UV using CH_3OH/H_2O (30:70, flow rate 0.8 mL/min) to yield naringin-4′-*O*-*β*-glucopyranoside (**6**, 1.3 mg, R_t 6.1 min), diosmetin-7-*O*-neohesperidoside (**23**, 1.2 mg, R_t 6.5 min), diosmetin-6,8-di-*C*-glucoside (**14**, 1.3 mg, R_t 11.2 min), 2-*O*-*β*-glucopyranose-2-hydroxy-4-methoxyhydrocinnamic acid (**36**, 1.3 mg, R_t 11.5 min), and melitidin (**4**, 5.9 mg, R_t 27.1 min). Fraction F was separated by RP-HPLC-UV using CH_3OH/H_2O (25:75, flow rate 0.8 mL/min) to yield chrysoeriol-8-*C*-glucoside (**17**, 1.0 mg, R_t 12.5 min) and diosmetin-8-*C*-glucoside (**19**, 0.9 mg, R_t 16.3 min). Fraction I was separated by normal phase HPLC with *n*-hexane/EtOAc (50:50 flow rate 0.8 mL/min) to yield compounds eriodictyol (**31**,1.1 mg, R_t 7.5 min) and sinensetin (**25**, 0.6 mg, R_t 16.3 min). Fraction K was separated by normal phase HPLC with *n*-hexane/EtOAc (40:60 flow rate 0.8 mL/min) to yield bergaptene (**28**, 0.5 mg, R_t = 6.5 min) and limonin (**37**, 1.2 mg, R_t = 16.3 min).

Bergamjuicin (35). Colorless amorphous solid. $[\alpha]_D$ = −31.8 (*c* 0.7, MeOH). ^{1}H and ^{13}C NMR data: see Table 4. ESIMS (negative ions): *m*/*z* 885 $[M - H]^-$. HR-ESIMS: *m*/*z* 885.2662 (calcd for $C_{39}H_{49}O_{23}$ 885.2665).

RESULTS AND DISCUSSION

In this study, we have investigated the bergamot-derived polyphenolic fraction (BPF), obtained from bergamot industrial juice after clarification by ultrafiltration (UF), removing the suspended solids, followed by purification on a polystyrene resin column eluted with a mildly basic solution, achieving removal of free sugars and small organic acids. This standardized procedure[6] results in a marked enrichment in polyphenols of the obtained powder without affecting their composition. The almost complete superimposability between the polyphenolic relative composition of BPF and that of filtered juice has been verified by comparing the UPLC profiles of these two matrices (see Supporting Information (SI)). It has been estimated[6] that 6 g of BPF (the amount used for chromatographic purification, see below) correspond to 1.0 L of bergamot juice in terms of flavonoid content. Very similar composition was also shown by hand-squeezed fresh fruits and from industrial juices used for BPF preparation (see SI, Figure S3).

UPLC-DAD-MS Analysis. UV chromatograms were recorded at 285, 340, and 310 nm in order to cover the absorption maxima of the main classes of secondary metabolites reported as bergamot juice components. UPLC conditions allowed a marked reduction of analytical times (total run time was 16 min) compared to other standard analyses: a crucial factor to manage large batch of samples but maintaining high separation efficiency. Identification of compounds reported in Tables 1−3 was based on HRMS data and MS/MS fragmentation pattern.

UV detection at λ_{max} = 285 nm, optimal to detect flavanones (Figure 1), gave the profile reported in Figure 2. Ten peaks were selected on the basis of their UV absorption maxima characteristic of the flavanone ring system. On the basis of UV, HRMS, and MS^n data, the major peaks could be easily assigned to the five 7-*O*-neohesperidoside flavanones neoeriocitrin (**1**), naringin (**2**), neohesperidin (**3**), melitidin (**4**), and brutieridin (**5**) (Table 1). In the case of **1**−**3**, this assignment was also supported by coinjection with pure standards available in our laboratories. Much lower amounts of the 7-*O*-rutinoside analogues eriocitrin (**7**) and narirutin (**8**), already reported as minor constituents of the bergamot juice,[10,12] could be also identified. The additional peaks nos. 6 and 9, also showing UV absorption maxima around λ_{max} = 283, were tentatively assigned to a monoglycosylated naringin and a monoglycosylated naringenin, respectively, although the linkage position of the additional sugar unit could not be deduced by MS data. On the other hand, on the basis of the available data, we could not formulate a likely structural hypothesis for peak no. 10 (MW = 636, $C_{29}H_{32}O_{16}$).

