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Flavonoids Detection by HPLC-DAD-MS-MS in Lemon Juices from Sicilian Cultivars

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High-performance liquid chromatography−diode array detector (HPLC-DAD) coupled with electrospray mass spectrometry (ESI-MS) was used to detect the flavonoid profile in lemon juices obtained from the main Sicilian cultivars *(Femminello comune*, *Monachello*, and *Interdonato*). Significant amounts of an unusual constituent were found in the lemon juice of the above-mentioned cultivars together with eriocitrin, hesperidin, and diosmin. Following purification by preparative HPLC, the structure was assigned as 6,8-di-*C*-glucopyranosyldiosmetin by means of DAD-UV, ESI-MS-MS, and ^{1}H NMR analysis. Three other minor components were also detected. One of these presented a flavone nature, and spectral data and literature references both suggested a 6,8-di-*C*-glucopyranosylapigenin structure. The different contents of eriocitrin, hesperidin, diosmin, and above minor components in the cultivars allow juices to be readily differentiated.

KEYWORDS: Lemon juice; flavonoids; HPLC-DAD-ESI-MS-MS; NMR

INTRODUCTION

Flavonoids have antiviral and antiinflammatory properties, affect capillary permeability and fragility, and observedly inhibit human platelet aggregation (*1*−*10*). Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants in vitro than vitamins E or C (*11*) and that their consumption is associated with a reduced risk of cancer (*12*−*14*).

Flavonoid glycosides, found in citrus leaf, flavedo, albedo, and juice, present a typical distribution pattern in different species or parts of fruits (*15*−*20*). Naringin and narirutin together with small amounts of hesperidin and neohesperidin characterize the grapefruit species (*21*). Naringin, neohesperidin, and, to some extent, neoeriocitrin are distinctive of sour orange (*22*). Hesperidin, narirutin, and small amounts of didymin are present in sweet oranges (*16*, *18*).

Lemon peel flavonoids are characterized by the presence of four groups of flavonoids: flavone-*O*-glycosides (luteolin-7-rutinoside and diosmin being the most important); flavone-*C*-glycosides (6,8-di-*C*-glucosyl-apigenin, 6,8-di-*C*-glucosyl-luteolin, 6,8-di-*C*-glucosyl-chrisoeriol, 6,8-di-*C*-glucosyl-diosmetin); flavonols (rutin and three polymethoxyflavones), and flavanones (hesperidin and eriocitrin) (*23*−*26*).

Lemon juice flavonoids are characterized by the presence of the flavanone glycosides, hesperidin and eriocitrin, and the flavone glycoside diosmin (*27*, *28*). Quercetin and myricetin are also known to be present in lemon juice in very low concentrations (*29*).

In the present work, flavonoid profiles of lemon juice from three cultivars (*Femminello comune*, *Monachello*, and *Interdonato*) were studied in order to develop a method by which these can be differentiated on the basis of their HPLC-DAD 325 nm chromatogram patterns. Analysis by HPLC-DAD, ESI-MS-MS, and NMR spectrometry was applied to identify unusual components present in lemon juice.

MATERIALS AND METHODS

Materials. The juices were obtained from commercial ripe fruits grown in different areas in Sicily (Italy). An investigation was carried out on 40 samples obtained from each of the cultivars: *Femminello comune*, *Monachello*, and *Interdonato*. The peeled fruits were carefully squeezed by hand to avoid contamination of the juices by components in the peel.

Reagents and Standard Solutions. HPLC grade acetonitrile and acetic acid were supplied by Sigma-Aldrich; DMF was supplied by Carlo Erba; and hesperidin, eriocitrin, and diosmin standards were supplied by Extrasynthèse (France). The standards were diluted in DMF, and calibration lines were obtained using solutions of known concentration (10−50 mg/L for diosmin; 25−250 mg/L for hesperidin and eriocitrin).

Preparation of Samples. DMF (10.0 mL) was added to the samples of juices (10.0 mL), and the resulting solutions were filtered through ISO-DISC P-34, 3 mm diameter PTFE membrane, 0.45 μm pore size.

