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Flavonoids and Biflavonoids in Tuscan Berries of *Juniperus communis* L.: Detection and Quantitation by HPLC/DAD/ESI/MS

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The aim of the present work was to develop a quali-quantitative investigation, using HPLC/DAD and HPLC/ESI/MS techniques, of the phenolic composition of berries collected from wild Tuscan plants of *Juniperus communis* L. and grown in three different geographical zones. The applied chromatographic elution method made it possible to well separate up to 16 different compounds belonging to flavonoids, such as isoscutellarein and 8-hydroxyluteolin or hypolaetin glycosides, and six biflavonoids, among them amentoflavone, hynokiflavone, cupressoflavone, and methyl-biflavones. To the best of the authors' knowledge this is the first report on the presence of these compounds in juniper berries. The flavonoidic content in the analyzed berries ranged between 1.46 and 3.79 mg/g of fresh pulp, whereas the amount of the biflavonoids was always lower, varying between 0.14 and 1.38 mg/g of fresh weight.

KEYWORDS: ***Juniperus communis* L.; berries; flavonoids; biflavonoids; HPLC/DAD/ESI/MS**

INTRODUCTION

Juniper (*Juniperus communis* L.) is an important spice in many European cuisines, especially in Alpine regions, where juniper grows abundantly. It is the only example of a spice in the botanic group of the Coniferae (Cupressaceae) and also one of the few examples of spices from cold climatic regions, although the best quality comes from southern European countries. It is a native evergreen shrub/tree of regions in the northern hemisphere with a substantial list of traditional uses owing to its food applications and medicinal properties. Whole dried berries of *Juniperus* are widely used in some regions of Italy to prepare meats with a particular flavor; the ripened fruits are also a base product in the formulation of some liqueurs such as the famous gin, aromatic grappa, gineprino and, sometimes, also bitters. The peculiar flavor is primarily associated with its volatile oil components. Even if all of the organs of this plant contain essential oil, it is, almost exclusively, obtained from the berries (0.2−3.42%) (*1*).

Juniper essential oil has widely documented uses as a remedy to enhance diuresis (*2*) and for dyspeptic complaints (*3*). It also acts as an anti-inflammatory, hypoglycemic (*4*, *5*), and antimicrobial agent (*6*).

Most of the works reported in the literature up to now are focused on the determination of the chemical composition of the berry's essential oil (*1*, *7*, *8*), whereas only limited data are reported on the nonvolatile components of leaves and/or twigs of other juniper varieties (*9*−*11*). These studies have shown that the main classes of metabolites are flavonoids and biflavonoids. Pentosidic derivatives of isoscutellarein and hypolaetin and mono- and diglycosides of quercetin and kaempferol were reported belonging to the first class, whereas, within biflavonoids, amentoflavone, and cupressoflavone have been cited.

The aim of the present work was to develop a quali-quantitative investigation, using HPLC/DAD and HPLC/ESI/MS techniques, of the phenolic composition of berries collected from wild Tuscan plants of *J. communis* L.

MATERIALS AND METHODS

Samples of fruits were collected from three natural populations of *J. communis* L. growing in three different geographical zones in Tuscany (Italy): Reggello (Firenze) (REG), Monte Benichi (Bucine, Arezzo) (MB), and La Verna (Arezzo) (VE). The number of plants for each provenance varied from 20 to 35. Green (unripe) and red berries (ripe) were collected at the same elevational and horizontal position in the crown of the plant and kept frozen at −20 °C until analyzed.

All solvents used were of HPLC grade; CH_3CN was from E. Merck (Darmstadt, Germany) The pure standards of amentoflavone, cupressoflavone, hynokiflavone, scutellarein, and rutin were purchased from Extrasynthese (Geney, France).

Sample Preparation. The juniper berries were crushed after contact with liquid nitrogen, and then 5 g of the coarse powdered sample was extracted with EtOH/H_2O (adjusted to pH 2 by HCOOH), 7:3 v/v, (50 mL × 2) for 1 h, at room temperature and under magnetic stirring.

