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Distribution of Flavonoids and Furocoumarins in Juices from Cultivars of *Citrus bergamia* Risso

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HPLC separation of flavonoids and furocoumarins in the crude juices of three cultivars of *Citrus bergamia* Risso ("Castagnaro", "Fantastico", and "Femminello") was carried out on a C18 reversed phase column. The analysis was performed in a single run using a DAD detector coupled with an ESI-MS-MS source. Two furocoumarins (bergapten and bergamottin) were detected and quantified simultaneously with the sixteen flavonoid components previously found in industrial bergamot juice. Full characterization of the furocoumarins was performed by ^{1}H NMR analysis on samples separated by means of preparative HPLC. The free-radical scavenging ability of cultivar juices was assessed by using DPPH radical. The data presented show that the "Femminello" cultivar, even though it is the least common of the three, is by far the richest in health-promoting bioactive compounds (both flavonoids and furocoumarins). Given the range of applications of furocoumarins, the preparative separation described herein is proposed as a simple and rapid method to obtain this class of compounds in good yield from crude juice.

KEYWORDS: *Citrus bergamia*; bergamot juice; HPLC-DAD-ESI-MS-MS; ^{1}H NMR; flavonoids; furocoumarins; preparative HPLC; antioxidant activity

INTRODUCTION

Bergamot (*Citrus bergamia* Risso) plants grow almost exclusively on a narrow coastal strip in the area of Reggio Calabria (southern Italy). This area presents favorable weather and pedoclimatic conditions for its cultivation. Three cultivars of bergamot ("Castagnaro", "Fantastico", and "Femminello") are commercially grown and then industrially processed, exclusively to extract their essential oils. The industrial processing uses an indiscriminate mix of the three cultivars. The oil is concentrated in the external pigmented part of the fruit peel (flavedo) and, owing to the abundant presence of terpenes, esters, and alcohols, possesses a very intense and characteristic fragrance (*1*). For many years, bergamot oil was highly sought after by the cosmetic industry, being the base for many a prized perfume. The advent of the synthetic reconstruction of the bouquet of bergamot oil led to a dramatic decrease in demand.

However, over the past few years, following the growing interest in antioxidant bioactive compounds and their dietary sources, such as *Citrus* juices (*2–8*), bergamot juice has attracted attention as a result of its remarkable flavonoid content. We have recently reported on the composition of the flavonoid fraction of bergamot industrial juice (*9*). Along with the most widely present components, neoeriocitrin, naringin, and neohesperidin, the following have also been identified and quantified: *C*-glucosides (lucenin-2, vicenin-2, stellarin-2, lucenin-2 4′-methyl ether, scoparin, and orientin 4′-methyl ether), flavone *O*-glycosides (rhoifolin 4′-*O*-glucoside, chrysoeriol 7-*O*-neohesperidoside-4′-*O*-glucoside, rhoifolin, chrysoeriol 7-*O*-neohesperidoside, neodiosmin), and flavanone *O*-glycosides (eriocitrin) (*8,9*). Both the wide variety of different types of flavonoids and the fact that some of these compounds are present in good amounts hints at possible future applications of a juice that has, to date, been little exploited.

Flavonoids do indeed show strong antioxidant and radical scavenging activity (*10–12*) and appear to be associated with reduced risk for certain chronic diseases, the prevention of some cardiovascular disorders, and certain types of cancerous processes (*13*). Flavonoids also exhibit antiviral, antimicrobial, and anti-inflammatory activities, effects on capillary fragility, an ability to inhibit human platelet aggregation, as well as antiulcer and antiallergenic properties (*14, 15*). The probable mechanism by which they act and potential clinical applications have been reviewed (*16*).

Linear furocoumarins (psoralens) are widely found in plants but are particularly abundant in the Umbelliferae/Apiaceae and Rutaceae (*17*). They can be troublesome to humans, since they can cause photosensitization toward UV light (resulting in sunburn or serious blistering) and promote skin pigmentation when they are administrated topically (*18*). When ingested, they can interfere with the oral bioavailability of certain prescription drugs. This could be due to their inhibition of intestinal

