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Liquid chromatography–electrospray ionization mass spectrometry study of the flavonoids of the roots of *Astragalus mongholicus* and *A. membranaceus*

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Abstract

High-performance liquid chromatography–electrospray ionization mass spectrometry has been applied to analyze the flavonoids of Huangqi, the roots of *Astagalus mongholicus* and *A. membranaceus*. Eight flavonoids were identified as calycosin-7-*O*-β-D-glucoside, calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate (**2**), ononin, (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside, calycosin, (3*R*)-7,2′-dihydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside, formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate and formononetin by direct comparison with the isolated standards from Huangqi. The existence of (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan, (3*R*)-7,2′-dihydroxy-3′,4′-dimethoxyisoflavan, astrapterocarpanglucoside-6′-*O*-malonate and astraisoflavanglucoside-6′-*O*-malonate was detected. This is the first report of flavonoid glycoside malonates in these two *Astragalus* species, and malonate **2** is a structurally completely identified new compound.

Keywords: *Astragalus mongholicus*; *Astragalus membranaceus*; Plant materials; Flavonoids; Malonates

1. Introduction

Astragali Radix, the dry roots of *Astragalus mongholicus* Bge. [*A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao] or *A. membranaceus* (Fisch.) Bge. (Leguminosae), known as Huangqi in China, is the most important tonic in the Traditional Chinese Medicine to reinforce ''qi'' (vital energy), to strengthen the superficial resistance, and to promote the discharge of pus and the growth of new tissue. It is also an antiperspirant, a diuretic, and is used for treatment of nephritis and diabetes [1–4]. Triterpene saponins, polysaccharides, and more than 10 flavonoids were isolated from the two plants [2–12]. The flavonoids were established to be among the beneficial components [2–4,12–15], and have suitable chromophores for UV detection. Thus, they have been chosen as ''maker compounds'' for the chemical evaluation or standardization of Huangqi and its products [4,16,17]. However, these methods did not

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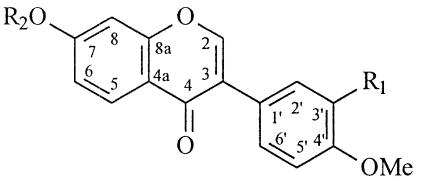
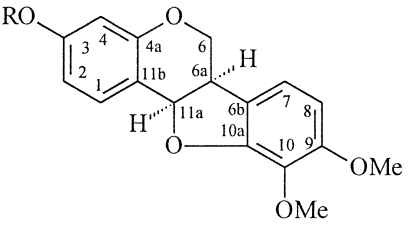
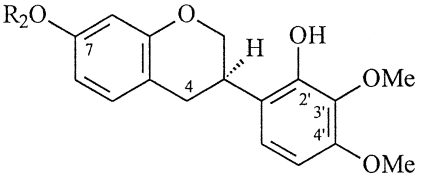
offer the correct information about the flavonoid profiles of Huangqi, and the calculated total flavonoid amounts [16] were at least 30% lower because of the omission of calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate (**2**) and formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (**7**), its two major flavonoids. The liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method presented in this report identified all of the major flavonoids of Huangqi, and also revealed the existence of more than 10 flavonoids in the extract of each of the two plants, respectively. Eight of them were identified as calycosin-7-*O*-β-D-glucoside (**1**), calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate (**2**), ononin (**3**), (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside (**4**), calycosin (**5**), (3*R*)-7,2′-dihydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside (**6**), formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (**7**) and formononetin (**8**) based on the direct comparison with the standards isolated from Huangqi. Four other flavonoids, (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan (**9**), (3*R*)-7,2′-dihydroxy-3′,4′-dimethoxyisoflavan (**10**), astrapterocarpanglucoside-6″-*O*-malonate (**11**) and astraisoflavanglucoside-6″-*O*-malonate (**12**) were also detected in these plant extracts. Malonates **2**, **11** and **12** are new compounds. Their structures are listed in Fig. 1.