UV detection at λ_{max} = 340 nm, selected to detect flavones, gave the profile reported in Figure 2. Bergamot juice has been already reported to contain relatively large amounts of flavone-*O*-glycosides (generally *O*-7), flavone-*C*-glycosides (C-6 or C-8, or both), and polymethoxyflavones.[10,12,15] Flavone aglycones are apigenin, luteolin, chrysoeriol, and diosmetin, the two last compounds differing for the *O*-methylation position (3′ and 4′, respectively). Although UV and MS data are unable to give information for the assignment of these isomeric aglycones, literature data indicate that peaks with lower RT (higher polarity) can be tentatively attributed to chrysoeriol derivatives.[16] Gattuso et al. have proposed that this effect is due to the different tendency to form hydrogen bonds with the glycosyl moiety.[16] Similarly, flavone-8-*C*-monoglycosides are described as eluting earlier than the corresponding 6-*C*-

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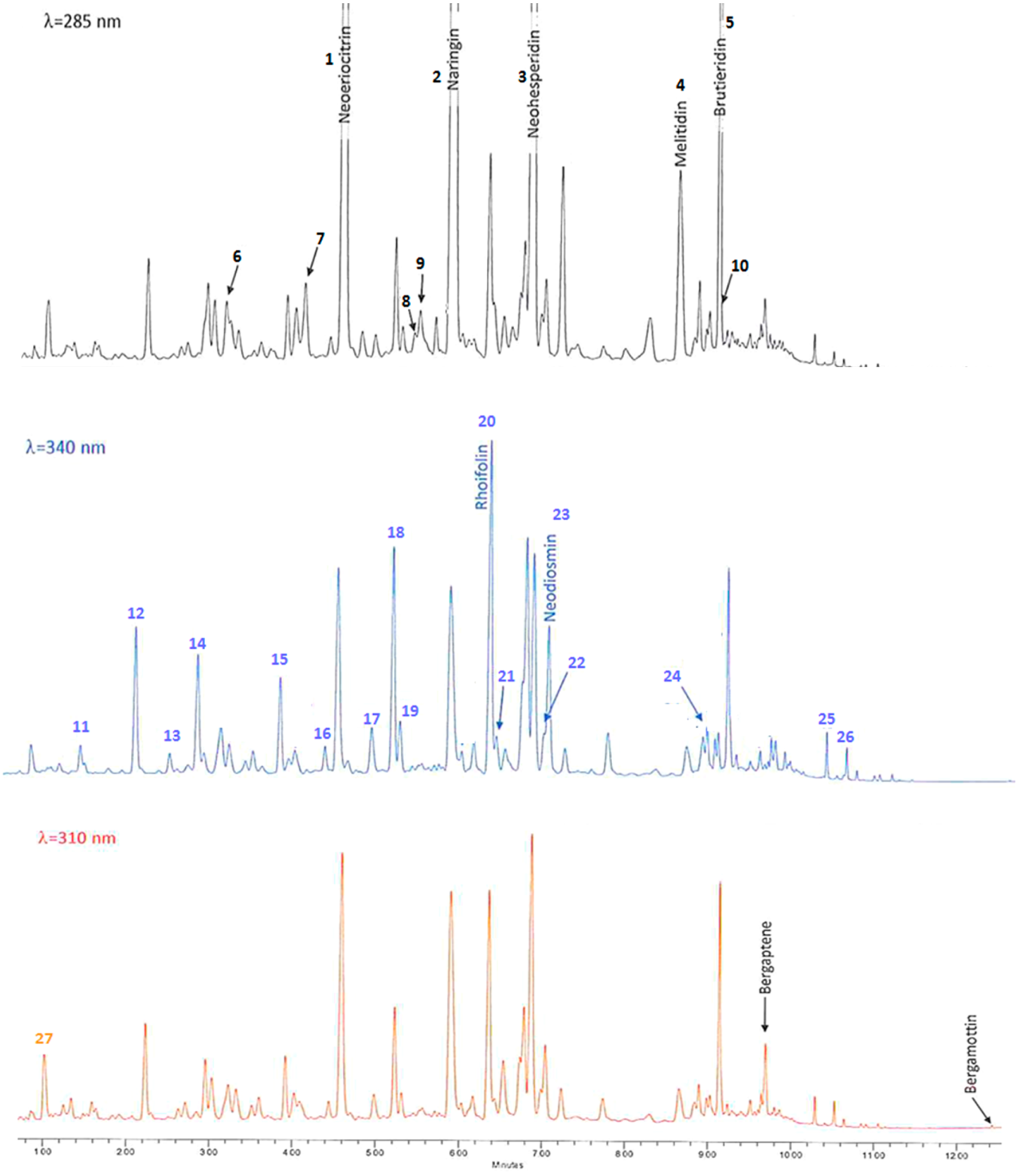