HPLC Analysis of Flavonoids. HPLC with gradient elution was used. The analyses were carried out using an HPLC instrument (Shimadzu model LC 10 AD) equipped with a diode array. Detection by diode array was performed simultaneously at three different wavelengths: 278, 325, and 350 nm. The UV spectra were recorded between 200 and 450 nm, as previously described (*20*). Each sample was tested three times and gave superimposable chromatograms. Peak

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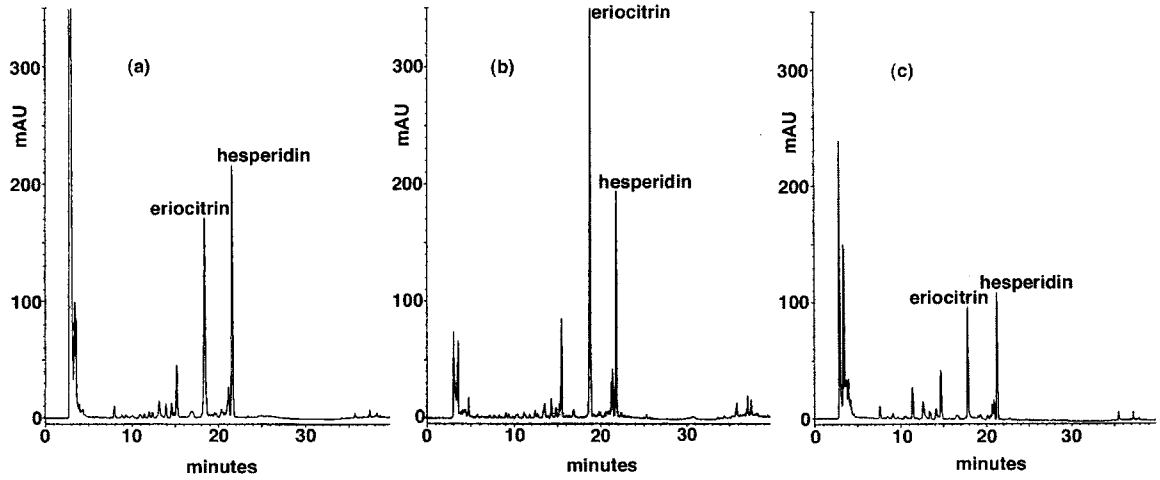


Figure 1. Typical chromatograms of lemon juices at 278 nm: (**a**) *Femminello comune*; (**b**) *Monachello*; (**c**) *Interdonato*.

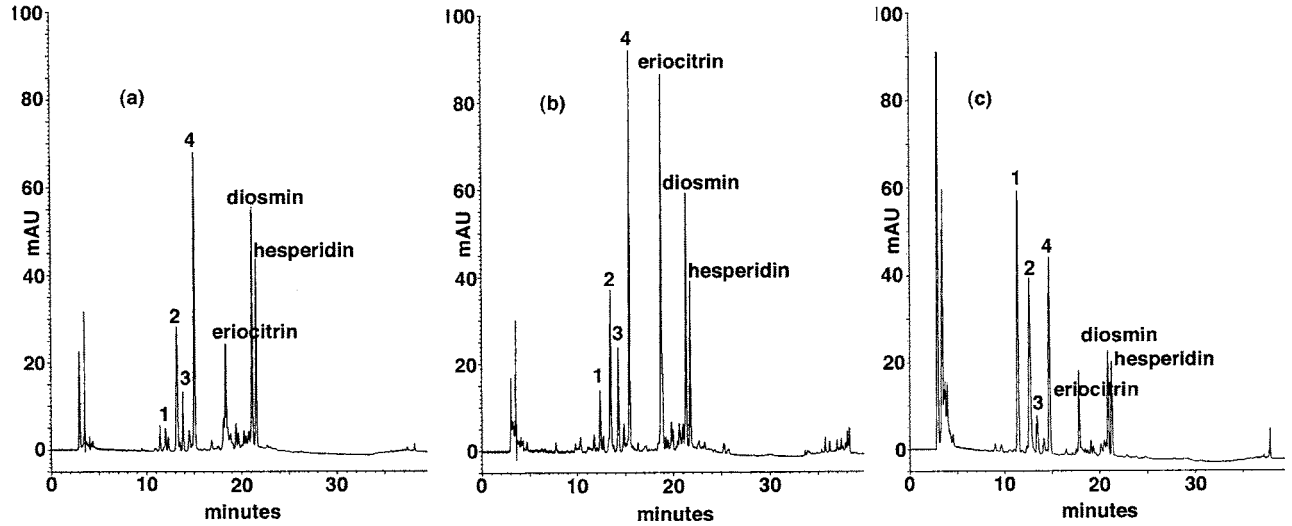


Figure 2. Typical chromatograms of lemon juices at 325 nm: (**a**) *Femminello comune*; (**b**) *Monachello*; (**c**) *Interdonato*.

