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High-performance liquid chromatography–electrospray mass spectrometry in phytochemical analysis of sour orange (*Citrus aurantium* L.)

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Abstract

High-performance liquid chromatography coupled with a UV photodiode-array detector and an electrospray mass spectrometer (HPLC–ES-MS) was used to analyze the phytochemical constituents of an extract of sour orange (*Citrus aurantium* L., family Rutaceae). Eight flavonoids were identified, as isonaringin (**1**), naringin (**3**), hesperidin (**5**), neohesperidin (**6**), naringenin (**2**), hesperitin (**4**), nobiletin (**8**) and tangeritin (**7**). The permethoxylated flavones tangeritin (**7**) and nobiletin (**8**) showed the most intense protonated molecules. This method can be used to detect trace amounts of these compounds in an orange extract. © 1997 Elsevier Science B.V.

Keywords: *Citrus aurantium* L.; Phytochemical analysis; Orange fruit; Flavonoids

1. Introduction

A number of citrus species have been recorded in the Chinese Pharmacopoeia as appropriate for medical use. *Zhi Shi* (sour orange, also known as bitter orange) is the dried, immature fruit of *Citrus aurantium* L. As a traditional Chinese remedy, it has been used to activate vital energy and circulation, eliminate phlegm, and disperse stagnation [1]. Due to its sour and bitter taste, it has not been used as an edible fruit.

The primary active biological constituents of sour orange are flavonoids, of which it has a high content, and a sympathomimetic amine, synephrine (**11**) [2]. Citrus flavonoids, which occur principally in the peel, have been studied for a century. More than 60 flavonoids have been isolated and structurally determined [3]. Three types of flavonoids occur in citrus spp.: flavanones, flavones, and flavonols. Table 1 lists the structures of flavonoids found in *Citrus aurantium*.

The flavanones predominate among the citrus flavonoids, with flavones and flavonols present in considerably smaller amounts. While the flavonoids in citrus usually occur as glycosides, the permethoxylated flavones are an exception: they occur as free aglycones.

Many potentially health promoting effects have been ascribed to the citrus flavonoids [4,5].

Hesperitin (**4**) and naringenin (**2**) are effective at inhibiting the in vitro proliferation of human breast cancer cells [6]. Tangeritin (**7**) and nobiletin (**8**) are the most active antimutagens of the flavonoids tested so far, and may have chemopreventive potential [7].

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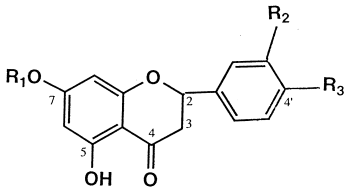
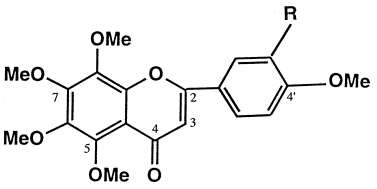
Table 1
Chemical structures of flavonoids and synephrine found in *Citrus aurantium*

Flavanones



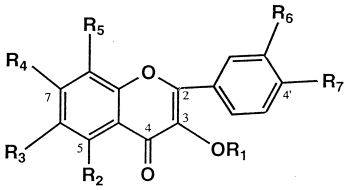
No.	Compound	R1	R2	R3	Mr
1.	Isonaringin	rutinose	H	OH	580
2.	Naringenin	H	H	OH	272
3.	Naringin	neohesperidose	H	OH	580
4.	Hesperitin	H	OH	OCH3	302
5.	Hesperidin	rutinose	OH	OCH3	610
6.	Neohesperidin	neohesperidose	OH	OCH3	610

Flavones



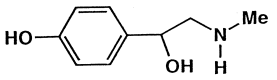
		R	Mr
7.	Tangeritin	H	372
8.	Nobiletin	OCH3	402

Flavonols



		R1	R2	R3	R4	R5	R6	R7	Mr
9	Auranetin	CH3	H	OCH3	OCH3	OCH3	H	OCH3	372
10	Quercetin	H	OH	H	OH	H	OH	OH	302

		Mr
11	Synephrine	167



The analysis of citrus flavonoids has become increasingly requisite. There have been several papers published on the HPLC analysis of citrus flavonoids [8–11]. However, each has paid attention only to an individual flavone. A recent paper reported the analysis of 25 standard citrus flavonoids using HPLC with a UV photodiode-array detector, but it did not show the HPLC chromatogram of the orange extract [12].