Calycosin (**5**): R1=OH, R2=H, Mr=284
Calycosin-7-*O*-β-D-glucoside (**1**): R1=OH, R2=glucosyl, Mr=446
Calycosin-7-*O*-β-D-glucoside-6"-*O*-malonate (**2**): R1=OH, R2=6"-*O*-malonylglucosyl, Mr=532
Formononetin (**8**): R1=R2=H, Mr=268
Ononin (**3**): R1=H, R2=glucosyl, Mr=430
Formononetin-7-*O*-β-D-glucoside-6"-*O*-malonate (**7**): R1=H, R2=6"-*O*-malonylglucosyl, Mr=516



(6a*R*,11a*R*)-3-Hydroxy-9,10-dimethoxypterocarpan (**9**): R=H, Mr=300
(6a*R*,11a*R*)-3-Hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside (**4**): R=glucosyl, Mr=462
Astrapterocarpanglucoside-6"-*O*-malonate (**11**): R=6"-*O*-malonylglucosyl, Mr=548



(3*R*)-7,2'-Dihydroxy-3'-4'-dimethoxyisoflavan (**10**): R=H, Mr=302
(3*R*)-7,2'-Dihydroxy-3',4'-dimethoxyisoflavan-7-*O*-β-D-glucoside (**6**): R=glucosyl, Mr=464
Astraisoflavanglucoside-6"-*O*-malonate (**12**): R=6"-*O*-malonylglucosyl, Mr=550

Fig. 1. The flavonoids of Huangqi extract.

This is the first paper to report the use of LC–ESI-MS for the identification of the flavonoids of *A. mongholicus* and *A. membranaceus*, and to establish the presence of flavonoid glycoside malonates in *Astragalus* species.

2. Materials and methods

2.1. Materials

The plant materials used in this LC–MS study were the dried roots of *A. mongholicus* and *A. membranaceus*, identified and obtained from Beijing Huayi Chinese Pharmaceutical Factory (Beijing, China). The roots of *A. mongholicus* for the isolation of the flavonoids were purchased from Asia Natural Products (San Francisco, CA, USA). Diaion HP-20 was purchased from Sigma (St. Louis, MO, USA). Other chemicals, chromatographic materials and all of the solvents were purchased from VWR Scientific (Seattle, WA, USA).

2.2. Isolation and identification of flavonoid standards

The standards were isolated in the following way. Powdered materials (5 kg) were extracted with 80% ethanol (6 l×3) by maceration at room temperature. The combined alcoholic solution was concentrated at 60°C under vacuum. The concentrated extract (1.5 l) was diluted with 6 l of water. The water solution was successively extracted with hexane, chloroform, ethyl acetate, and finally *n*-butanol. Compounds **2** and **7** were mainly obtained by chromatography on a Diaion HP-20 column (eluted with water containing 0, 30, 50 and finally 100% ethanol) and a C_{18} column (water containing 0, 10, 20, 30, 50, 100% acetonitrile) of the remaining water solution. Compounds **5** and **8** were observed in the chloroform fraction, and were obtained by preparative thin-layer chromatography (TLC) (silica gel 60 F_{254} plates, 20×20 cm, 0.5 mm thickness, chloroform–methanol, 9:1, v/v), washing each zone scraped from the plate with methanol. The *n*-butanol fraction was repeatedly subjected to silica gel column chromatography (chloroform–methanol–water, 10:3:1 to 5:5:1, v/v), and preparative TLC to yield flavonoids **1**–**7**. For the separation and purification of flavonoid glycosides **1**, **3**, **4** and **6**, the solvents used in normal-phase TLC were chloroform–methanol–water (10:3:1 or 8:2:1, v/v), and in reversed-phase TLC were methanol–water (7:3 or 8:2, v/v). The isolated flavonoids were identified as calycosin-7-*O*-β-D-glucoside (astraisoflavoneglucoside, **1**) [12,16], calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate (**2**), ononin (formononetin-7-*O*-β-D-glucoside, **3**) [9], (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside (astrapterocarpanglucoside, **4**) [7,16], calycosin (7,3′-dihydroxy-4′-methoxyisoflavone, **5**) [9], (3*R*)-7,2′-dihydroxy-3′, 4′-dimethoxyisoflavan-7-*O*-β-D-glucoside [(3*R*)-isomucronulatol-7-*O*-β-D-glucoside, astraisoflavan-7-*O*-β-D-glucoside, **6**] [8,10,16], formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (ononin-6″-*O*-malonate, malonylononin, **7**) [18,19] and formononetin (7-hydroxy-4′-methoxyisoflavone, **8**) [9], by comparison of their UV, mass, proton and carbon nuclear magnetic resonance (NMR) data with those published in the cited references, respectively. The NMR spectra were taken in [$^2H_6$]pyridine or [$^2H_6$]dimethyl sulfoxide (DMSO) solution with a Bruker AM-400 NMR instrument, using tetramethylsilane (TMS) as internal standard. The structure of the new compound **2** was established as calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate through the use of a set of one- and two-dimensional NMR techniques as mentioned previously [20]. The detailed separation procedures and identification of these compounds will be reported in the near future.