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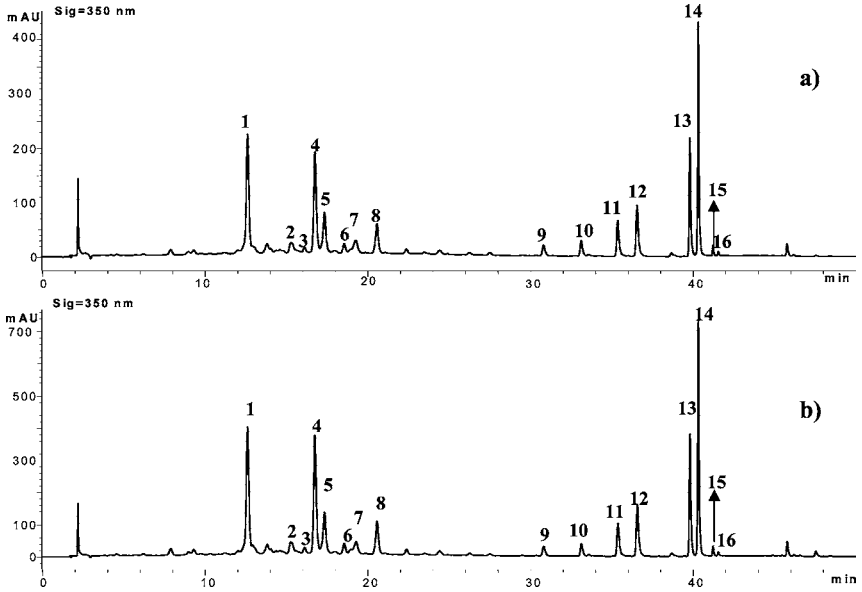

Figure 1. Chromatographic profiles at 350 nm of the red (**a**) and green (**b**) juniper berries of the MB sample. The numeration is that of **Table 1**.

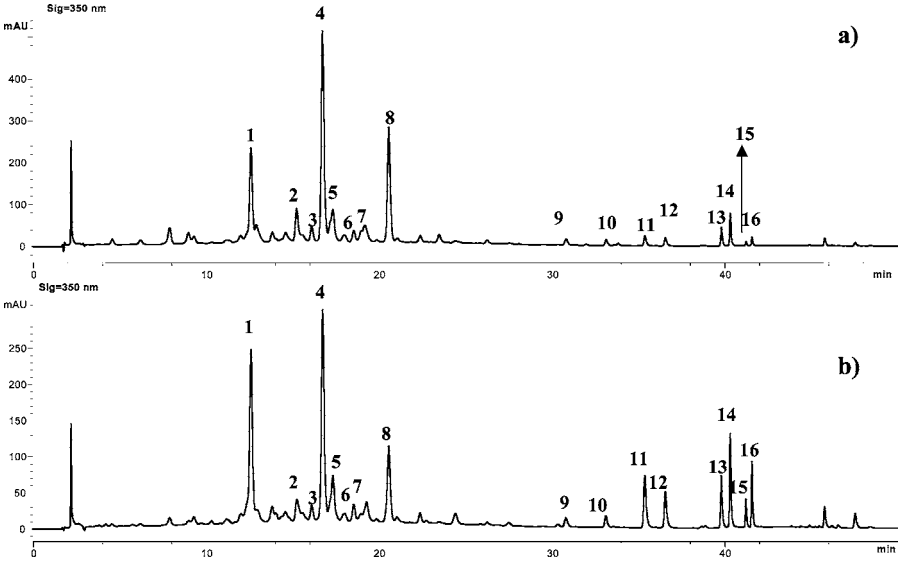

Figure 2. Chromatographic profiles at 350 nm of the red (**a**) and green (**b**) juniper berries of the REG sample. The numeration is that of **Table 1**.

After filtration, the obtained extracts were concentrated under vacuum to a final concentration of 25 mL. These samples were directly analyzed by HPLC/DAD and HPLC/ESI/MS.

Quantitative Determination. Quantitative evaluation of each compound was performed using five-point regression curves (r^2 = 0.9998) through the use of two authentic standards. All of the flavonoids were calculated at 350 nm using a standard of rutin in a range of concentration between 0 and 7.3 μg. The biflavonoid amounts were calculated at 350 nm using amentoflavone as reference compound in the range of 0−3.4 μg.