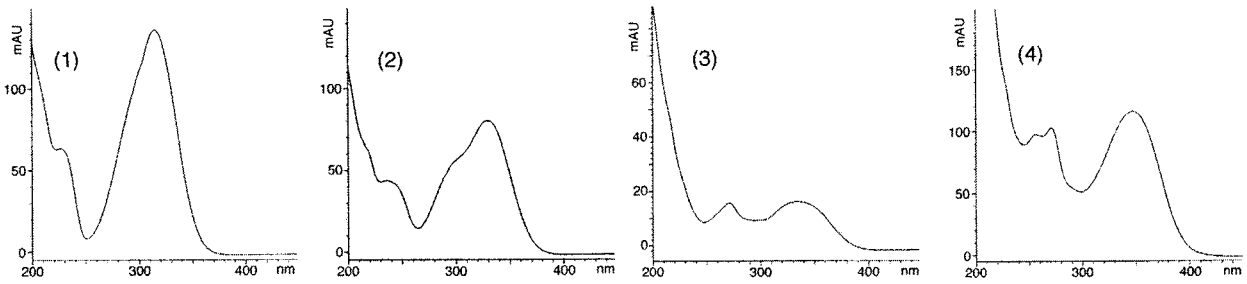


Figure 3. UV spectra of 1–4 components.

identification was carried out by direct on-line comparison of retention time (RT) and UV spectra of authentic samples. In the absence of commercial standards, UV peak areas were used to compare the flavonoid contents.

Liquid Chromatography (LC)−MS. HPLC-MS analyses of flavonoids were carried out on a ThermoQuest model LCQ-DUO ion trap mass spectrometer with an ESI source equipped with a diode array to perform an MS-MS analysis. Detection by diode array was performed simultaneously at three different wavelengths: 278, 325, and 350 nm. The UV spectra were recorded between 200 and 450 nm. The LC conditions have also been described previously (*20*). The mass analyzer was set to simultaneously record both positive and negative spectra in addition to the TIC. The mass analysis was carried out in full-scan mode in the range of 250−700 amu, using 3 ms for collection of the ions in the trap; 15 microscans were summed. Helium was used as the collision gas, and the collision energy was set at 25% for MS-MS spectra.

Preparative HPLC. Preparative HPLC with isocratic elution was carried out using an HPLC instrument (Shimadzu model LC 8 A) equipped with a UV detector. The column used was a Discovery C18 Supelco 250 mm × 21.2 mm, particle size 5 μm. The injection loop was 2.0 mL, and the flow rate was 10.0 mL/min at room temperature (20 °C). The mobile phase consisted of water (80%) and acetonitrile (20%). One component was eluted at RT 8.65 min and collected (about 5 mL) between RT 8.3 and 8.8 min.

Acid Hydrolysis. Four fractions of the above components (RT 8.65) recovered from 8 mL of juice were dried under vacuum. The residue was dissolved in methanol/water (1:1, v/v, 2.0 mL). HCl (2 N, 0.5

Table 1. Average Values ($\bar{x}$), Standard Deviations (s), and Ranges (mg/L) of Eriocitrin, Hesperidin, and Diosmin in Single Strength Lemon Juice, Based on 8° Brix

	eriocitrin		hesperidin		diosmin		eriocitrin/hesperidin	
	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)
Femminello comune	138 ± 20	105–188	153 ± 28	101–197	47 ± 8	32–67	0.9 ± 0.2	0.6–1.3
Monachello	228 ± 30	190–298	126 ± 15	102–162	44 ± 6	33–55	1.8 ± 0.3	1.5–2.6
Interdonato	103 ± 13	84–139	107 ± 12	88–140	32 ± 4	25–39	1.0 ± 0.1	0.9–1.2

Table 2. Peak Areas of Components 1–4 at 325 nm

	Femminello comune	*Monachello*	*Interdonato*
1	84	124	582
2	326	405	450
3	150	163	112
4	600	678	485

mL) was added, and the solution was refluxed for 8 h. After it was filtered through ISO-DISC P-34, 3 mm diameter, PTFE membrane, 0.45 μm pore size, the solution was analyzed by HPLC.

^{1}H NMR. ^{1}H spectra were recorded in DMSO, containing tetramethylsilane as an internal standard, with a Varian Gemini 300 spectrometer at 300 MHz.

RESULTS AND DISCUSSION

The profiles of flavonoids (**Figures 1** and **2**) in single strength lemon juice of the Sicilian cultivars *Femminello comune*, *Monachello*, and *Interdonato* were obtained by direct HPLC injection and DAD detection at 278 and 325 nm, respectively. Eriocitrin, diosmin, and hesperidin were identified. In all cultivars, four peaks (1−4) were also detected at 325 nm. Although diosmin and peak 4 have a higher absorption at 350 nm, we report the chromatograms at 325 nm in order to enhance, within the same profile, components 1 and 2, which have maximums at 315 and 330 nm, respectively.