Figure 2. DAD chromatograms of BPF at 285 nm (top), 340 nm (middle), and 310 nm (bottom).

monoglycosides.[16] Our analysis revealed 16 members of this class (Table 2), and UV, HRMS, and MS^n data gave information to propose a structural assignment for the majority of them. Eight out of the 16 peaks were attributed to flavone-*C*-glycosides (**11**−**17** and **19**, Figure 3), with apigenin and diosmetin being the most abundant aglycones (Table 2). To our knowledge, vitexin (**15**, apigenin-8-*C*-glucoside) had never been reported before for bergamot juice.

Four peaks were assigned to flavone-*O*-glycosides, three of which being 7-*O*-neohesperidoside derivatives of luteolin (**18**), apigenin (**20**), and diosmetin (**23**) (Figure 3). The assignment of the two last compounds was unambiguously supported by coinjection of available pure standards. Compound **21** could

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Table 1. UPLC-DAD-MS Data of Flavanones in BPF

compd	ret time (min)	$[M - H]_{obs}$	$[M - H]_{calc}$	molecular formula	diagnostic MS^n (m/z)	UV maxima (nm)	structural assignment
1	4.75	595.1666	595.1668	$C_{27}H_{32}O_{15}$	(+) 579, 451, 289	283.9, 227.0	neoeriocitrin
2	5.88	579.1717	579.1719	$C_{27}H_{32}O_{14}$	(+) 563, 435, 273	282.7, 227.0	naringin
3	6.92	609.1822	609.1825	$C_{28}H_{34}O_{15}$	(+) 593, 465, 303	283. 9, 228.2	neohesperidin
4	8.73	723.2148	723.2142	$C_{33}H_{40}O_{18}$	(−) 621, 579, 271	282.7, 225.8	melitidin
5	9.20	753.2243	753.2248	$C_{34}H_{42}O_{19}$	(−) 691, 609, 301	283.9. 227.0	brutieridin
6	3.05	741.2248	741.2242	$C_{33}H_{42}O_{19}$	(−) 579, 459, 271	285.1, 222.3	*not assigned*
7	4.01	595.1670	595.1668	$C_{27}H_{32}O_{15}$	(+) 579, 451, 289	283.9	eriocitrin
8	5.77	579.1721	579.1719	$C_{27}H_{32}O_{14}$	(+) 419, 273	281.5, 223.4	narirutin
9	5.82	433.1133	433.1134	$C_{21}H_{22}O_{10}$	(−) 271	282.7, 223.4	*not assigned*
10	9.22	635.1616	635.1612	$C_{29}H_{32}O_{16}$	(−) 329, 314, 299	335.2, 283.9	*not assigned*