HPLC coupled with a UV photodiode-array detector and mass spectrometer provides more structural information on the compounds, thus enabling us to identify peaks more reliably. So far, there are no reports on the HPLC–ES-MS analysis of citrus flavonoids. Individual citrus flavonoids such as rutin (quercetin 3-β-rutinoside) and hesperidin (**5**) have been mentioned in papers dealing with the HPLC–thermospray-MS research [13]. Hesperidin and rutin

primarily showed aglycone molecular ions, but very weak glycoside molecular ions in thermospray-MS [14].

Our continuing research interests are focused on the HPLC–ES-MS analysis of botanical extracts, so that we can observe which types of natural products molecules are appropriate to be ionized for ES-MS analysis.

2. Experimental

2.1. Instrumentation

An HP 1090 Series II HPLC (Hewlett-Packard, CA, USA) with a photodiode-array detector set at 290 nm was coupled with a HP 5989 B quadrupole mass spectrometer. UV spectra were taken in the region of 200–500 nm. Chromatographic conditions were as follows: column, Waters Symmetry C_{18}, 2.1×150 mm, 5 μm (Waters, Milford, MA, USA); eluent, (A) water (0.6% HOAc), (B) methanol. The gradient elution had the following profile: 0–12 min 20–40% B; 12–19 min 40% B; 19–30 min 40–100% B; 30–33 min 100% B; 33–35 min 100–20% B. The flow-rate was 0.2 ml/min; temperature was 45°C.

Mass range measured, 150–900 u; quadrupole temperature, 150°C; EM, 2173 V. The spectra were acquired in the positive mode. The ES interface was an HP 59987A; drying N_2, temperature 360°C, 40 ml/min; nebulizing N_2, 5.5×10^5 Pa (80 p.s.i.). HPLC–MS connection was through an I.D. 0.007″ stainless steel tubing. The tubing length from outlet of UV detector to MS inlet was 0.6 m.

2.2. Standard citrus flavonoids and reagents

Naringenin (**2**), naringin (**3**), hesperidin (**5**), neohesperidin (**6**) and synephrine (**11**) were purchased from Sigma (St. Louis, MO, USA). Tangeritin (**7**) was purchased from Indofine (Somerville, NJ, USA). MeOH and H_2O are HPLC grade (VWR, Seattle, WA, USA).

2.3. Plant material and sample preparation

A 4.3-mg quantity of naringin (**3**), naringenin (**2**), and hesperidin (**5**), 5.0 mg of neohesperidin (**6**) and 2.6 mg of tangeritin (**7**) were dissolved in 50 ml methanol. A 3-μl volume of the mixed sample solution was injected into the HPLC column. Five mg of synephrine (**11**) was dissolved in 50 ml methanol.

Dried *Zhi Shi* fruit was purchased from Tai Sang Trading Co. (San Francisco, CA, USA), and 1.2 g was ground in a mill. Samples were extracted with 50 ml of 80% ethanol at 90°C for 2 h. The ethanol solution was filtered and concentrated to dryness at vacuum. The total 120 mg extract was dissolved in 50 ml methanol and filtered through a 0.45-μm nylon Acrodisk 13 mm filter. A 3-μl volume of the sample solution was injected into the HPLC column.

3. Results and discussion

3.1. HPLC–ES-MS of five standard citrus flavonoids

It is extremely difficult to obtain the molecular ions of flavonoid glycosides in electron impact (EI) mass spectra. Fast atom bombardment (FAB) enables the direct MS determination of molecular weights of flavonoid glycosides. Pseudomolecular ions of flavonoids with no more than two sugars can be observed in desorptional chemical ionization MS (D/CI-MS) [15]. Electrospray MS is also a soft ionization technique that mainly forms protonated molecules $[M+H]^+$ or adduct ions; in most cases, no fragment ions are observed.

Fig. 1 shows the simultaneous HPLC–ES-MS and HPLC–UV chromatogram of five standard citrus flavonoids. Peaks 1, 2 and 3 are the saturated flavanone diglycosides naringin (**3**), hesperidin (**5**) and neohesperidin (**6**). They showed less ionization than the aglycone naringenin (**2**) of peak 4, but their molecular ions are still strong.