Apparatus. *HPLC-DAD Analysis.* The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA). A 150 × 3.9 mm i.d., 4 μm Fusion, RP18 column (Phenomenex, Torrance, CA) equipped with a precolumn of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH_3CN. The multistep linear solvent gradient used was as follows: 0−15 min, 85−75% A; 15−25 min, 75−65% A; 25−35 min, 45−50% B; 35−40 min, 50−100% B; 40−50 min, 100% B. Equilibration time was 10 min. The flow rate was 0.4 mL min^{-1} and the oven temperature, 26 °C.

The injection volumes were in the range of 10−25 μL for all of the samples. UV−vis spectra were recorded in the range of 200−500 nm, and the chromatograms were acquired at 240, 280, 330, and 350 nm.

HPLC-MS Analysis. The HPLC/MS analyses were performed using a HP 1100L liquid chromatograph coupled to a HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies). Spectra were recorded in negative ion mode, setting the fragmentation energy between 80 and 180 V and applying the same chromatographic conditions as described previously. The mass spectrometer operating conditions were as follows: gas temperature, 350 °C; nitrogen flow rate, 7 L min^{-1}; nebulizer pressure, 30 psi; quadrupole temperature, 40 °C; and capillary voltage, 3500 V.

RESULTS AND DISCUSSION

Qualitative Investigation. The first step of this work was focused on performing the optimization of the experimental conditions to quali-quantitatively investigate the phenolic composition of juniper berry samples by HPLC/DAD and HPLC/MS. Toward this aim, a pool of green and red berry samples, purchased from three different areas of Tuscany, were considered.

The applied chromatographic elution method (see Materials and Methods) allowed the separation of 16 different compounds