2.3. Solution of flavonoid standards

A reference methanol solution (10 ml) contained **1** (0.3 mg), **2** (0.4 mg), **3** (0.2 mg), **4** (1.2 mg), **5** (0.2 mg), **6** (1.2 mg), **7** (0.4 mg) and **8** (0.2 mg).

2.4. Sample preparation for LC–MS study

At room temperature, the ground dried material (1 g) was extracted with methanol–water (10 ml, 9:1, v/v) using sonication for 60 min. The extract was filtered through a 0.45-μm nylon acrodisk 13 filter (Gelman, Ann Arbor, MI, USA). A 10-μl volume of the extract was injected onto the analytical column for analysis. In order to detect the original flavonoids in the plant, the sample preparation and high-per-

formance liquid chromatography (HPLC) analytical condition were adjusted to avoid any detectable degradation of these compounds during the performance. The extraction method appears suitable and effective for the complete extraction of all the flavonoids. This conclusion was based on the high recoveries (>95% calculated by LC peak area, mAU) which were checked by spiking a known amount (1.00 g) of plant sample with known amounts (1.0 mg) of four different standards (**1**, **2**, **4**, **5**).

2.5. LC–ESI-MS instrumentation

A HP 1090 Series II LC system (Hewlett-Packard, Palo Alto, CA, USA) with a photodiode-array detector set at 260 nm was coupled to an HP 5989B quadrupole mass spectrometer. UV spectra were also obtained by scanning from 200 nm to 500 nm. A Waters Symmetry C_{18} column (5 μm, 150×2.1 mm) with a sentry guard column (Symmetry C_{18}, 5 μm, 20×3.9 mm) was used. The mobile phase consisted of (A) water containing 0.25% (v/v) acetic acid and (B) acetonitrile containing 0.25% (v/v) acetic acid, using a linear gradient of 18–42% (v/v) B in 40 min. The flow-rate was 0.2 ml/min, and the temperature of column oven was 45°C. The ESI-MS spectra were acquired in the positive ion mode, using an electrospray interface Model HP 59987A. The temperature of the drying gas (N_2) was 350°C, at a gas flow-rate of 40 ml/min, and a nebulizing pressure (N_2) of $5.5 \cdot 10^5$ Pa (80 p.s.i.). The LC system was directly connected to the mass spectrometer without stream splitting.

3. Results and discussion

3.1. LC–ESI-MS analysis of flavonoid standards

The solution of eight flavonoids (**1–8**) was chromatographed in order to determine their retention times (t_R), UV and MS data for comparison with the chromatograms of the plant extracts and other samples. LC–UV and total ion chromatograms are shown in Fig. 2, and t_R, $[M+H]^+$ and UV λ_{max} values are shown in Table 1.