Table 2. UPLC-DAD-MS Data of Flavones in BPF

compd	ret time (min)	$[M - H]_{obs}$	$[M - H]_{calc}$	molecular formula	diagnostic $MS^n(m/z)$	UV maxima (nm)	structural assignment
11	1.66	609.1451	609.1456	$C_{27}H_{30}O_{16}$	(−) 519, 489, 399	341.0, 216.4	luteolin-6,8-di-*C*-glucoside
12	2.04	593.1518	593.1512	$C_{27}H_{30}O_{15}$	(−) 503, 473, 353	334.0, 270.8	apigenin-6,8-di-*C*-glucoside
13	2.55	623.1621	623.1618	$C_{28}H_{32}O_{16}$	(−) 503, 413, 383	347.9, 270.8	chrysoeriol-6,8-di-*C*-glucoside
14	2.82	623.1622	623.1618	$C_{28}H_{32}O_{16}$	(−) 503, 413, 383	346.7, 254.2	diosmetin-6,8-di-*C*-glucoside
15	3.90	431.0988	431.0984	$C_{21}H_{20}O_{10}$	(−) 311, 283	335.2, 268.4	apigenin-8-*C*-glucoside
16	4.33	431.0982	431.0984	$C_{21}H_{20}O_{10}$	(−) 311, 283	335.2, 269.6	apigenin-6-*C*-glucoside
17	5.11	461.1092	461.1089	$C_{22}H_{22}O_{11}$	(−) 341	346.7, 254.2	chrysoeriol-8-*C*-glucoside
18	5.26	593.1509	593.1512	$C_{27}H_{30}O_{15}$	(−) 477, 285	347.9, 254.2	luteolin-7-*O*-neohesperidoside
19	5.31	461.1090	461.1089	$C_{22}H_{22}O_{11}$	(−) 371, 341	347.9, 269.6	diosmetin-8-*C*-glucoside
20	6.39	577.1566	577.1563	$C_{27}H_{30}O_{14}$	(−) 269	336.4, 266.1	aigenin-7-*O*-neohesperidoside
21	6.41	461.1083	461.1089	$C_{22}H_{22}O_{11}$	(−) 299	346.7, 269.6	*not assigned*
22	7.01	491.1193	491.1190	$C_{23}H_{24}O_{12}$	(+) 331	334.0, 274.4	*not assigned*
23	7.06	607.1692	607.1688	$C_{28}H_{32}O_{15}$	(−) 299	346.7, 253.0	diosmetin-7-*O*-neohesperidoside
24	9.10	609.1828	609.1825	$C_{28}H_{34}O_{15}$	(−) 489, 343, 301	364.7, 285.1	rutin
25	10.30	373.1288[a]	373.1287[a]	$C_{20}H_{20}O_7$	(+) 358, 343, 315	341.0, 269.6	sinensetin
26	10.53	373.1290[a]	373.1287[a]	$C_{20}H_{20}O_7$	(+) 358, 343	330.5, 240.0	tangeretin

[a][M + H] data.

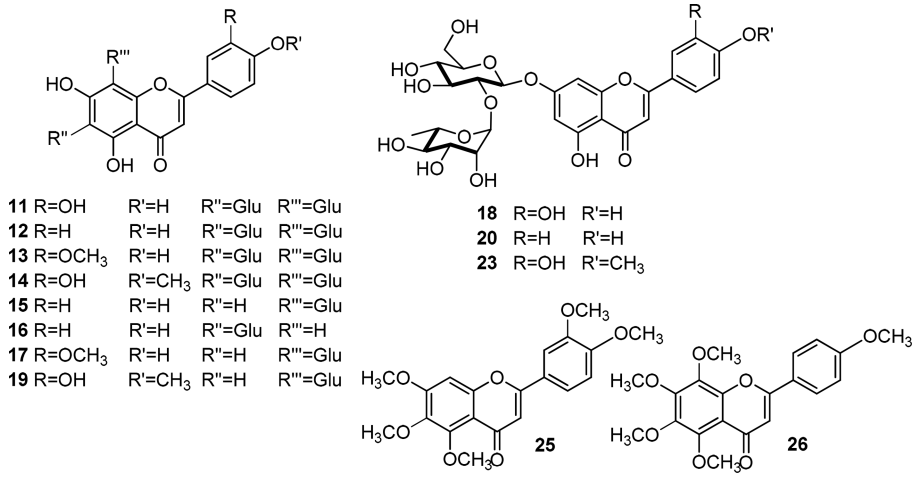

Figure 3. Chemical structures of the main flavones of BPF.

Table 3. UPLC-DAD-MS Data of Coumarins in BPF

compd	ret time (min)	$[M + H]_{obs}$	$[M + H]_{calc}$	molecular formula	diagnostic $MS^n(m/z)$	UV maxima (nm)	structural assignment
27	1.03	325.0922	325.0923	$C_{15}H_{16}O_8$			*not assigned*
28	9.77	217.0501	217.0500	$C_{12}H_8O_4$	(+) 202, 174	308.9, 250.6	bergaptene
29	12.42	339.1599	339.1596	$C_{21}H_{22}O_4$	(+) 203, 159	311.3, 267.3	bergamottin

be assigned as a monoglycosylated derivative of chrysoeriol or diosmetin. Because neither the nature of the aglycone nor the position of glycosylation can be inferred from the available data, we have left this peak unassigned. Similarly, compound **22** (MW 492), likely a monoglycosylated derivative of an aglycone with MW = 330, could not be assigned. Finally,

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compound **24** was identified as the flavonol glycoside rutin and compounds **25** and **26** as the two nonglycosylated flavones sinensetin and tangeretin, respectively. These two isomeric pentamethoxylated flavones, already reported as constituents of bergamot peel oil,[17] could be discriminated on the basis of their different UV profiles.