Peak 5 is the unsaturated flavone aglycone tangeritin (**7**), which has five methoxy groups. It was the most ionized, showing a very intense peak on the HPLC–ES-MS chromatogram. In comparison with our previous work, unsaturated flavone mono-glycosides from red clover were more ionized than flavanone diglycosides, showing very abundant protonated molecules [16].

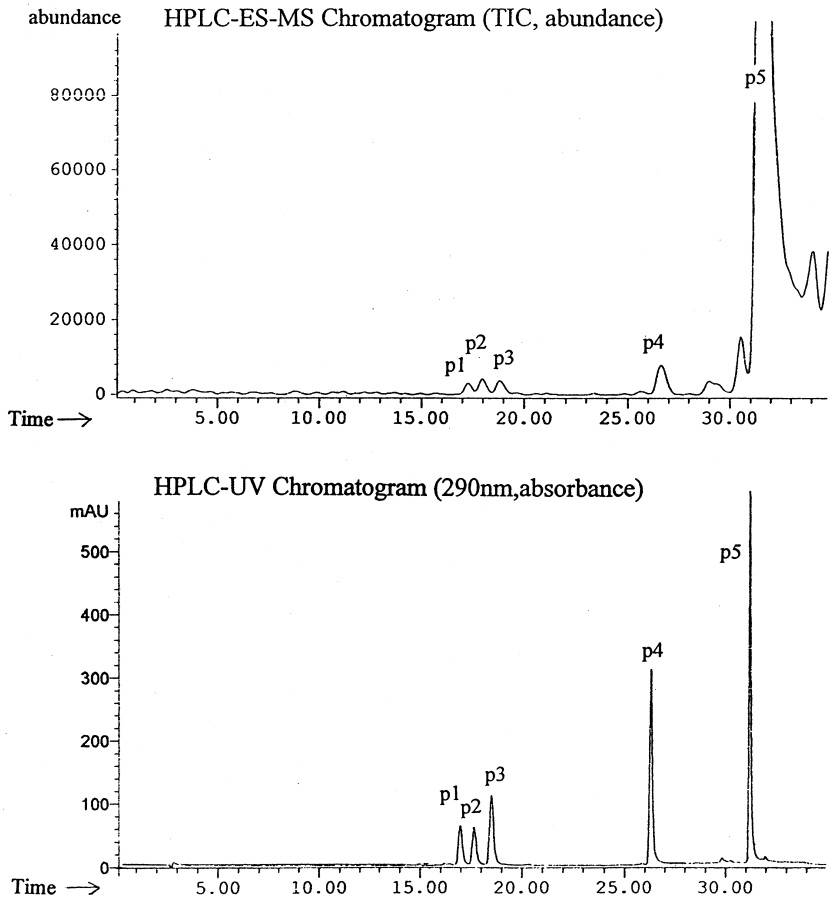


Fig. 1. Simultaneous HPLC–UV and HPLC–ES-MS chromatogram of citrus flavonoid standards, without post-column stream splitting. Chromatographic conditions were as described in Section 2. The following compounds are indicated: p1, naringin; p2, hesperidin; p3, neohesperidin; p4, naringenin; and p5, tangeritin.

Table 2 shows the retention time (t_R), UV λ_{max} values, and molecular ions of five standard flavonoids. Their mass spectra are shown in Fig. 2. Naringin (**3**) shows both an intense molecular ion $[M+H]^+$ at m/z 581 and an intense aglycone molecular ion $[A+H]^+$ at m/z 273, resulting from the elimination of two sugars. An ion at m/z 356 was unexpected, probably resulting from $[A+H+HOAc+Na]^{2+}$. Hesperidin (**5**) shows an intense molecular ion $[M+H]^+$ at m/z 611, a weak adduct ion $[M+Na]^+$ at m/z 633, and no aglycone molecular ion $[A+H]^+$ at m/z 303. An ion at m/z 371

Table 2
The values of t_R, $[M+H]^+$, $[M+Na]^+$, UV λ_{max} of citrus standard flavonoids