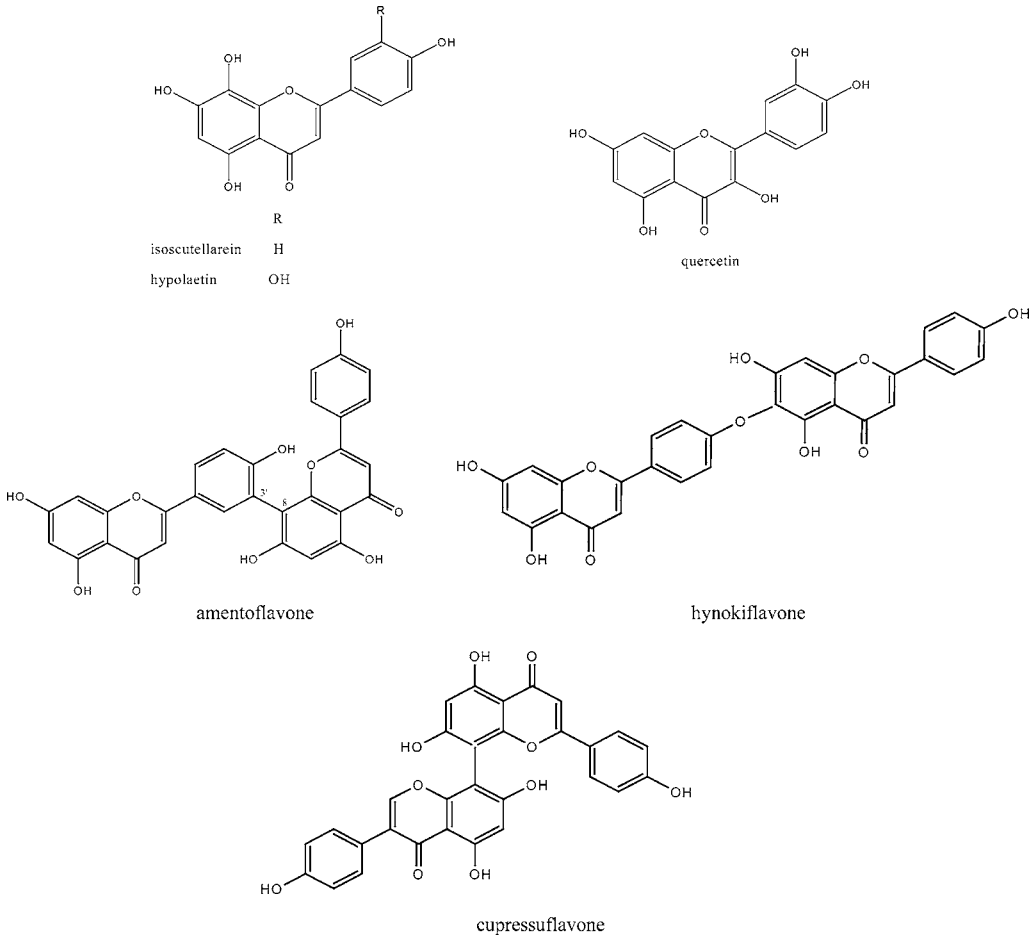

Figure 3. Chemical structures of the main identified compounds.

Table 1.

peak	compound	t_R	λ_{max} (nm)	$[M - H]^-$; fragment ions	refs
1	quercetin-hexoside-deoxyhexoside	12.7	254; 348	609; 463 $[M - H - 146]^-$ 301 [quercetin – H]$^-$	
2	quercetin-3-*O*-pentoside	15.3	254; 350	433; 301 [quercetin – H]$^-$	
3	isoscutellarein-8-*O*-hexoside[b]	15.9	270; 300; 330	447; 285 [isoscutellarein – H]$^-$	*13*
4	hypolaetin 7-pentoside	16.8	257; 276; 300, 342	433; 867 $[2M - H]^-$; 301 [8-hydroxyluteolin – H]$^-$	*11*; *14*; *15*
5	apigenin-hexoside	17.4	268; 340	431; 269 [apigenin – H]$^-$	
6	luteolin-pentoside	18.6	254; 348	417; 285 [luteolin – H]$^-$	
7	hypolaetin-hexoside[c]	19.3	254; 272; 298; 346	463; 867 $[2M - H]^-$; 301 [8-hydroxyluteolin – H]$^-$	*11*; *14*; *15*
8	isoscutellarein-7-*O*-pentoside	20.6	275; 303; 328	417; 285 [isoscutellarein – H]$^-$; 835 $[2M - H]^-$	*12*; *14*
9	apigenin	30.9	269; 338	269	standard
10	cupressoflavone	33.2	272; 328	537	standard
11	amentoflavone	35.4	269; 338	537	standard
12	biflavone	36.6	270; 340	537	
13	methyl-biflavone	39.8	268; 332	551	
14	hynokiflavone	40.3	270; 338	537	standard
15		41.2	290; 332	319	
16	methyl-biflavone	41.5	272; 334	551	

[a] In mix H_2O/CH_3CN. [b] Not in agreement with data from refs *12* and *14*. [c] The link position is not indicated due to the impurity of the chromatographic peak (see **Figures 1** and **2**).

(**Figures 1** and **2**), and among them isoscutellarein and 8-hydroxyluteolin glycosides together with six biflavonoids were detected.

All of the flavonoids and biflavonoids were identified through the use of pure commercial standards and by comparison with literature data relating to their UV−vis, MS spectra, and retention times in reversed phase. All of the detected metabolites are shown in **Table 1**, and **Figure 3** gives their chemical structures.

Our discussion focuses on the identification of the most interesting compounds.

Compounds **3** and **8** were identified as two glycosides of isoscutellarein with the sugar moiety linked to the hydroxyl group in the 8- or 7-position, respectively. Due to the absence of pure standards, for attribution of the glycoside bond positions, comparison of their UV−vis spectra with literature data (*12*, *13*) was diagnostic. Due to a certain disagreement in the literature on this matter, we selected as reference a paper that describes a complete structural characterization of the isolated isoscutellarein 8-*O*-β-glucuronopyranoside (*13*). The λ_{max} values of absorption (271, 300, 330 nm) of this compound are nearly superimposible with those of compound **3** identified in juniper