Table 1 shows that the UV data of the pterocarpan **4** and isoflavan **6** are quite different from those of isoflavones (**1–3**, **5**, **7** and **8**). Due to using dilute acetic acid in the LC–MS solvent system, the UV detector was set at 260 nm (Fig. 2b), which is less sensitive for the detection of pterocarpans and isoflavans than using 230 nm (Fig. 2c). However, acetic acid caused a split solvent peak and drifting baseline in the LC chromatogram below 230 nm (Fig. 2c), which was undesirable for the direct coupling to the mass spectrometer.

As shown in Fig. 2 and Table 1, each of the standards showed significant and distinctive $[M+H]^+$ ion, fragment ion, UV spectra, and quite different retention time, consequently, they could be unambiguously identified in Huangqi extracts. Flavonoid glycoside malonate **2** (or **7**) showed the same UV data as its related glycoside **1** (or **3**). This fact indicated that the malonyl group was at the sugar moiety of the molecule [21], which is consistent with its structure deduced from NMR spectral analysis. The $[M+H]^+$ ion of the malonate is 86 u larger than that of its related glycoside, and both of them showed same fragment ion which is equal to the $[M+H]^+$ ion of their aglycone. These observations are important for the direct detection of the malonates in Huangqi extract by using the LC–UV–MS method.

3.2. LC–ESI-MS of the extract of the roots of A. mongholicus

The LC and total ion chromatograms of the extract are shown in Fig. 3. The retention time (t_R), $[M+H]^+$, UV λ_{max} values and the identification of individual peaks are listed in Table 2. Based on the comparison of these values with those of the standards, 8 peaks were identified as **1** (P1; P to represent the peak in the text), **2** (P3), **3** (P5), **4** (P6), **5** (P7), **6** (P8), **7** (P9) and **8** (P13). LC–MS studies also determined that peak 14 was (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarp (**9**), the aglycone of **4**, and peak 15 was (3*R*)-7,2′-dihydroxy-3′,4′-dimethoxyisoflavan [(3*R*)-isomucronulatol, **10**], the aglycone of **6**. Each pair of them also showed similar UV spectra and 162 u of mass difference on their $[M+H]^+$ ion due to only the glycoside having glucose moiety. Both aglycones were previously isolated from Huangqi [7,8,10].