Peak no.	Compound	t_R(min)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	$[A+H]^+$ (m/z)	Other ions (m/z)	UV λ_{max} (nm)
p1	Naringin	16.9	581	—	273	356	284, 328 low
p2	Hesperidin	17.6	611	633	—	371	283, 326 low
p3	Neohesperidin	18.5	611	633	303	371	285, 330 low
p4	Naringenin	26.3	273	—	—	—	289, 326 sh
p5	Tangeritin	31.1	373	395	—	—	270, 325

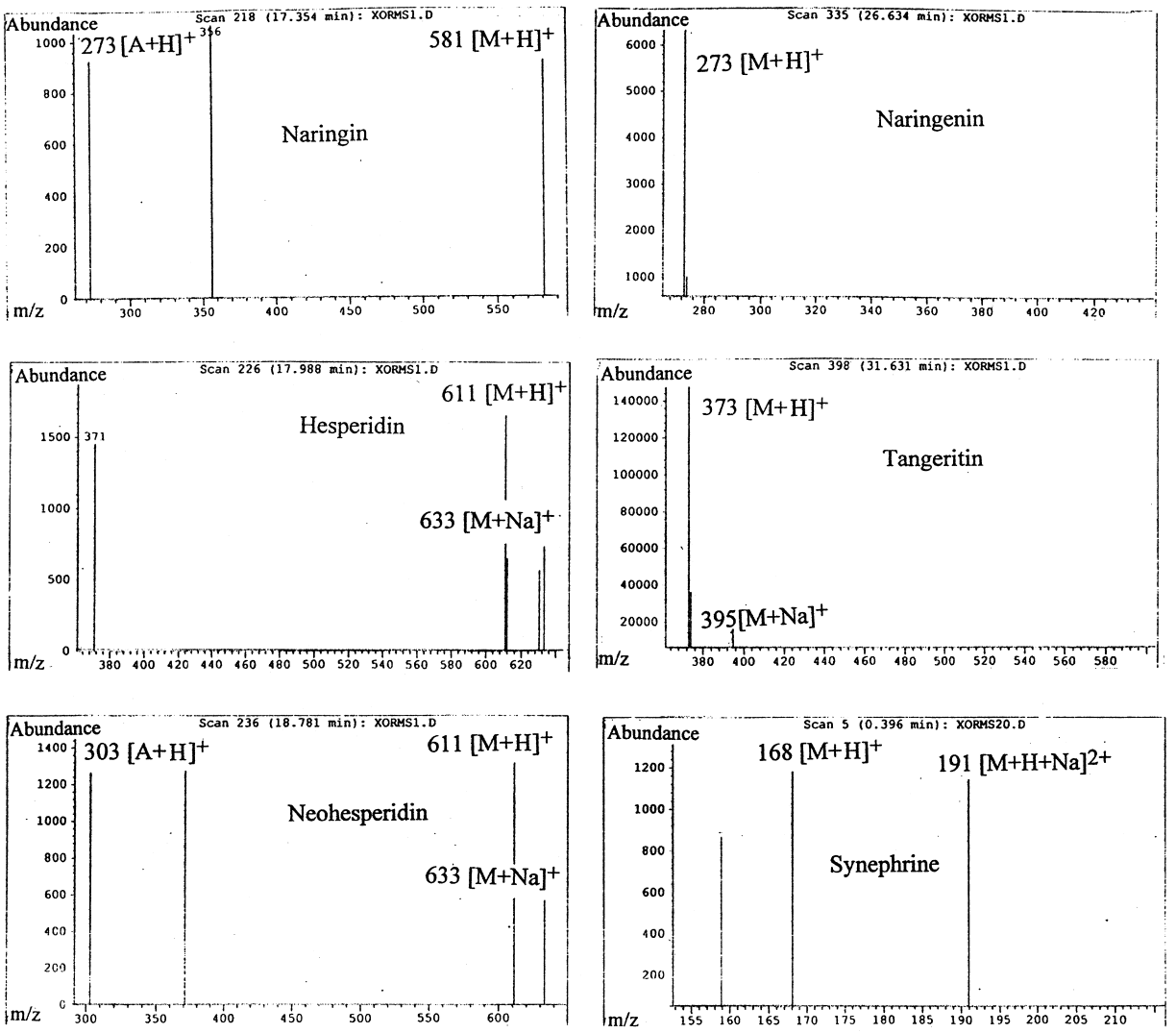


Fig. 2. Mass spectra of naringin, hesperidin, neohesperidin, naringenin, tangeritin, and synephrine.

probably resulted from $[A+H+CH_3OH+2H_2O]^+$. Neohesperidin (**6**) shows an intense molecular ion $[M+H]^+$ at m/z 611, a weak adduct ion $[M+Na]^+$ at m/z 633, an intense aglycone molecular ion $[A+H]^+$ at m/z 303, and an ion $[A+H+CH_3OH+2H_2O]^+$ at m/z 371 as well.