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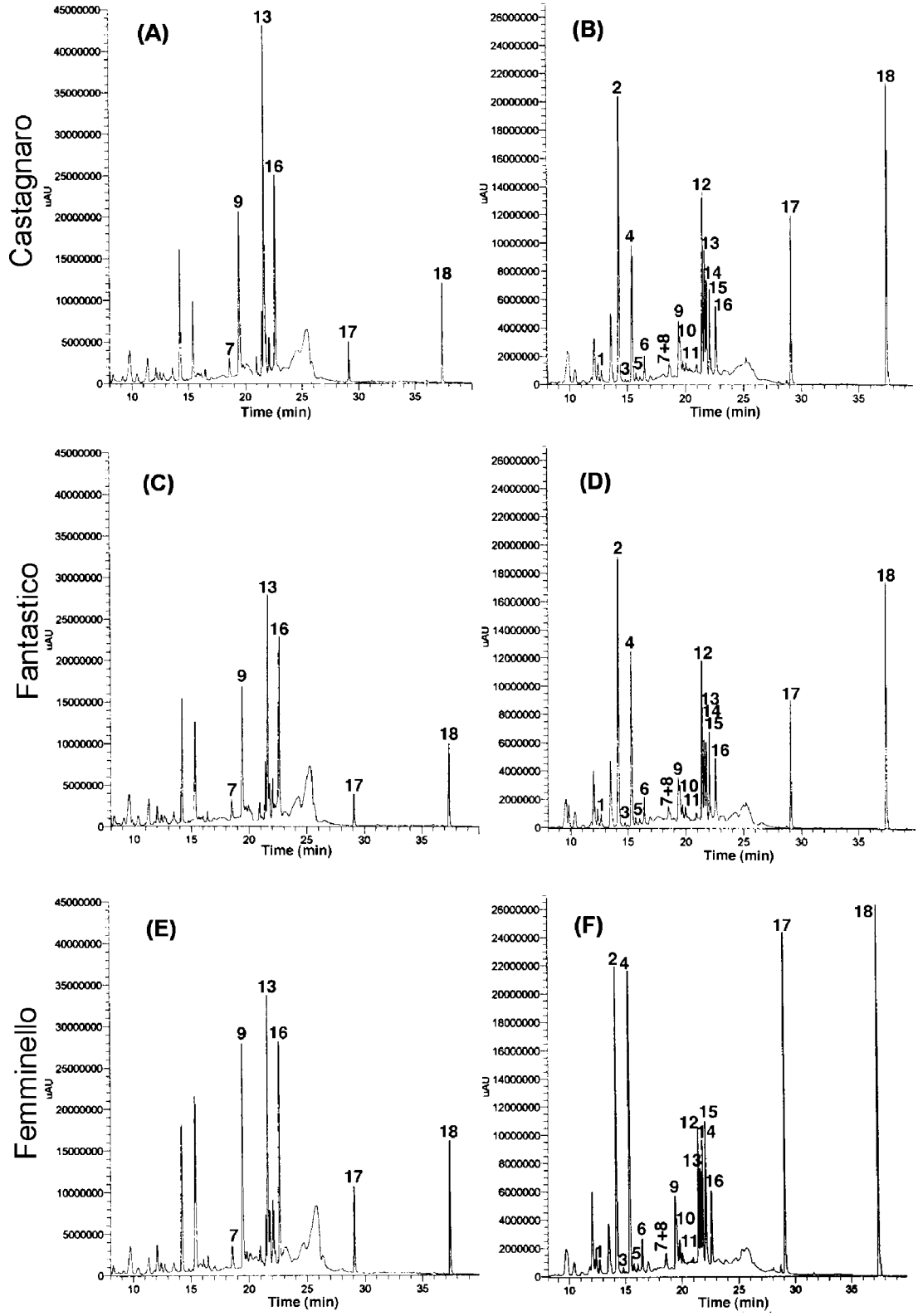


Figure 1. Typical DAD chromatograms of bergamot juices recorded at 278 nm (left) and 325 nm (right) from (**A**, **B**) "Castagnaro", (**C**, **D**) "Fantastico", and (**E**, **F**) "Femminello". Components **1**–**18** were identified as follows: **1**, lucenin-2; **2**, vicenin-2; **3**, stellarin-2; **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; **16**, neohesperidin; **17**, bergapten; **18**, bergamottin.

cytochrome P450 3A4 (CYP3A4) (*19*), and this effect has actually been patented as a means to obtain increased oral bioavailability of drugs (*20*). Furocoumarins can also mediate drug efflux, possibly via the P-glycoprotein transporter (*21*). Used therapeutically, they are helpful for treating psoriasis and vitiligo, stimulate melanin pigment formation, and have a broad spectrum of biological activities, including antimicrobial, antiplatelet-aggregation, and antimutagenic activities (*22, 23*). Previous studies reported the presence of health-promoting bioactive compounds such as furocoumarins and coumarins in

Citrus species, e.g. grapefruit juice. The isolation of furocoumarins from bergamot fruits has also been reported (*20*, *24*, *25*).