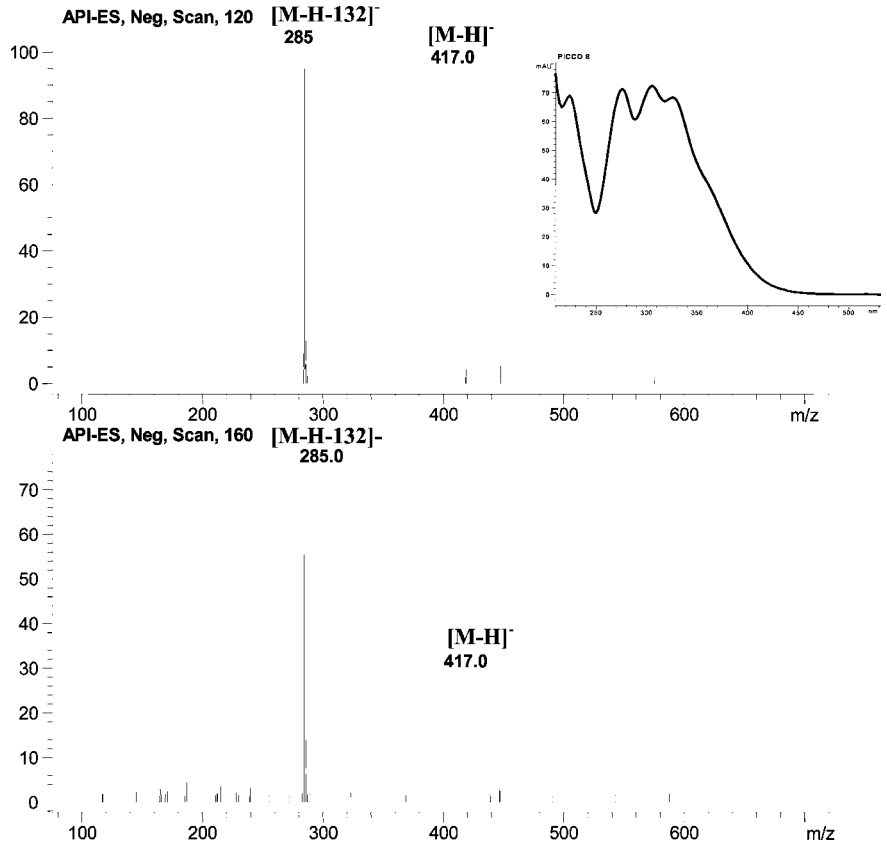

Figure 4. MS spectra in negative ionization mode at fragmentors 120 V (**a**) and 160 V (**b**) of the isoscutellarein 7-pentoside (**8**).