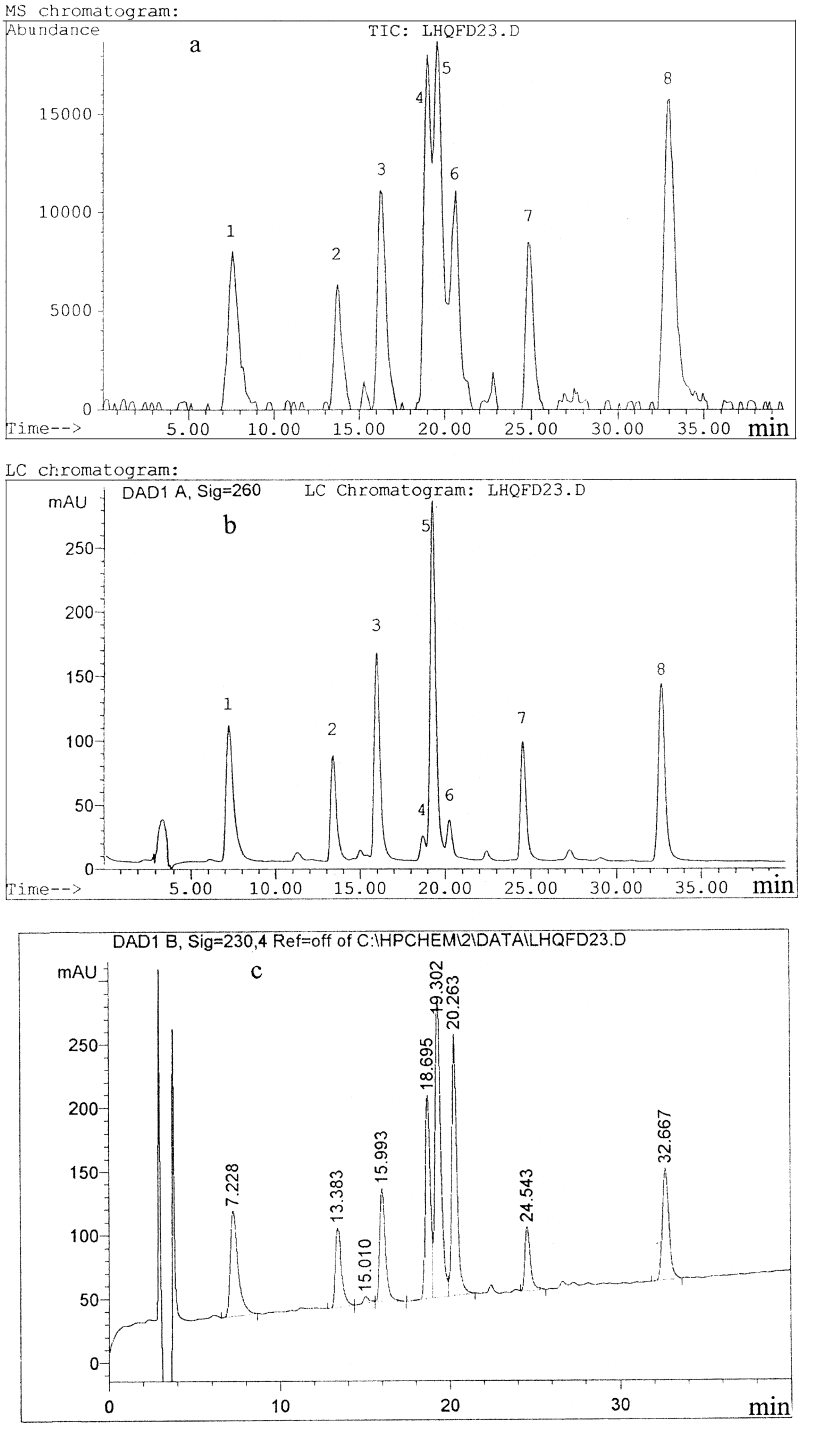


Fig. 2. Simultaneous LC–UV and LC–ESI-MS chromatograms of the Huangqi standard compounds. Chromatographic conditions are described in Materials and methods. (a) Mass chromatogram, (b and c) LC chromatograms recorded at 260 and 230 nm, respectively. The t_R value, MS and UV λ_{max} of each compound are listed in Table 1.

Table 1
Retention time (t_R) values, $[M+H]^+$, $[M+Na]^+$, other MS ions, UV λ_{max} of the standards

Peak no.	Compound name	t_R (min)	$[M+H]^+$ (m/z)	Fragment ion (m/z)	λ_{max} (nm)
1	Calycosin-7-*O*-β-D-glucoside (**1**)	7.2	447	285	260, 290sh
2	Calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate (**2**)	13.1	533	285	260, 290sh
3	Ononin (**3**)	16.0	431	269	255, 301sh
4	(6a*R*,-11a*R*)-3-Hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside (**4**)	18.6	463	301	282
5	Calycon (**5**)	19.3	285	–	250, 290
6	(3*R*)-7,2′-Dihydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside (**6**)	20.3	465	303	282
7	Formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (**7**)	24.3	517	269	255, 301sh
8	Formononetin (**8**)	32.6	269	–	250, 303