Table 1 summarizes the contents of eriocitrin, hesperidin, and diosmin found. All values are based on 8° Brix. The flavonoid concentrations in *Femminello comune* and *Monachello* appear higher than in *Interdonato*. In all cultivars, the amounts of eriocitrin and hesperidin are greater than diosmin. The observed pattern of flavonoids allows the three cultivars to be easily characterized. In *Femminello comune*, hesperidin and eriocitrin are present in similar concentrations (about 150 mg/L). In *Interdonato*, the hesperidin and eriocitrin concentrations have the same 1:1 ratio but with lower overall values (100 mg/L). In *Monachello*, eriocitrin is the main component (about 220 mg/L) with an eriocitrin/hesperidin ratio of 2:1. Diosmin concentration is about the same in all cultivars. **Table 2** shows the peak areas at 325 nm of components 1−4 in the three cultivars. Peak 4 was the major component in *Femminello comune* and *Monachello*, while the *Interdonato* cultivar was characterized by a specific pattern showing components 1, 2, and 4 in a close ratio of 1:1:1.

Figure 3 shows the UV spectra of components 1−4. The flavonic nature of components 3 and 4 may be inferred because of the typical pattern of band I (associated with absorption due to the B ring cinnamoyl system, centered at 334 and 347 nm, respectively) and band II (due to the A ring benzoyl system centered at 270 and 271 nm, respectively).

The ESI-MS analysis of DAD peaks 3 (RT 13.8) and 4 (RT 14.6) showed two single components with molecular ions $[M + H]^+$ 595 and 625, respectively. The MS spectra of 1 (RT 12.1) and 2 (RT 13.1) appear more complex and suggest the presence of a mixture of components. Components 3 and 4 were

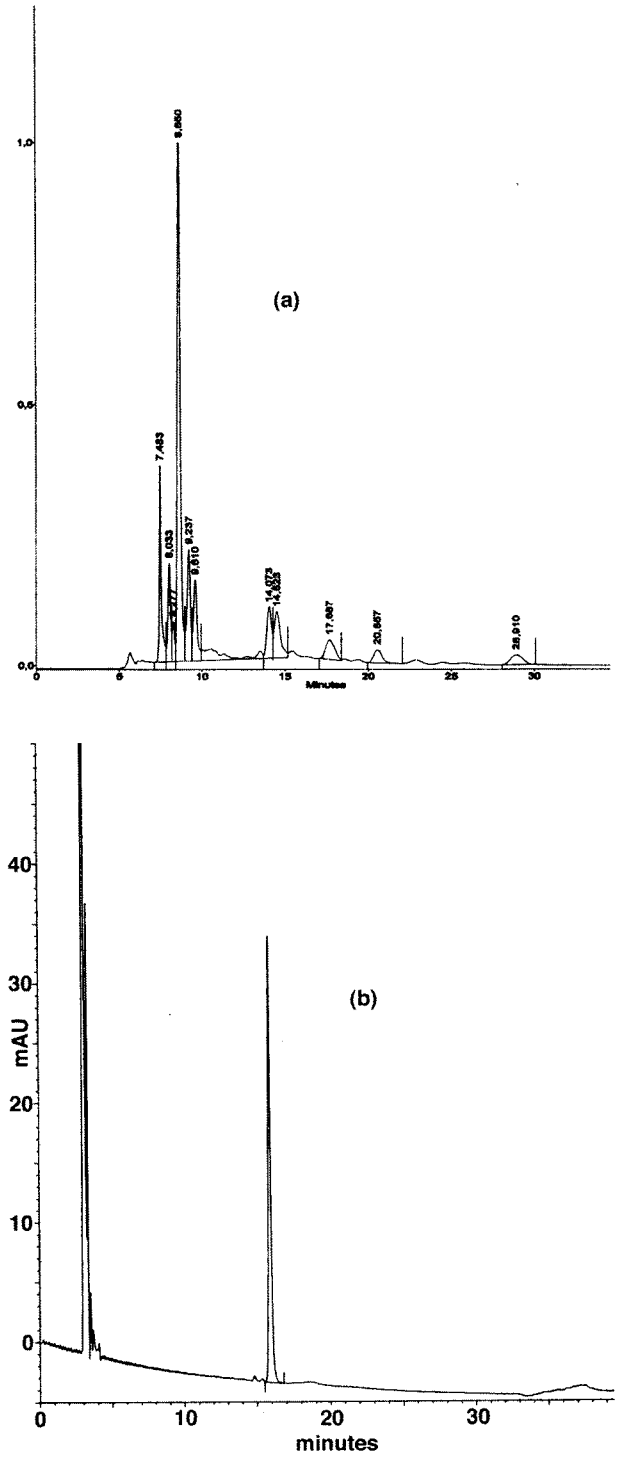


Figure 4. (**a**) Preparative chromatogram of lemon juice. (**b**) Analytical HPLC chromatogram of fraction collected between RT 8.3 and 8.8 min.