With a view to the potential utilization of bergamot juice as a dietary aid to promote human health as well as potential pharmaceutical applications, we focus in the present study on the different distributions of flavonoids and furocoumarins in the three cultivars, "Castagnaro", "Fantastico", and "Femminello". The main aim was to identify the cultivar that presents the highest availability of these substances that are beneficial to health.

EXPERIMENTAL PROCEDURES

Material and Reagents. Bergamot fruits were supplied by Ditta Visalli Diego Estrazione Essenze e Derivati (Melito di Porto Salvo, RC, Italy). Juice samples from each cultivar were prepared by hand squeezing the fresh fruit, from which the peel had previously been removed. The juices were stored at −20 °C until required for our study. The investigation was carried out on ten samples of each cultivar from the 2006–2007 fruit season.

Reagents and Standard Solutions. HPLC-grade acetonitrile and methanol, DPPH (2,2-diphenyl-1-picrylhydrazyl), bergapten, and bergamottin were supplied by Sigma-Aldrich (St. Louis, MO), and dimethylformamide (DMF) was supplied by Carlo Erba (Milan, Italy). Neodiosmin, eriocitrin, neoeriocitrin, naringin, and neohesperidin were from Extrasynthèse (Genay, France). Vicenin-2 and lucenin-2 4′-methyl ether were separated from orange and citron juices, respectively (*8*).

Preparative HPLC. Preparative HPLC was carried out using a LC 8 A HPLC instrument (Shimadzu) equipped with a Shimadzu SPD-10 A vp UV detector. The column used was a 250 mm × 21.2 mm i.d., size 5 μm Discovery C18 Supelco column. The injection loop was 2.0 mL, and the flow rate was 20.0 mL/min at room temperature (20 °C). Detection was carried out at 310 nm. The mobile phase consisted of a linear gradient of acetonitrile in H_2O as follows: 40–100% (0–30 min), 100% (30–35 min), and 100–40% (35–40 min). The first component was eluted at a t_R of 9.3 min, and the second, at a t_R of 25.2 min.

Sample Preparation. DMF (10.0 mL) was added to the juice sample (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 with a 0.45 μm pore size (Supelco, Bellefonte, PA).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra were recorded at 300 Hz on a Varian Mercury 300 instrument (Varian, Palo Alto, CA) in $CDCl_3$. Chemical shifts were reported using residual TMS as internal standard.

LC-MS-MS Analyses of Flavonoids and Furocoumarins. LC-MS-MS analyses of samples were carried out with a ThermoQuest model LCQ-Duo instrument equipped with a diode array spectrophotometer and an ion trap mass spectrometer with an electrospray ionization source (ESI). Separation of each compound 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, 310, and 325 nm. The operating parameters of the mass spectrometer were as follows: 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 spectrometry 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 the instrument maximum. A source fragmentation of 20 V as 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 and Furocoumarins Content. Bergapten, bergamottin, neodiosmin, eriocitrin, neoeriocitrin, naringin, neohesperidin, vicenin-2, and lucenin-2 4′-methyl ether were all used as standards. The calibration curves were obtained using DMF solutions of known concentration (10–100 mg/L). The values for lucenin-2, stellarin-2, isovitexin, scoparin, are orientin 4′-methyl ether are expressed as lucenin-2 4′-methyl ether equivalents. The values for rhoifolin 4′-*O*-glucoside, chrysoeriol 7-*O*-neohesperidoside 4′-*O*-glucoside, rhoifolin, and chrysoeriol 7-*O*-neohesperidoside are expressed as neodiosmin equivalents. Quantitative analysis was carried out by integration of the areas of the peaks from the chromatogram at 325 nm for flavonoids and at 310 nm for furocoumarins, by using the Genesis peak detection algorithm integrated in the ThermoQuest software.

Identification of Compounds. Compounds **1**–**18** were eluted in the same order as observed in LC analysis of industrially pressed juices: UV, MS, and MS-MS data, recorded in correspondence to each peak, are superimposable with the ones previously reported (*9*). MS-MS data are available as Supporting Information.

Compound **1.** t_R, 12.7 min; UV, 258 (sh), 270, 349 nm; MS, 611 $[M + H]^+$, 609 $[M - H]^-$.

Compound **2.** t_R, 14.2 min; UV, 271, 335 nm; MS, 595 $[M + H]^+$, 593 $[M - H]^-$.