UV detection at λ_{max} = 310 nm, selected for coumarins, gave the profile reported in Figure 2 and led to the detection of three further peaks (Table 3). The furanocoumarins bergaptene (**28**) and bergamottin (**29**) were assigned by coinjection of standard compounds, while peak **27** (MW = 324), likely a glycosylated coumarin, could not be assigned.

In summary, the UPLC-DAD-MS analysis of bergamot polyphenolic fraction disclosed the presence of 29 compounds and allowed the assignment of the chemical structure for 23 of them. The remaining six compounds could not be unambiguously assigned, although structural hypotheses were formulated.

Chromatographic Purification and NMR Analysis. An intrinsic limitation of the UPLC-DAD-UV analysis is its unsuitability to reveal the presence of molecules lacking chromophores or absorbing far from the selected wavelengths even though they are present in discrete amounts. In addition, the UV detection cannot give reliable information about the relative abundance of the mixture components, unless suitable standards are available for all the compounds. Thus, the UPLC-DAD-MS analysis of BPF was complemented with an NMR-based investigation. This approach was intended first to support the identification of phenolic constituents postulated by means of UPLC-DAD-MS but also to allow the identification of compounds not assigned or not detected in the MS-based experiment.

BPF was chromatographed on two different stationary phases, C-18 reversed phase silica gel and Sephadex LH-20. These two phases can give complementary results, and their combination fulfills the needs of separating different classes of compounds with different glycosylation rates. The RP18 stationary phase efficiently separates based on the overall polarity of the components: thus, nonglycosylated analogues can be easily purified, but it is poorly able to separate mono- from di- and triglycosylated flavonoids. Conversely, the Sephadex LH-20 column, isocratically eluted with MeOH, mainly behaves as a size-exclusion chromatography, more efficiently separating compounds characterized by different glycosylation levels. In both cases, crude fractions obtained from the chromatographic columns were purified by HPLC on SI60 or RP18 stationary phases using refractive index (RI) detection.

As expected, the analyzed material resulted to be dominated by the flavanone neohesperidosides **1**−**5** ("the big five") and, based on the amount collected after the chromatographic purifications in the two procedures, we can estimate that these compounds account for at least 45% of BPF, distributed as illustrated in Figure 4.

Several other compounds, previously identified via UPLC-DAD-MS, were obtained in the pure form and their structural assignment was supported by comparison of experimental NMR data with those reported in the literature: eriocitrin (**7**),[18] narirutin (**8**),[19] luteolin-6,8-di-*C*-glucoside (**11**),[20] apigenin-6,8-di-*C*-glucoside (**12**),[21] diosmetin-6,8-di-*C*-glucoside (**14**),[21] apigenin-6-*C*-glucoside (**16**),[21] chrysoeriol-8-*C*-glucoside (**17**),[21] diosmetin-8-*C*-glucoside (**19**),[22] rutin (**24**),[19] sinensetin (**25**),[23] tangeretin (**26**),[24] and bergaptene (**28**).[12]

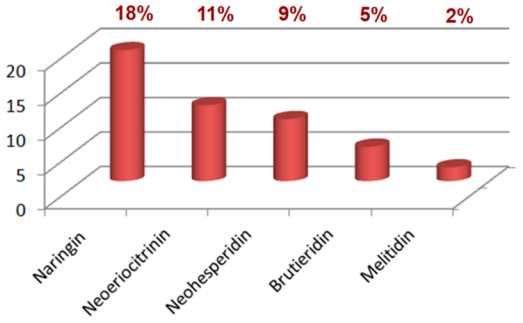

Figure 4. Percentage distribution of the five major flavanone glycosides in BPF.