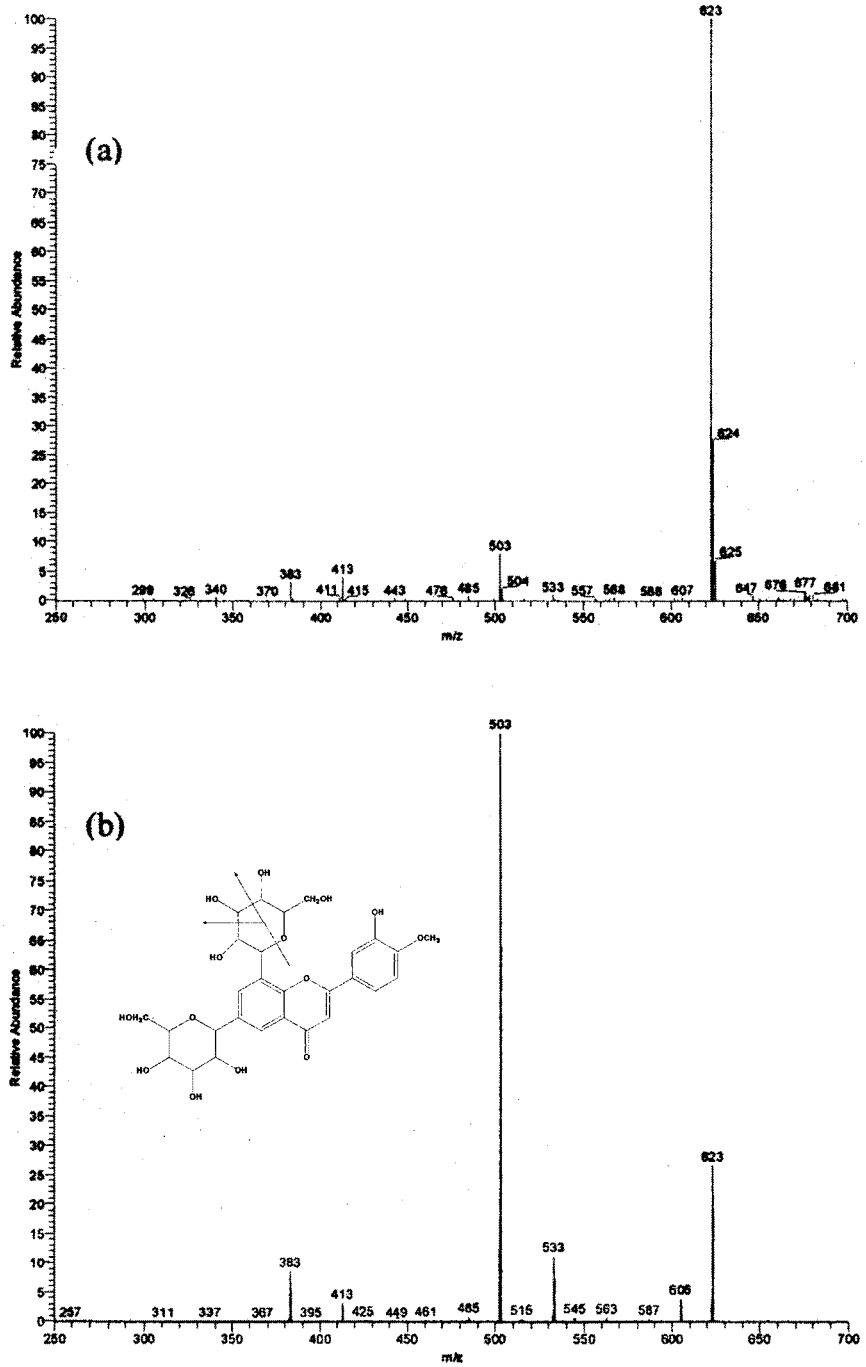


Figure 5. (**a**) Negative ions ESI-MS and (**b**) ESI-MS-MS spectrum of component 4.

tentatively separated by preparative HPLC. The purification of 3 failed because of the nature of minor components. However, 4 was recovered as a viscous oil.