Compound **3.** t_R, 14.8 min; UV, 256 (sh), 271, 347 nm; MS, 625 $[M + H]^+$, 623 $[M - H]^-$.

Compound **4.** t_R, 15.4 min; UV, 257 (sh), 271, 348 nm; MS, 625 $[M + H]^+$, 623 $[M - H]^-$.

Compound **5.** t_R, 15.8 min; UV, 268, 324 nm; MS, 741 $[M + H]^+$, 739 $[M - H]^-$.

Compound **6.** t_R, 16.4 min; UV, 249 (sh), 268, 337 nm; MS, 771 $[M + H]^+$, 769 $[M - H]^-$.

Compound **7.** t_R, 18.6 min; UV, 283 nm; MS, 597 $[M + H]^+$, 595 $[M - H]^-$.

Compound **8.** t_R, 18.6 min; UV, 269, 336 nm; MS, 433 $[M + H]^+$, 431 $[M - H]^-$.

Compound **9.** t_R, 19.4 min; UV, 283 nm; MS, 597 $[M + H]^+$, 595 $[M - H]^-$.

Compound **10.** t_R, 19.6 min; UV, 256, 268, 347 nm; MS, 463 $[M + H]^+$, 461 $[M - H]^-$.

Compound **11.** t_R, 19.8 min; UV, 253 (sh), 270, 348 nm; MS, 463 $[M + H]^+$, 461 $[M - H]^-$.

Compound **12.** t_R, 21.4 min; UV, 267, 337 nm; MS, 579 $[M + H]^+$, 577 $[M - H]^-$.

Compound **13.** t_R, 21.6 min; UV, 281 nm; MS, 581 $[M + H]^+$, 579 $[M - H]^-$.

Compound **14.** t_R, 21.8 min; UV, 252, 268, 347 nm; MS, 609 $[M + H]^+$, 607 $[M - H]^-$.

Compound **15.** t_R, 22.1 min; UV, 253, 268, 345 nm; MS, 609 $[M + H]^+$, 607 $[M - H]^-$.

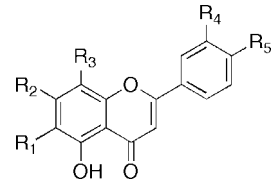
Compound **16.** t_R, 22.6 min; UV, 283 nm; MS, 611 $[M + H]^+$, 609 $[M - H]^-$.

Compound **17.** Mp, 189–193 °C; t_R, 29.1 min; UV, 221, 249, 260, 268, 311 nm; MS, 217 $[M + H]^+$, 215 $[M - H]^-$; ^{1}H NMR ($CDCl_3$) δ 8.14 (d, 1, J = 9.7 Hz, H-4), 7.58 (d, 1, J = 2.4 Hz, H-2′), 7.12 (broad s, 1, H-8), 7.00 (dd, 1, J = 0.9, 2.4 Hz, H-3′), 6.26 (d, 1, J = 9.8 Hz, H-3), 4.25 (s, 3, 5-OCH_3) ppm.

Compound **18.** Mp, 74−79 °C; t_R, 37.3 min; UV, 221, 250, 268 (sh), 307 nm; MS, 339 $[M + H]^+$, 337 $[M - H]^-$; ^{1}H NMR ($CDCl_3$) δ 8.14 (d, 1, J = 9.8 Hz, H-4), 7.57 (d, 1, J = 2.4 Hz, H-2′), 7.13 (broad s, 1, H-8), 6.94 (dd, 1, J = 0.9, 2.4 Hz, H-3′), 6.25 (d, 1, J = 9.8 Hz, H-3), 5.51 (m, 1, H-2″), 5.05 (m, 1, H-6″), 4.93 (d, 2, J = 6.8 Hz, H-1″), 2.08 (m, 4, H-4″, H-5″), 1.67 (s, 3, H-10″), 1.66 (s, 3, H-8″), 1.58 (s, 3, H-9″) ppm.

DPPH Assay. The antioxidant ability of the crude juice from each cultivar, "Castagnaro", "Fantastico", and "Femminello", was assessed using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The assay was carried out following a procedure described in the literature (*26*), with minor modifications. The bergamot juice was centrifuged at 4000 rpm for 10 min, and the supernatant of the samples was collected for analysis. Twenty-five microliters of supernatant were mixed with 63 μM of DPPH in methanol, in a final volume of 4.0 mL. The control

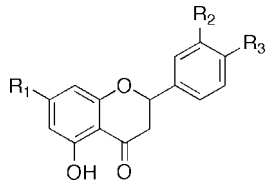
Table 1. Flavone-*C*-glucosides (**1**–**4**, **8**, **10**, and **11**) and Flavone-*O*-glycosides (**5**, **6**, **12**, **14**, and **15**)



	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	OH	O-Glu	apigenin 7-*O*-neohesperidoside-4′-glucoside (rhoifolin 4′-glucoside)
6	H	O-Nh[a]	H	OMe	OH	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	OH	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.