Our attention was next attracted by the six compounds detected but not assignable on the basis of UV-MS available information. Some of them could be obtained in the pure form and were easily identified by analysis of their NMR data (Figure 5). Thus, compound **9** was identified as naringenin-7-*O*-*β*-glucopyranoside (prunasin)[25] and compound **6** as naringin-4′-*O*-*β*-glucopyranoside.[26] Both of these compounds had never been described before as constituent of bergamot fruit, although their presence had been detected in other *Citrus* species, e.g. *C. aurantium* and *Citrus sinensis*.[27] Compound **21** was identified as diosmetin-7-*O*-*β*-glucoside:[28] diosmetin can be easily discriminated by chrysoeriol on the basis of ring C resonances in the ^{1}H NMR spectrum.

Compound **22** (MW = 492) was assigned as demethoxycentaureidin-7-*O*-*β*-glucoside, a very rare 6-methoxylated analogue of **21**. This compound is not only unprecedented in *Citrus bergamia* but more generally in Rutaceae, and only a handful of plants have been reported to contain it.[29] Interestingly, the unassigned compound **10** (MW = 636) shares with **22** the same aglycone with MW = 330 (see Table 1) and differs for an additional 144 uma unit, which corresponds to an hydroxymethylglutaric acid residue. Although we did not succeed in its isolation, we can postulate that compound **10** is a HMG ester of **22** and will defer its isolation to future studies.

A significant result obtained by the combination of double chromatographic separation and NMR-based identification approach was the disclosure of 10 additional compounds that had not been detected via UPLC-DAD-MS, probably due to coelution problems (six additional flavanone derivatives) or to the lack of chromophores absorbing at the selected wavelengths (a phenylpropanoic derivative and three limonoids). The free flavanones naringenin (**30**), eriodictyol (**31**), and hesperetin (**32**) were obtained from the RP18 column and were easily identified by their NMR profiles. These nonglycosylated compounds could arise from the BPF preparation process, leading to the loss of sugar moieties. Moreover, the Sephadex LH-20 column efficiently separated two additional monoglycosylated flavanones, eriodictyol-7-*O*-*β*-glucoside (**33**) and hesperetin-7-*O*-*β*-glucoside (**34**), both never reported for bergamot, but found in other *Citrus* species.[30]

A pure triglycosylated flavanone with MW = 886 ($C_{39}H_{50}O_{23}$) was also obtained, which turned out to be an unprecedented compound for which we propose the trivial name bergamjuicin (**35**) (Figure 6).

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J. Agric. Food Chem. 2019, 67, 3159−3167

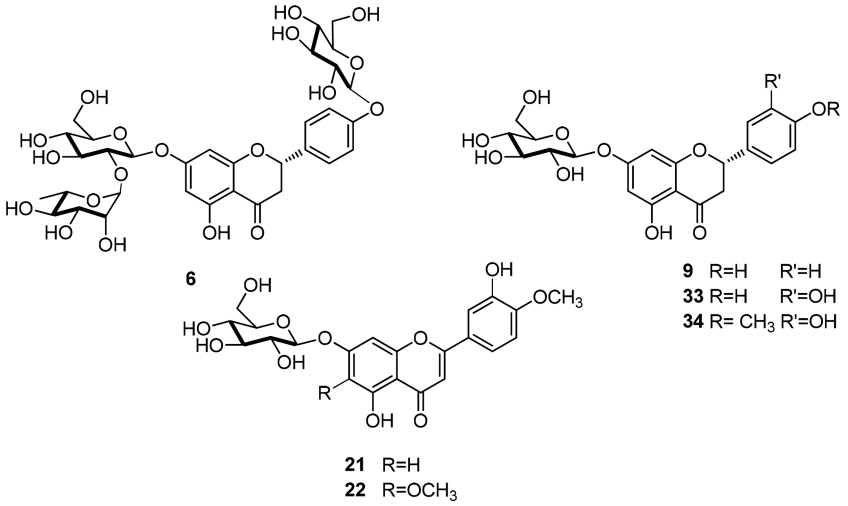

Figure 5. Chemical structures of flavanone and flavone derivatives identified through NMR analysis.

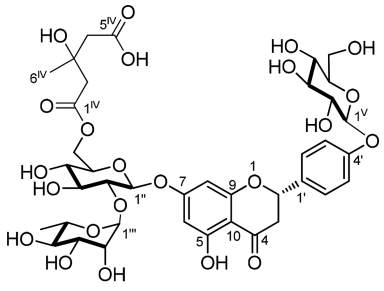

Figure 6. Chemical structure of the new flavanone derivative bergamjuicin (**35**).