Figure 4a shows a strong peak centered at RT 8.65 min together with three minor others on the chromatogram at 350 nm carried out on the preparative HPLC. The analysis of collected fractions between RT 8.3 and 8.8 min on the analytical HPLC showed the presence of one only peak centered at RT 15.83 min (**Figure 4b**), which presented the UV pattern reported in **Figure 3**. Both the above RT and the UV spectrum were superimposable with those related to the component 4 as recorded during the direct on line analysis of juice and demonstrated an effective purification of HPLC degree.

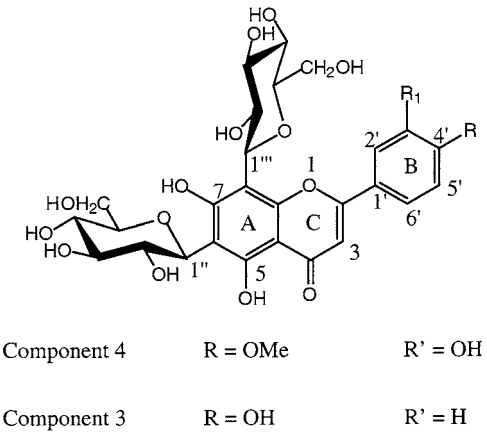
Separation of the very low peak of component 3 (corresponding to the peak 3 in **Figure 4a**) with respect to the neighboring bigger peaks was not useful to obtain fractions of a purified compound. Therefore, fractions collected in correspondence to the peaks 1 and 2 (**Figure 4a**) analyzed by the analytical HPLC showed the presence of several further peaks, which have so far eluted, at present, separation and identification. Because these components appear to be potential markers of the *Interdonato* cultivar, we are currently attempting to enounce their characterization.

The negative ion ESI-MS spectrum of 4, obtained by direct injection into the source (**Figure 5a**), shows an intense peak at 623 for the molecular mass $[M-H]^-$. **Table 3** reports the ions and their relative abundance originated by ESI-MS-MS in negative mode from the precursor ion *m*/*z* 623 (**Figure 5b**).

The MS-MS spectrum of $[M-H]^-$ presents some interesting evidence regarding the Di-glycosidic flavonoid structure of 4.

Table 3. Ions and Relative Abundance Originates from ESI-MS-MS in Negative Mode of the Precursor Ions *m/z* 593 and 623

negative ions	*m/z* 593	*m/z* 623
$[M–H]^-$	593 (18, 5)	623 (26, 5)
$[M–H – 18]^-$	575 (10, 5)	605 (3, 5)
$[M–H – 60]^-$	533 (2, 5)	
$[M–H – 90]^-$	503 (25, 5)	533 (11)
$[M–H – 120]^-$	473 (100)	503 (100)
$[M–H – 120 – 90]^-$	383 (4, 8)	413 (3)
$[M–H – 120 – 120]^-$	353 (11)	383 (8, 5)



Component 4 R = OMe R' = OH

Component 3 R = OH R' = H

Figure 6. Component 3: R = –OH, R_1 = –H. Component 4: R = $–OCH_3$, R_1 = –OH.

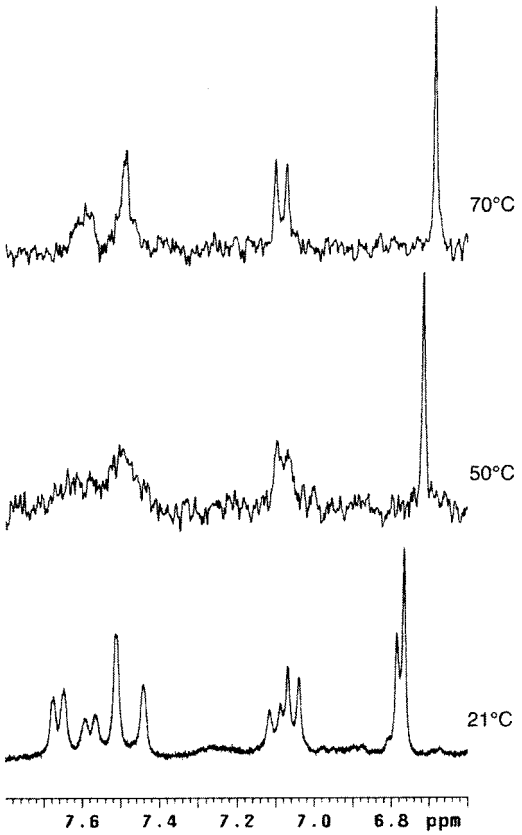


Figure 7. ^{1}H NMR spectrum of component 4 at 21, 50, and 70 °C.