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.

contained all of the components except juice supernatant. The changes in absorbance at 517 nm were monitored over 50 min. All tests were run in triplicate, and the results were averaged. Results were expressed as percentage decrease with respect to control.

Statistical Analysis. Data were expressed as means of least three independent experiments. Statistical comparisons of the results were performed by one-way ANOVA using SPSS 11.5 (Chicago, IL). Significant differences ($P < 0.05$) between the three cultivars were analyzed by Duncan's multiple range test.

RESULTS AND DISCUSSION

Hand-squeezed crude bergamot juice from each of the cultivars to be investigated was mixed with DMF, centrifuged, filtered, and analyzed by reversed-phase HPLC-DAD-MS-MS. **Figure 1** shows the DAD chromatograms of samples of bergamot juice from "Castagnaro" (**A**, **B**), "Fantastico" (**C**, **D**), and "Femminello" (**E**, **F**) at 278 and 325 nm, respectively.

Analysis of the first order ESI-MS spectra recorded for each peak along with MS-MS experiments, UV comparison, and retention time (t_R), led to assignment of the peaks **1**–**16** to the structures of flavonoid glycosides previously found in industrially pressed bergamot juice (**Tables 1** and **2**).

Compound **17**, t_R 29.08 min, showed a very simple ESI-MS spectrum in positive mode, where only a pseudomolecular ion $[M + H]^+$ *m/z* 217 was present. The MS-MS spectrum in the positive mode (focused on *m/z* 217) showed a *m/z* 202 fragment, highlighting the loss of one methyl group. The ESI-MS spectrum in positive mode of compound **18**, t_R 37.37 min, also showed the presence of only a pseudomolecular ion $[M + H]^+$ *m/z* 339. The MS-MS spectrum in positive mode (focused on *m/z* 339) showed an abundant *m/z* 203 fragment. Both compounds **17** and **18** showed DAD-UV absorption maxima at ca. 310 and 221 nm (**Figure 2**), indicating the probable presence of furocoumarin nuclei (*27*).

As the data recorded in the on line chromatographic analysis of compounds **17** and **18** was not sufficient for the elucidation of the structure, we optimized a preparative HPLC method to enable them to be collected separately. The separation was carried out on a C18 column with a linear gradient of water–acetonitrile as mobile phase. The HPLC chromatogram in **Figure 3** shows the excellent separation of the peaks of interest from the other components of the crude juice ("Femminello").

The fractions collected in correspondence with each peak were recrystallized from methanol to obtain compounds **17** and **18** as white solids, mp 189–193 and 74–79 °C respectively, and were subjected to ^{1}H NMR spectroscopy. From the spectral data recorded, compounds **17** and **18** were identified as bergapten (5-methoxypsoralen) and bergamottin (5-geranyloxypsoralen), respectively (**Figure 4**). The assignments were confirmed by comparison of MS spectra, melting points, and UV and ^{1}H NMR data, obtained from authentic samples (*25*).

Table 3 shows the flavonoid and furocoumarin contents in the juices of the three cultivars as range and mean values. A preliminary point is that the contents found for all compounds **1**–**16** are lower than those previously reported for an industrial juice produced by the indiscriminate processing of fruits from all three cultivars (*9*). This finding is explained by the differences in the distribution of flavonoids and furocumarins between the peel and the endocarp of the fruits (*25*). The compositions of industrially pressed juices are therefore enriched by the release of components from the albedo and flavedo (*3*). However, the purpose of the present study was to investigate the real differences between the cultivars using a controlled procedure (hand squeezed production of the juice). In agreement with literature data (*6*, *9*), the flavanone-*O*-glycosides naringin (**13**), neohesperidin (**16**), and neoeriocitrin (**9**) were found as the most