The ^{1}H NMR spectrum of **35** (CD_3OD, Table 4) clearly evidenced the presence of the naringenin aglycone, highlighted by the characteristic peaks of rings C (a methine at δ_H5.49, and a diastereotopic methylene at δ_H3.14 and 2.81) and A (δ_H 6.14 and 6.17, both bs) and of the two 2H doublets of ring B. The midfield region of the ^{1}H NMR spectrum also contained three signals between δ_H 4.90 and 5.25, which, on the basis of the cross-peaks of the 2D NMR HSQC spectrum, were assigned to three anomeric protons (δ_H 4.94, δ_C 101.6; δ_H 5.08, δ_C 98.4; δ_H 5.24, δ_C 101.9), thus indicating the triglycosylated nature of **35**.

Inspection of 2D COSY and TOCSY NMR spectra allowed us to build up the three sugar spin systems, which, on the basis of the available proton−proton coupling constants and ^{13}C NMR values could be confidently assigned as two β-glucopyranosyl units and one α-rhamnopyranosyl. The remaining signals of the ^{1}H NMR spectrum of **35** were easily assigned (Table 4) to a 3-hydroxy-3-methylglutaryl moiety on the basis of the comparison of H/C NMR data with those of compounds **4** and **5**. Cross-peaks of the 2D NMR HMBC spectrum not only supported the structural assignment of these moieties but also provided key evidence to link them together. Thus, correlation H-1‴/C-2″ defined the neohesperidoside unit, while correlation H-1″/C-7 indicated its attachment at C-7. Ester linkage of the HMG unit at C-6″ was indicated by the relative downfield shift of H_2-6″ (δ_H 4.44 and 4.19) and unambiguously confirmed by the HMBC cross-peak H_2-6″/C-1^{IV}. Finally, the second β-glucopyranose unit was attached at C-4′ on the basis of the key HMBC cross-peak H-1^{V}/C-4′. Accordingly, glycosylation at C-4′ caused a significant downfield shift at H-2′ and H-3′ compared to parallel signal of reference compounds **4** and **5** (δ_H 7.44 and 7.14 in place of δ_H 6.96 and 6.70, respectively). Absolute configurations of the sugar moieties and of the naringenin aglycone were not determined experimentally, but they have been reported as those found in the other BPF compounds. Thus, bergamjuicin (**35**) is a new naringin derivative including both HMG esterification at C-6″ and a further glycosylation at C-4′ and adds to melitidin and brutieridin as HMG-esterified flavanone of bergamot.

Finally, concerning compounds lacking extended chromophores, and therefore not detected in the previous UPLC-DAD-MS analysis, relatively high amounts of **36** (MW 358, $C_{16}H_{22}O_9$) (Figure 7) were obtained from both Sephadex LH-20 and RP18 fractions and identified as 2-*O*-β-glucopyranose-2-hydroxy-4-methoxyhydrocinnamic acid. This phenylpropanoid derivative is absolutely unprecedented in plants of the Rutaceae family and has been isolated only from *Tagetes lucida* (Asteraceae),[31] *Gnidia polycephala* (Thymeleaceae),[32] and *Caesalpinia bonduc* (Fabaceae).[33] It is interesting to note that *Gnidia polycephala*, along with **36**, also contained skimmin, a glycosylated coumarin (umbelliferone glucoside) with MW = 324, corresponding to the unassigned compound **27**.

The less polar fractions obtained from RP18 contained the limonoid derivatives limonin (**37**), nomilinic acid (**38**), and its glycoside **39** (Figure 7). Nonglycosylated limonoids have been reported to be more abundant in seeds, while their glycosylated analogues show higher distribution in the juice.[13] Nonglycosylated limonoids, along with flavanones, contribute to the bitterness of the juice, but anticancer and antimicrobial activities, along with an activity on metabolic disease symptoms, have also been reported for these compounds.[13] It is interesting to notice that our finding that **37**−**39** are the main limonoids of bergamot juice, based on a LC-NMR approach on BPF, is in full agreement with recent results obtained by Russo et al. by using an HPLC/PDA/MS preparative approach directly on the fruit juice.[13]

In conclusion, in spite of the recent interest in its several health promoting properties, a systematic characterization of bergamot fruit preparations based on a comprehensive phytochemical approach was still lacking. In the present work, we have tried to fill this gap, and a thorough