The observed loss of H_2O $[M-H-18]^-$ as well as the $[M-H-90]^-$ and $[M-H-120]^-$ fragments appear characteristic of a *C*-glucosyl presence (*30*). Therefore, fragments *m/z* 413 and 383 are related to the fragmentation of a second glucosyl unit. Di-*C*-glucosyl substitution in component 4 rather than the di-*O*-glucosyl nature was clearly supported by the results of acid hydrolysis. In fact, because of the acid, hydrolysis did not break the C bond between cycle A and the sugar moiety, and the HPLC chromatograms of component 4 appeared identical before and after the hydrolysis step.

This evidence, together with the NMR spectra analysis described below, suggests the structure shown in **Figure 6** for component 4. The ^{1}H NMR spectrum of 4 in DMSO showed a pairing of major and minor peaks. The duplication of these signals can be attributed to the presence of two rotamers, one of which is present in a larger proportion. A similar pattern generated by the slow rotation around sugar *C*-aglycon bond behavior has been observed previously for synthetic *C*-8-glucosylflavones (*31*). **Figure 7** shows the ^{1}H NMR spectra recorded at 50 and 70 °C in the region of 6.7−7.8 δ.

Table 4 reports ^{1}H NMR spectral data for the two rotamers of 4. The ratio for the two rotamer populations by interpretation of the H3 peaks was calculated as ca. 1.5:1. Assignments were confirmed by homodecoupling experiments at both ambient and higher temperatures. The duplicated signals coalesced at higher temperatures. The spectra of the major rotamer at ambient temperature displayed signals of methoxyl protons at δ 3.88 (3H, s), an olefinic proton at δ 6.78 (1H, s), and a singlet and multiplet due to aromatic protons at δ 7.51 (1H, bs) and δ 7.07 (1H, bd) and δ 7.66 (1H, bd) respectively, which overall suggests the tetrasubstitution of the A ring and para/meta substitution of the B ring. The presence of two anomeric signals provides evidence for a disaccharide derivative. The large H1‴−H2‴ and H1″−H2″ coupling constants for the anomeric protons indicate β-configurations of the two glucosyl units. The ^{1}H NMR spectrum of the major rotamer was also in agreement with spectral data reported in the literature for 6,8-di-*C*-β-glucosyl-diosmetin isolated from lemon peel, although no other rotamers were cited as being present (*23*).

Table 4. ^{1}H NMR Data for Rotamers of Component 4 in DMSO at Ambient Temperature[a]

major rotamer (60%) (δ)	minor rotamer (40%) (δ)
7.66 (1H, bd, $J_{5',6'}$ = 8.0, H6′)	7.58 (1H, bd, $J_{5',6'}$ = 8.8, H6′)
7.51 (1H, bs, H2′)	7.44 (1H, bs, H2′)
7.05 (1H, bd, $J_{5',6'}$ = 8.0, H5′)	7.10 (1H, bd, $J_{5',6'}$ = 8.8, H5′)
6.77 (1H, s, H3)	6.78 (1H, s, H3)
4.79 (1H, d, $J_{1''',2'''}$ = 9.4, H1‴)[a]	4.96 (1H, d, $J_{1''',2'''}$ = 8.8, H1‴)[c]
4.74 (1H, d, $J_{1'',2''}$ = 10.4, H1″)[b]	4.65 (1H, d, $J_{1'',2''}$ = 10.4, H1″)[d]
3.88 (3H, s, –OMe)	3.86 (3H, s, –OMe)

[a] Calculated ratio of rotamers population (1, 5:1) was based on H3 signals. Coupling constants are given in Hz. *a*, *b* and *c*, *d* are interchangeable, respectively.

Therefore, UV data of 4 were well superimposable with the reported absorptions of above-described diosmetin derivative (*23*). Thus, the location of the methoxyl moiety in 4 was supported by the analysis of band I, which showed the presence of a shoulder centered at 243 nm rather than a strong absorption as in a chrysoeriol derivative (*32*).