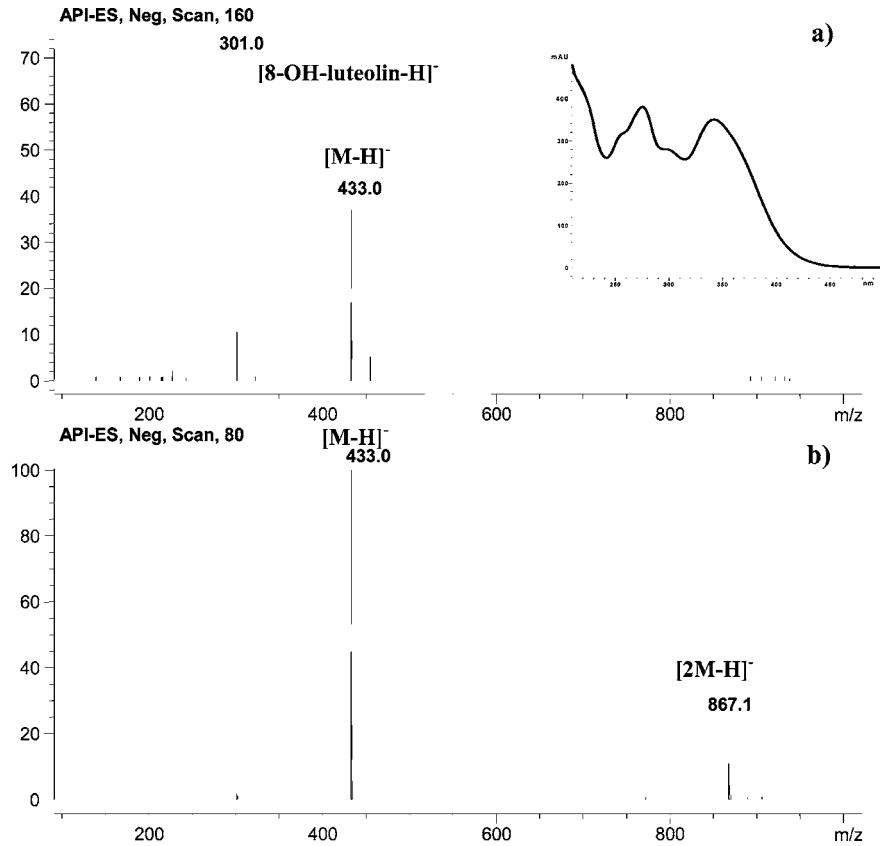

Figure 5. MS spectra in negative ionization mode at fragmentors 160V (**a**) and 80 V (**b**) of the hypolaetin 7-pentoside (**4**).

and, thus, in accordance with our data. Their mass spectra showed the presence of the ions attributable to the aglycone isoscutellarein, *m*/*z* 285, and the quasi-molecular ions at *m*/*z* 447 and 417, for the mono-*O*-hexosyl (**3**) and mono-*O*-pentosyl (**8**), respectively. Moreover, for compound **8**, probably due to its higher concentration with respect to compound **3**, the ion