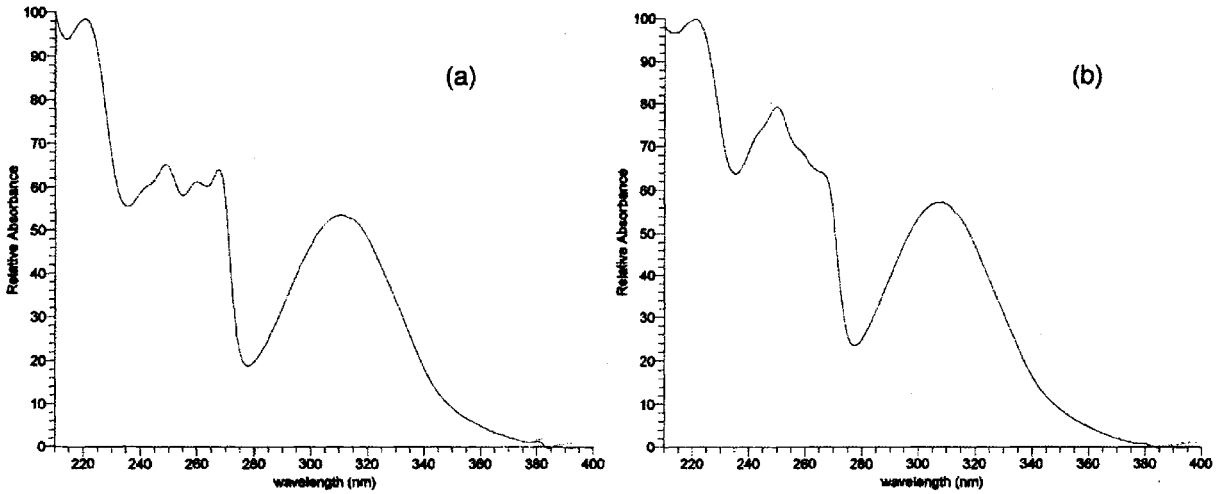


Figure 2. DAD-UV spectra for **17** (**a**) and **18** (**b**).

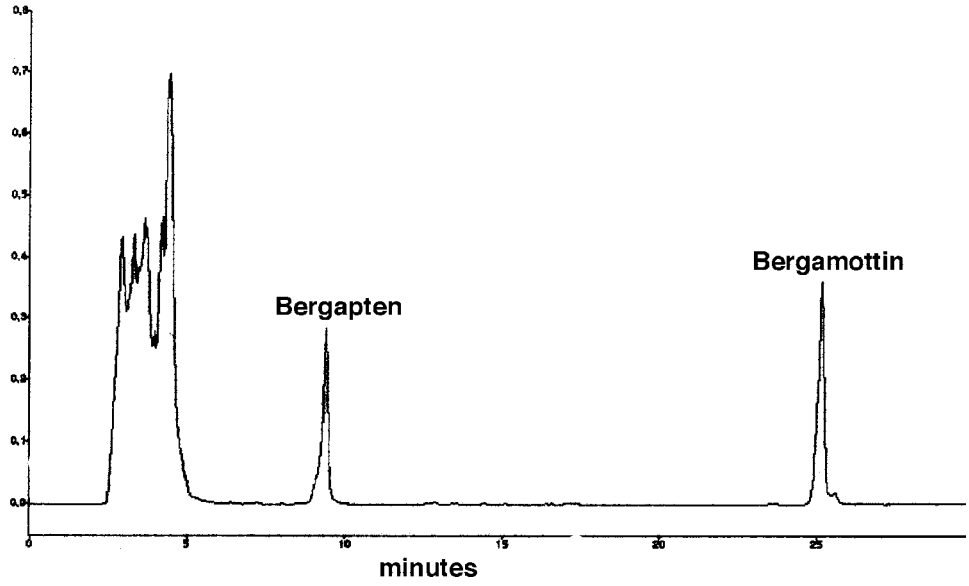


Figure 3. Preparative HPLC chromatogram for the collection of compounds **17** and **18** from "Femminello" bergamot juice.

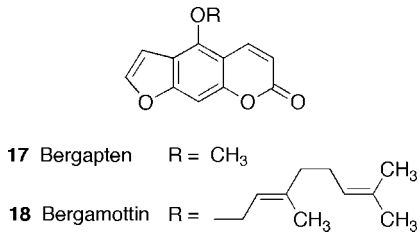


Figure 4. Assigned structures for compounds **17** and **18**.