As shown in Table 1 (for the standards) and Table 2 (for the extract), the $[M+H]^+$ ion of malonate **2** (or **7**) is always 86 u more than that of its glycoside **1** (or **3**), and the malonate and its glycoside have similar UV spectra. Malonates **2** and **7** were gradually converted to their related glycosides during the malonate-free heating process [24]. Similarly, the $[M+H]^+$ ion of peak 10 (m/z 549) was at 86 u more than that of (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside (**4**) ($[M+H]^+$ ion at m/z 463), and both peaks (6 and 10) have similar UV spectra. Peak 10 decreased while the peak 6 increased during the heating process. These observations indicated that this peak was astrapterocarpan-glucoside-6″-*O*-malonate (**11**), the malonyl derivative of **4**. In the same way, peak 11 could be considered to be astraisoflavanglucoside-6″-*O*-malonate (**12**), the malonate of **6**. Because they exist in same plant, malonates **11** and **12**, the major malonates of **4** and **6**, might be probably formed through the same biogenetic pathway as that for malanates **2** and **7**, the major malonates of **1** and **3**. This suggestion is also supported by the fact that so far only 6″-*O*-malonates of the flavonoid glucosides were reported from plants [18,19,22–27]. Thus, the malonyl group of malonates **11** and **12** is most probable at 6′-position of their sugar part, as shown. There are still three small peaks remaining for identification. Peak 4 showed the same UV and mass data as those of calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate (**2**), and was also converted to calycosin-7-*O*-β-D-glucoside (**1**), which suggested that the minor malonate was the isomer of **2**, but its structure was not determined by LC–MS detection. For the same reason, peak 12 was a minor isomer of malonate **7**. Peak 2 was an unidentified minor flavonoid.

This study also found that the malonates **2** and **7** were not very stable in the extract liquid and alcoholic solution, especially, at high temperature (85–90°C) or long term storage at room temperature. A detailed LC determination with the malonate-free process of the extract showed that the malonates of this extract were almost completely converted to their related glycosides by heating the aqueous ethanol (80%) extract at reflux for 16 h, or by storing it at room temperature for about four weeks. Consequently, the malonates could only be found in trace amounts in the extracts made using aqueous alcohol through a lengthy heating process. This finding is in agreement with the published information about the stability of flavonoid glycoside malonates [18,19,23–27].

As shown in Fig. 3, the LC–UV and MS peaks of the four minor flavonoids (**9**–**12**) are not very significant because of their low concentrations in this extract. Thus, their existences have been confirmed by analyzing some fractions from the column and TLC separation, which contained the compounds in higher concentration for unambiguous LC–MS identification. However, some of the previously reporting Huangqi flavonoids, such as, 8,3′-dihydroxy-7,4′-dimethoxyisoflavone, 10-hydroxy-3,9-dimethoxyptercarpan, 3,9,10-trimethoxypterocarpan, (3*R*)-8,2′-dihydroxy-7,4′-dimethoxyisoflavan, 7-*O*-methylisomucronulatol, isomucronulatol-7,2′-di-*O*-glucoside, 5′-hydroxyisomucronulatol-7,2′-di-*O*-