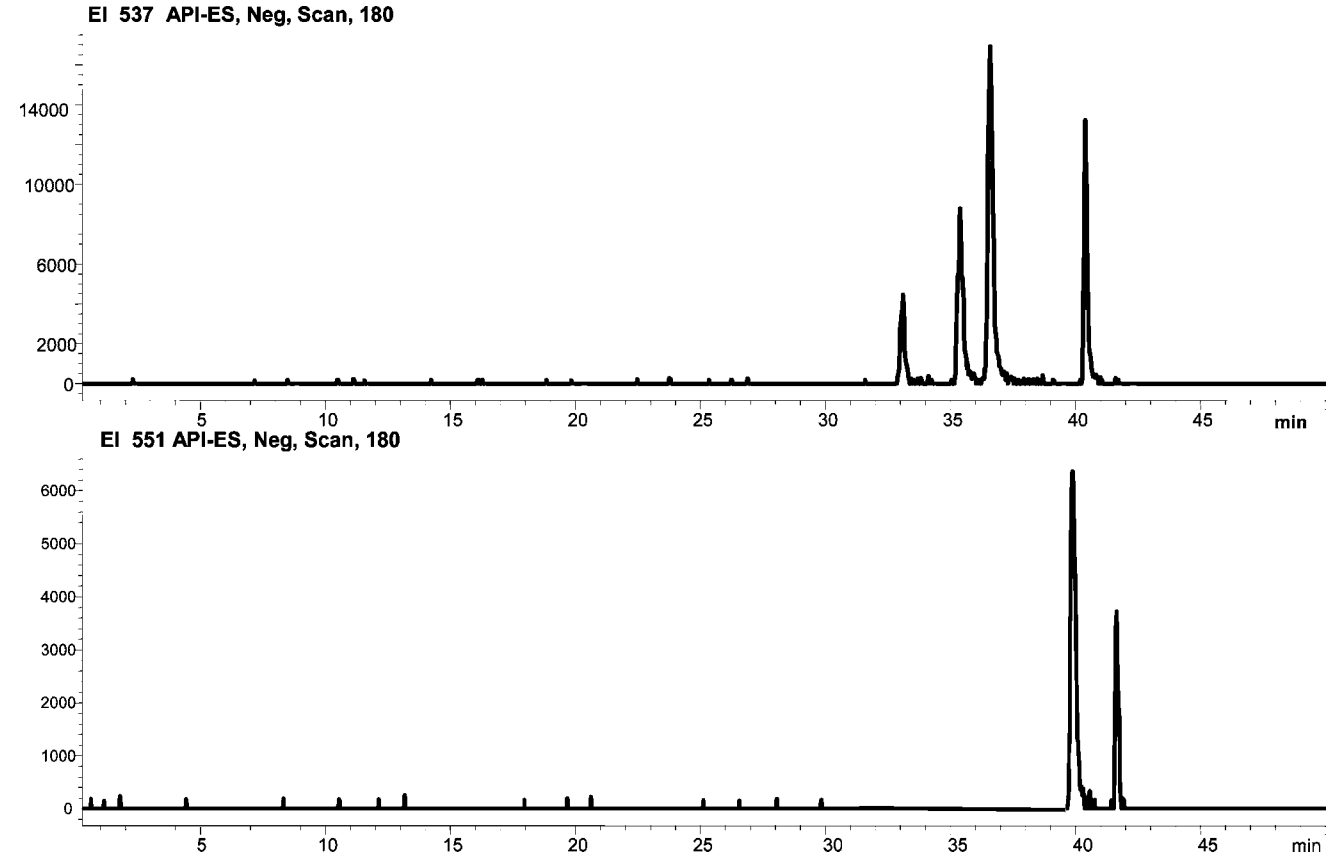

Figure 6. EI profiles at *m*/*z* 537 and 551 of the biflavonoidic compounds in ripe REG berries.

Table 2. Quantitation of Flavonoids (Calculated as Rutin) and Biflavonods (Calculated as Amentoflavone), Expressed in Micrograms per Gram of Fresh Weight with RSD Percent of Three Different Determinations

	flavonoids											biflavonoids								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	total	RSD %	**10**	**11**	**12**	**13**	**14**	**15**	**16**	total	RSD %
VE g[a]	490	14	10	658	91	10	50	346	0	1670	2.28	28	61	26	25	0,061	0	21	220	2.47
VE r[a]	1100	61	40	1455	169	10	177	75	32	3790	0.34	44	150	70	46	0,141	0	63	510	0.82
MB g	1180	72	30	893	263	17	37	257	33	2790	4.5	46	133	230	360	0,6	11	0	1370	1.00
MB r	692	26	0	429	13	8	17	149	0	1460	2.56	33	91	120	200	0,343	6	0	800	2.41
REG g	783	55	10	697	105	0	19	255	0	1920	0.29	10	91	61	61	0,092	21	60	400	2.58
REG r	783	162	0	1245	163	0	91	722	0	3170	4.52	33	91	120	200	0,343	6	0	140	1.18

[a] g, green (unripe berries); r, red (ripe berries).

relative to the dimeric form ($[2M - H]^-$ at *m*/*z* 835) was also detected (**Table 1**).

Analogously, compounds **4** and **7** were tentatively identified as two monoglycosides of 8-hydroxyluteolin or hypolaetin. Again, the MS spectra were a very diagnostic tool for the discrimination of the kind of sugar linked to the aglycone. In addition to the ion of the aglycone, at *m*/*z* 301 (8-hydroxyluteolin), the presence of the $[M - H]^-$ ions, at *m*/*z* 433 and 463, confirmed the presence of a pentosyl group for compound **4** (**Figure 5**) and of a hexosyl group for compound **7**. Moreover, our spectroscopic measurements were in agreement with the λ_{max} values reported in the literature by several authors (*11*, *14*, *15*) for different glycosides of the same aglycone.

With regard to the class of biflavonoids, the overlapping of the UV−vis spectrum of apigenin (**9**) with those of the biflavonoid derivatives (**10**−**16**) confirmed the presence of bi-apigenin compounds. Due to their difficult ionization the MS spectra for this class of compounds were not very diagnostic. In all cases and also applying higher fragmentation energies, only the molecular ion $[M - H]^-$, without any other fragments useful for the structural identification, was detected.

As an example, **Figure 6** shows the EI profiles of the ripe berry extract of the REG sample, at *m*/*z* 537 for detecting biflavonoids, obtained by applying a high fragmentation energy.