abundant flavonoids, and between the cultivars, they all show significant differences ($P < 0.05$) by statistical analysis (one-way ANOVA). Naringin (**13**) was present in the highest amount in "Castagnaro" (129.8 mg/L), whereas "Fantastico" juice (83.5 mg/L) contained the lowest amount. The naringin content (**13**, 104.5 mg/L) in "Femminello" juice was between the other two. However, "Femminello" juice was the richest in neoeriocitrin (**9**) (88.3 mg/L). Among the flavone *C*-glycosides, lucenin-2 4′-methyl ether (**4**), vicenin-2 (**2**), and isovitexin (**8**) showed significant differences between the three cultivars, whereas lucenin-2 (**1**), scoparin (**10**), and orientin 4′-methyl ether (**11**) were found in similar amounts in "Castagnaro" and "Fantastico", but with significant differences from "Femminello" (see **Table 3**). Lucenin-2 4′-methyl ether (**4**) and vicenin-2 (**2**) were highest in "Femminello" juice (62.8 and 55.2 mg/L, respectively) with respect to "Fantastico" (32.7 and 44.1 mg/L, respectively) and "Castagnaro" juices (25.4 and 47.5 mg/L, respectively). The other flavonoids, chrysoeriol 7-*O*-neohesperidoside (**14**) and neodiosmin (**15**), were distributed in the cultivar juices in the following order: "Femminello" (17.2 and 27.1 mg/L), "Castagnaro" (10.6 and 15.3 mg/L), and "Fantastico" (9.3 and 15.5 mg/L). "Femminello" juice was also found to be the richest (512 mg/L) as regards the total amount of flavonoids in juices, followed by "Castagnaro" (435 mg/L) and, last, "Fantastico" (373 mg/L). Significant differences between the cultivars were found for furocoumarins **17** and **18**. A higher amount (10.0 mg/L) of bergapten (**17**) was present in "Femminello" juice, while it appeared lower in "Castagnaro" (6.7 mg/L) and "Fantastico" (7.6 mg/L) juices. The bergamottin (**18**) content was 40.2 mg/L in "Femminello" juice and 27.2 mg/L and 23.5 mg/L in "Castagnaro" and "Fantastico" juices. The total amount of furocoumarins in the three cultivars, like the case of the flavonoids, was once again found to be the highest in "Femminello" juice (50.2 mg/L) compared with the juices from "Castagnaro" (34.2 mg/L) and "Fantastico" (31.1 mg/L).

Statistical analysis showed that fifteen out of eighteen components quantified were significantly different ($P < 0.05$) between "Femminello" and the other two cultivars, whereas only

Table 3. Flavone-*C*-glucosides (**1**–**4**, **8**, **10**, and **11**), Flavone-*O*-glycosides (**5**, **6**, **12**, **14**, and **15**), Flavanone-*O*-glycosides (**7**, **9**, **13**, and **16**), and Furocoumarins (**17** and **18**): Contents of Bergamot Juice (mg/L)[a]

		"Castagnaro"		"Fantastico"		"Femminello"	
		mean	range	mean	range	mean	range
Flavonoids							
1	lucenin-2	2.2 a	2.1–2.4	2.5 a	2.3–2.6	3.3 b	3.1–3.5
2	vicenin-2	47.5 a	46.5–49.2	44.1 b	42.6–44.8	55.2 c	54.6–58.4
3	stellarin-2	0.6 ns	0.5–0.6	0.7 ns	0.6–0.8	1.1 ns	0.9–1.3
4	lucenin-2 4′-OMe	25.4 a	22.1–27.5	32.7 b	30.5–34.6	62.8 c	60.5–66.8
5	rhoifolin 4′-*O*-Glu	1.2 ns	1.0–1.4	1.2 ns	1.1–1.3	1.3 ns	1.2–1.3
6	chrysoeriol 7-*O*-Nh-4′-*O*-Glu[b]	3.8 a	3.6–3.9	4.1 a	3.8–4.4	5.3 b	4.8–5.3
7	eriocitrin	8.4 ns	7.9–9.4	10.8 ns	9.6–11.7	10.2 ns	10.2–13.5
8	isovitexin	2.5 b	2.4–2.6	2.2 a	2.1–2.3	3.1 c	2.8–3.2
9	neoeriocitrin	62.6 b	59.8–64.5	52.2 a	50.3–57.8	88.3 c	85.4–94.5
10	scoparin	5.4 a	5.1–5.9	5.9 a	5.5–6.8	9.1 b	8.4–10.1
11	orientin 4′-OMe	1.7 a	1.3–1.8	1.9 a	1.5–2.1	4.2 b	3.9–4.8
12	rhoifolin	28.9 b	26.5–29.8	26.2 b	25.8–30.4	22.8 a	19.8–23.8
13	naringin	129.8 c	125.8–136.7	83.5 a	79.5–85.4	104.5 b	101.2–109.7
14	chrysoeriol 7-*O*-Nh[b]	10.6 a	9.5–11.4	9.3 a	8.6–10.1	17.2 b	15.6–20.5
15	neodiosmin	15.3 a	14.5–16.8	15.5 a	14.7–16.1	27.1 b	26.1–29.7
16	neohesperidin	89.5 b	85.7–91.2	80.0 a	76.2–82.4	96.4 c	95.4–101.2
Furocoumarins							
17	bergapten	6.7 a	6.4–7.2	7.6 b	7.2–8.5	10.0 c	9.5–11.8
18	bergamottin	27.2 b	26.4–29.5	23.5 a	22.5–24.6	40.2 c	38.5–43.7