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J. Agric. Food Chem. 2019, 67, 3159−3167

Table 4. ^{1}H and ^{13}C NMR Assignments for Bergamjuicin (35) in CD_3OD

position	δ_H mult (J in Hz)	δ_C mult
2	5.49 dd (13.2, 2.4)	79.0, CH
3	3.14 dd (17.2, 13.2)	42.9, CH_2
	2.81 dd (17.2, 2.4)	
4		196.8, C
5		164.2, C
6	6.17 bs	95.6, CH
7		165.1, C
8	6.14 bs	96.7, CH
9		166.4, C
10		105.0, C
1′		132.6, C
2′═6′	7.44 d (7.9)	128.2, CH
3′═5′	7.14 d (7.9)	117.2, CH
4′		158.4, C
1″	5.08 d (7.5)	98.4, CH
2″	3.65 overlapped	77.6, CH
3″	3.39 overlapped	72.7, CH
4″	3.30 overlapped	70.3, CH
5″	3.65 overlapped	74.8, CH
6″	4.44 dd (11.9, 2.0)	63.5, CH_2
	4.19 dd (11.9, 7.1)	
1‴	5.24 d (1.6)	101.9, CH
2‴	3.90 overlapped	71.3, CH
3‴	3.65 overlapped	70.9, CH
4‴	3.47 overlapped	77.0, CH
5‴	3.87 overlapped	68.9, CH
6‴	1.28 d (6.2)	17.3, CH_3
1^{IV}		171.4, C
2^{IV}	2.65−2.50 m	44.6, CH_2
3^{IV}		70.8, C
4^{IV}	2.65−2.50 m	44.9, CH_2
5^{IV}		174.2, C
6^{IV}	1.25 s	26.3, CH_3
1^{V}	4.94 d (6.8)	101.6, CH
2^{V}	3.48 overlapped	77.5, CH
3^{V}	3.65 overlapped	77.3, CH
4^{V}	3.52 overlapped	70.1, CH
5^{V}	3.69 overlapped	74.1, CH
$6a^{V}$	3.87 dd (12.0, 1.9)	61.2, CH_2
$6b^{V}$	3.68 dd (12.0, 9.0)	

investigation of BPF, obtained from bergamot juice, by UPLC-DAD-MS has been complemented by preparative chromatographic separation, followed by NMR characterization of the isolated compounds. The combination of these techniques proved to be very efficient at covering different classes of compounds, leading to the identification of 39 components, divided into 32 flavanone/flavone/flavonol derivatives, three coumarins, three limonoids, and one phenylpropanoid, several of which had never been reported from bergamot or, more generally, for Rutaceae. One of these compounds, named bergamjuicin (**35**), is a new natural product for which the full NMR characterization has been reported. The detailed analysis of composition provided for BPF, a material of great nutraceutical and industrial interest, could be a convenient guide for future routine analyses.

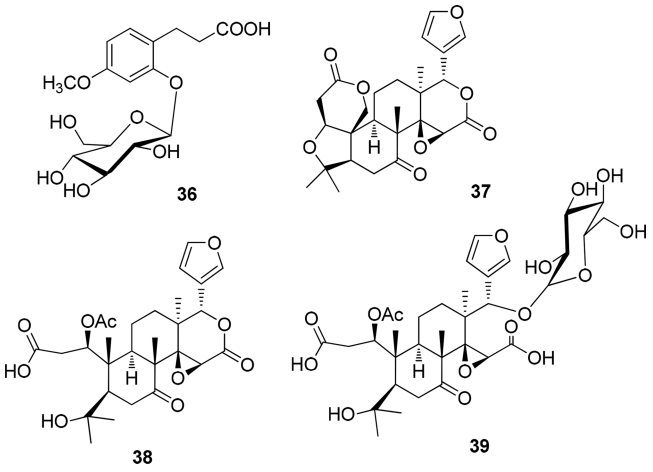

Figure 7. Chemical structures of a phenylpropanoid and three limonoids identified through NMR analysis

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b06591.

Comparison between UPLC profiles of bergamot juice and BPF. ^{1}H NMR spectra for selected pure compounds. ^{1}H and 2D NMR (COSY, HSQC and HMBC) spectra for bergamjuicin (35) (PDF)

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Notes

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