Y.Y. Lin et al. [13] reported that hesperidin (**5**) in thermospray MS showed a weak ion $[M+H]^+$ at m/z 611 (10% relative abundance), a major aglycone ion [M−rhamnoglucosyl moieties]$^+$ at m/z 303 (100% relative abundance) and [M−rhamnosyl]$^+$ at m/z 465 (8% relative abundance). This was probably due to the heating of thermospray, resulting in loss of the first sugar from the hesperidin molecule. However, electrospray operates without heat input into the spray ionization step. Therefore, there was no ion at m/z 465 to be produced.

Naringenin (**2**) only shows an intense molecular ion $[M+H]^+$ at m/z 273. Tangeritin (**7**) shows a very intense molecular ion $[M+H]^+$ at m/z 373 and a weak adduct ion $[M+Na]^+$ at m/z 395. Although the tangeritin sample concentration is less than that

of the other compounds, it shows the highest signal intensity.

3.2. HPLC–ES-MS of an 80% ethanol extract of Citrus aurantium

The HPLC–ES-MS of ethanol extract of *Citrus aurantium* is shown in Fig. 3. The identification of individual peaks is shown in Table 3. Peaks 2, 3, 4, 5, 6, and 11 are easily identified as isonaringin (**1**), naringin (**3**), hesperidin (**5**), neohesperidin (**6**), naringenin (**2**), and tangeritin (**7**), based on the comparison of their retention times, UV spectra and molecular ions with those of the standard compounds or literature data [9].

The isomeric pairs naringin (**3**) and isonaringin (**1**), hesperidin (**5**) and neohesperidin (**6**) differ in the structure of the disaccharides attached at the 7 position. The rutinosides eluted prior to the respective neohesperidosides; these results are the same as those reported in the literature [9]. Peak 10 shows an intense molecular ion $[M+H]^+$ at m/z 403 and a weak adduct ion $[M+Na]^+$ at m/z 425. It was identified as nobiletin (**8**). Peak 8 shows only one intense molecular ion $[M+H]^+$ at m/z 303, the hesperitin (**4**) molecule. The identity of peak 8 was confirmed as hesperitin (**4**) by hydrolysis of hesperidin (**5**) and HPLC analysis of the hydrolyzed product.