[a] Different letters in the same row represent significant differences at $P < 0.05$ by Duncan's multiple range test (ns = not significant). [b] *O*-Neohesperidose.

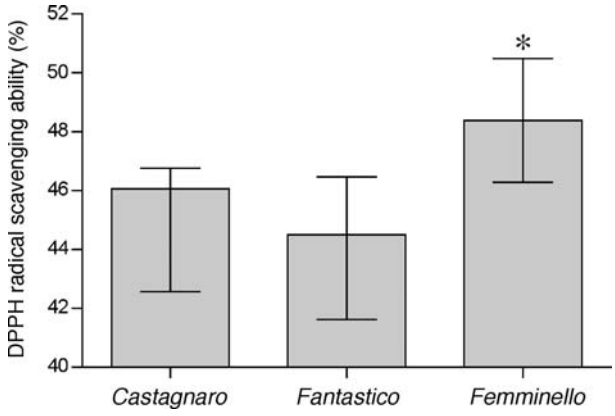


Figure 5. Free radical scavenging activities of juices from "Castagnaro", "Fantastico", and "Femminello" cultivars. Values are expressed as percentages with respect to a control experiment and represent means of at least three different experiments. The asterisk (*) indicates a significant difference between the cultivars at $P < 0.05$.

eight out of eighteen components showed significant differences between "Castagnaro" and "Fantastico".

The free-radical-scavenging activity of fresh hand squeezed "Castagnaro", "Fantastico", and "Femminello" juices was evaluated using a DPPH radical, which forms a violet solution and reacts with antioxidants and loses color. All the juices analyzed reacted with and quenched DPPH radicals, and they showed powerful antioxidant effects. As reported in **Figure 5**, "Femminello" juice shows the highest capacity to bleach DPPH free radicals, reaching 49% of radical scavenging activity. Nevertheless, "Fantastico" and "Castagnaro" juices also show good antioxidant activity (46 and 44% activity, respectively). Statistical analysis revealed that there is a significant difference ($P < 0.05$) between "Femminello" and the other two cultivars.

It can be observed, however, that although such ability parallels the total flavonoid amount (435, 373, and 512 mg/L for "Castagnaro", "Fantastico", and "Femminello", respectively), it has been proposed that DPPH quenching is mostly due to the four flavonoids bearing a cathecolic B-ring (i.e., **1**, **7**, **9**, and **12**), since all the other phenolic OH's are less reactive toward DPPH radical (*28*–*30*). Indeed, the amount of these cathecol containing flavonoids (102.1, 91.7, and 124.6 mg/L for "Castagnaro", "Fantastico", and "Femminello", respectively; see **Table 3**) reflects the observed overall antiradical activity of the three cultivars.

The total content of flavonoids and furocoumarins in "Femminello" juice suggests that the "Femminello" cultivar represents a better source for these classes of compounds rather than "Castagnaro" and "Fantastico" juices, although all of them have been shown to be very rich in bioactive components. The antioxidant activity of the three juices was tested by DPPH radical bleaching and showed "Fantastico" and "Castagnaro" juices to be significantly less active than "Femminello" juice. Given the great commercial interest in research on antioxidant-rich products to be used as dietary supplements for humans, possible industrial or pharmaceutical applications for bergamot juices are likely. In light of this, the results reported in this study will hopefully encourage producers to favor the cultivation of the "Femminello" cultivar. The "Castagnaro" cultivar also appears quite interesting in this respect, whereas the most widely grown cultivar to date, the "Fantastico", seems to be the poorest in health-promoting compounds. Furthermore, considering the economic advantages to be gained by the availability of chemicals of pharmaceutical interest from low-cost natural sources, compared to synthetic products, we propose that the preparative HPLC method developed in this study provides a quick, easy, and convenient way to obtain pure bergapten and bergamottin on a preparative scale.

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Supporting Information Available: Complete MS-MS data for compounds **1**–**16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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