Structural information on component 3 was derived from on line MS analysis of DAD peaks obtained by HPLC-MS injection of the juice together with UV spectral data. The negative ESI-MS spectrum presented a strong *m/z* 493 peak as $[M-H]^-$ (**Figure 8**). The ESI-MS-MS fragments originated from this ion by less of 90 and 120 mass counts (**Table 3**), which suggested

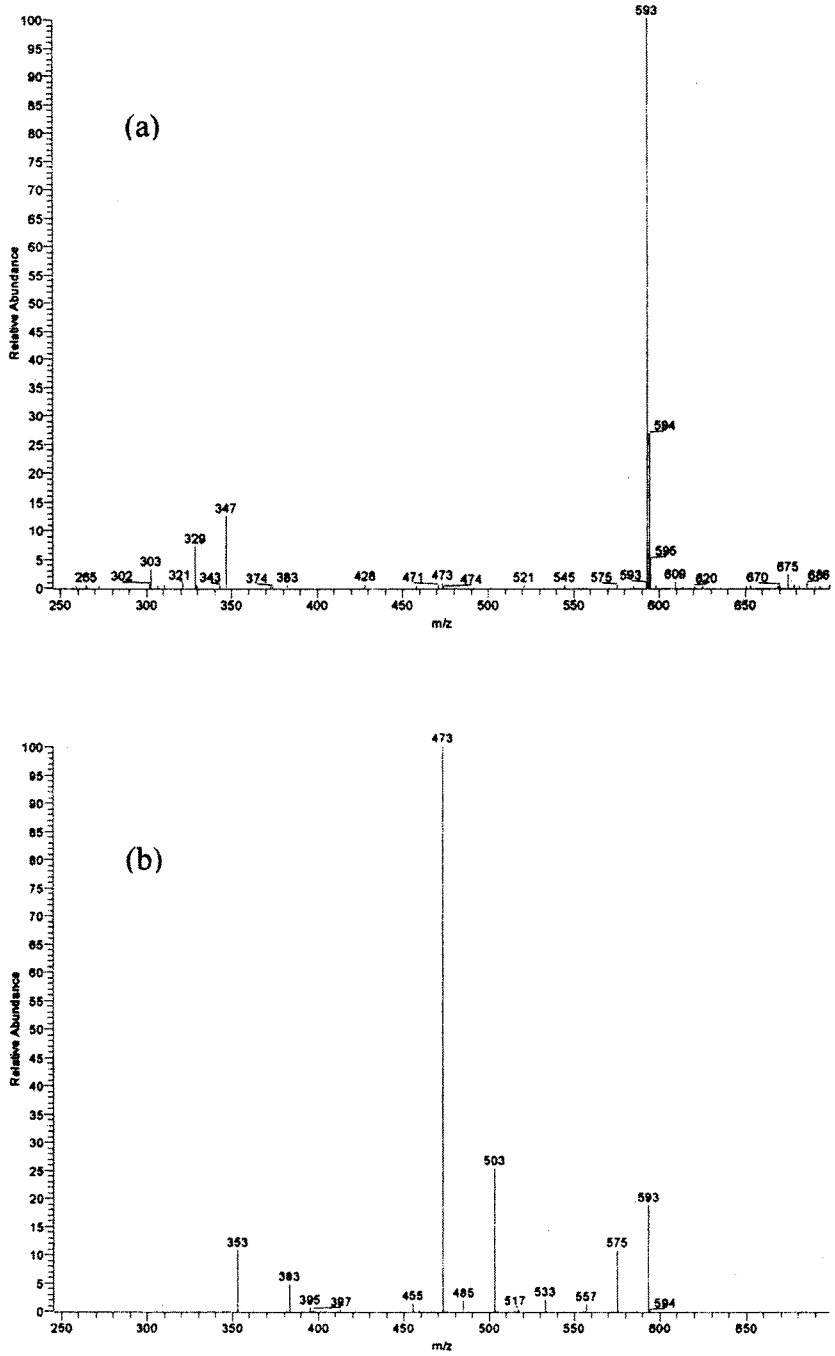


Figure 8. (**a**) Negative ions ESI-MS and (**b**) ESI-MS-MS spectrum of component 3.

the presence of two *C*-glucosyl moieties according to the fragmentation pattern shown in **Figure 5**. The comparison of molecular peaks of components 3 and 4 showed that in 3 one methoxyl group was missing. Thus, on the basis of the reported presence in Citrus species (*25*, *31*), the structure of 6,8-di-*C*-glucopyranosylapigenin is also suggested for component 3 (**Figure 6**).

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; DAD, diode array detector; ESI, electrospray ionization; MS, mass spectroscopy; ^{1}H NMR, proton magnetic resonance; DMF, dimethylformamide; PTFE, poly(tetrafluoroethylene); TIC, total ion current; DMSO, dimethyl sulfoxide.

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