Both peaks 6 and 9 have three intense ions, with

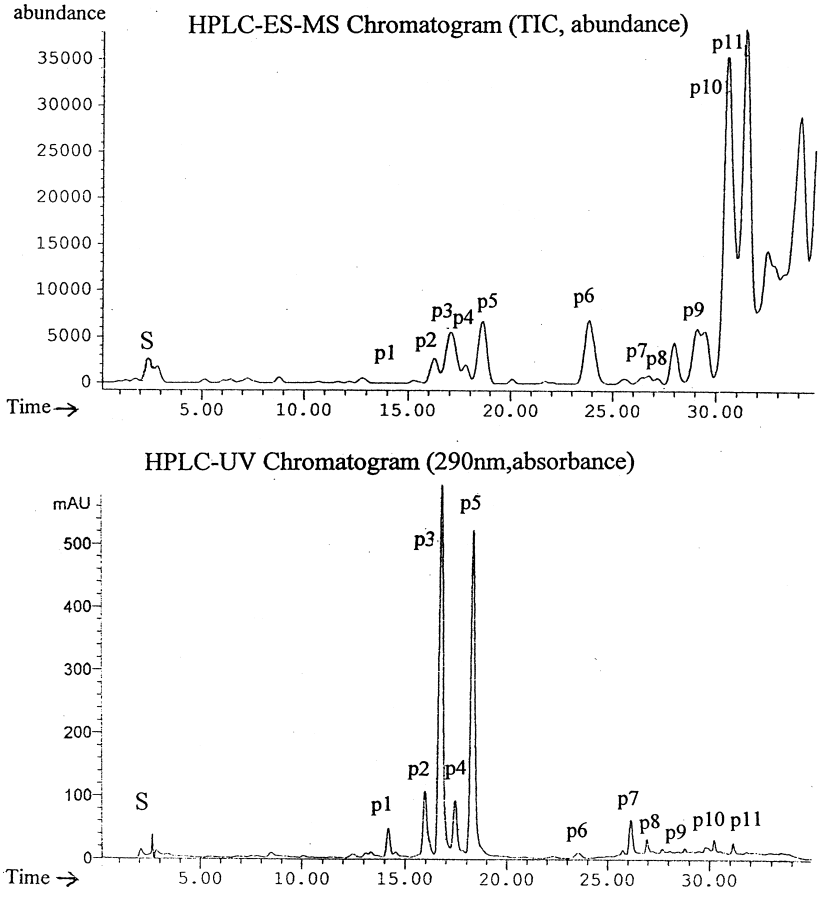


Fig. 3. Simultaneous HPLC–UV and HPLC–ES-MS chromatogram of 80% ethanol extract of sour orange, without post-column stream splitting. Chromatographic conditions were as described in Section 2. The following compounds are indicated: S, synephrine; p2, isonaringin; p3, naringin; p4, hesperidin; p5, neohesperidin; p7, naringenin; p8, hesperitin; p10, nobiletin; and p11, tangeritin.

Table 3
Peak assignments for analysis of *Citrus aurantium* extract

Peak no.	t_R (min)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	$[A+H]^+$ (m/z)	Other ions (m/z)	UV λ_{max} (nm)	Identification
p1	14.2	—	—	—	—	285, 330 low	nd
p2	15.9	581	—	—	356	285, 330 low	Isonaringin
p3	16.8	581	603	273	356	285, 330 low	Naringin
p4	17.5	611	—	—	371	283, 330 low	Hesperidin
p5	18.3	611	—	303	371	283, 330 low	Neohesperidin
p6	23.5	—	—	—	261,301,352	255 low, 325	nd
p7	26.1	273	—	—	—	285, 326 sh	Naringenin
p8	27.0	303	—	—	—	280, 330	Hesperitin
p9	28.9	—	—	—	253,355,373	—	nd
p10	30.2	403	425	—	—	250, 272,330	Nobiletin
p11	31.1	373	395	—	—	270, 325	Tangeritin

nd, not determined.

no dominant ion; therefore, neither peak is pure. Peak 1 is not identified. No ions were evident in this peak; hence, the compound was not ionized in the present condition. We have tried to modify ionization conditions by increasing EM voltage or increasing HOAc concentration in the mobile phase in order to obtain ionization of peak 1, but these efforts have not been successful. The retention time and UV spectrum of peak 1 was very close to that of a naringenin tri-glycoside [9]. Auranetin (**9**) and quercetin (**10**) were not detected in this extract.

3.3. Detection of synephrine in an 80% ethanol extract of Citrus aurantium

Synephrine is a highly water-soluble sympathomimetic amine which can increase cardiac output and blood pressure [17]. In the present HPLC condition, it could not be retained on the stationary phase and eluted almost immediately with the solvent front at t_0=2.1 min. However, HPLC–ES-MS still can be used to detect its existence in the extract. The mass spectrum of peak S in Fig. 2 shows an intense molecular ion $[M+H]^+$ at m/z 168, an intense adduct ion $[M+H+Na]^{2+}$ at m/z 191 and an ion at m/z 159. Using HPLC as a pump and sample inlet, the standard synephrine solution was directly injected into the MS without going through the column. Its mass spectrum is the same as that of peak S.

Our results show that rapid and reliable peak identification can be obtained with minute amounts of a botanical extract, using HPLC–ES-MS method. In addition, unknown compounds can be localized for further isolation. The flavonoid diglycosides exhibited not only intense protonated molecules and sodium adduct ions, but also important protonated aglycone molecules in the spectra. The permethoxylated flavones tangeritin (**7**) and nobiletin (**8**) showed very abundant protonated molecules. This method can be used to detect trace amounts of these flavonoids in orange extract. For the analysis of the flavone glycoside naringin (**3**), the detection limit was 10 ng; for the analysis of the flavone aglycone tangeritin (**7**), it was 1 ng, if using the UV detector at an absorbance of 290 nm.

Acknowledgments

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