Compounds **10**, **11**, and **14**, by comparison of their retention times (t_R), UV−vis spectra, and molecular ions with respect to those of authentic standards, were identified as cupressoflavone, amentoflavone, and hynokiflavone, respectively. Compound **12** is another isobaric isomer (MW = 538) of the previous biflavonoids. Compounds **13** and **16** showed higher molecular weights, 552 Da, and for these molecules, according to their t_R values indicating higher lipophilicity, the presence of two methyl-biflavones has been attributed. Moreover, these latter compounds were previously reported in the literature for other Cupressaceae plants (*16*, *17*).

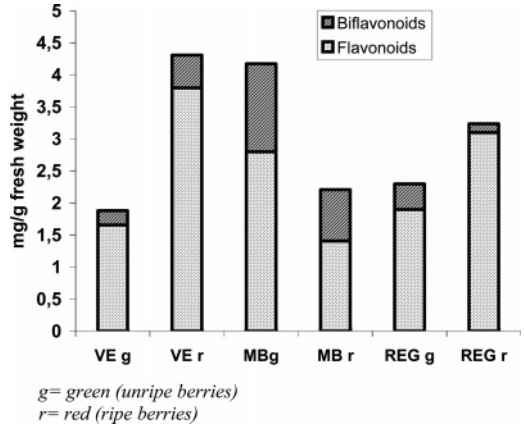

Figure 7. Histogram of the comparison between the flavonoidic and biflavonoidic content in the juniper berries expressed as milligrams per gram of fresh weight.

Quantitative Evaluation. Evaluation and expression of the quantitative results for a complex matrix, such as an herbal extract having the co-presence of several different structures, are often neither easy nor univocal. In this case we arbitrarily chose only two reference compounds that are easy to find on the market and not too expensive. From this perspective, rutin was believed to be suitable to quantify compound **1** (one of the main flavonoids of juniper) and all of the other flavonoids. **Table 2** reports the total flavonoid and biflavonoid contents of each sample expressed as milligrams of rutin and milligrams of amentoflavone per gram of fresh weight, respectively. For each sample the analyses were performed in triplicate, and their relative standard deviations (RSD %) were calculated and ranged between 0.3 and 4.5%.

To better highlight the most relevant differences between the analyzed samples, the total phenolic content is compared in the histogram in **Figure 7** showing each sample subdivided into flavonoids and biflavonoids. The flavonoid content in the analyzed berries ranged between 1.46 and 3.79 mg/g of fresh weight, whereas the amount of the biflavonoids was always lower, varying between 0.14 and 1.38 mg/g of fresh weight.

The chromatographic profile of the berries from MB was different from those for berries from the other two provenances; the unripe fruits showed higher flavonoid and biflavonoid contents compared to the corresponding ripe berries. In contrast, REG and VE varieties were more similar, and both showed a higher concentration of total phenols in the red berries. From **Figure 7** it emerges that the ripe fruits of MB and REG provenances showed lower amounts of biflavonoids with respect to the corresponding green berries.

These preliminary data suggest that phenols can discriminate among different populations, although clearly more extensive sampling will be required to study geographic variation of this species.

To the best of the authors' knowledge, this is the first report on the quali-quantitative estimation of flavonoids and biflavonoids in juniper berries. These latter compounds show noteworthy biological properties. Antifungal activities were highlighted for amentoflavone, cupressoflavone, and their methyl derivatives (*16*); antiviral properties against influenza and herpes viruses were showed for several biflavones (*18*); amentoflavone was recognized as an anti-inflammatory agent mainly by the inhibition of the phospolipase A2 and cyclooxygenase (*19*). More recently, also a DNA topoisomerase inhibitor activity was evidenced for amentoflavone and other methyl-biflavones (*20*). To date no data are available in the literature on the absorption and metabolism of these molecules. Our findings indicate that the juniper fruits are a new and inexpensive source of these metabolites not widely diffused in the vegetal kingdom.

Knowledge on the inheritance of phenols in this plant will greatly increase the utility of phenolic profiles as biochemical markers to define the pattern of differentiation of *J. communis* L. and to select superior chemotypes for specific commercial applications in the pharmaceutical and food and flavoring industries.

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