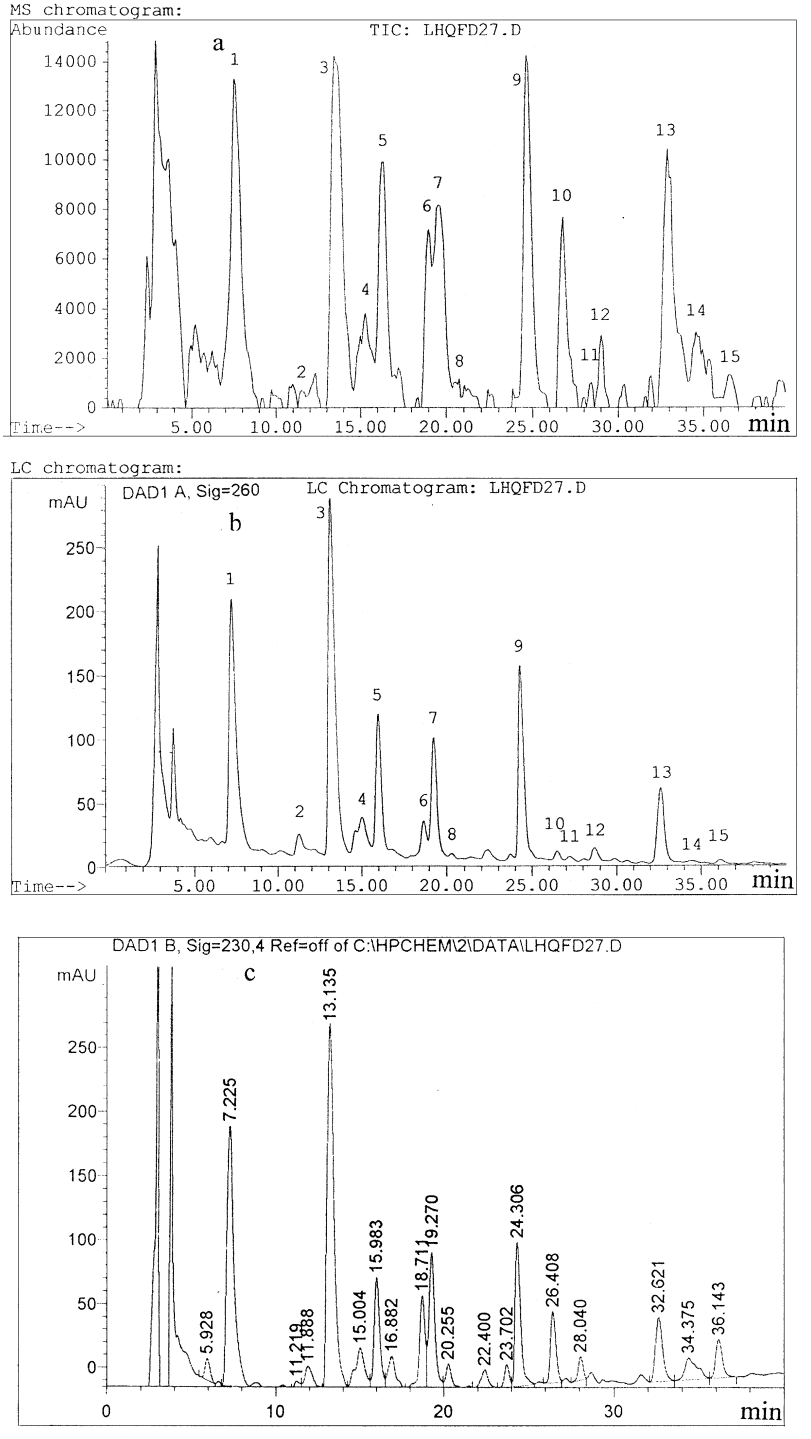


Fig. 3. Simultaneous LC–UV and LC–ESI-MS chromatograms of an extract of *A. mongholicus*. Chromatographic conditions are described in Materials and methods. (a) Mass chromatogram, and (b and c) LC chromatograms recorded at 260 and 230 nm, respectively. Peak assignments are listed in Table 2.

Table 2
Peak assignment for the analysis of the aqueous methanol extract of *A. mongholicus*

Peak no.	t_R (min)	$[M+H]^+$ (m/z)	Fragment ion (m/z)	λ_{max} (nm)	Identification
1	7.2	447	285	260, 290sh	Calycosin-7-*O*-β-D-glucoside (**1**)
2	11.9	463[a]	[a,b]	[b]	Unknown flavonoid
3	13.1	533	285	260, 290sh	Calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate (**2**)
4	15.0	533[a]	285	260, 290sh	Unknown malonate (isomer of **2**)
5	16.0	431	269	255, 301sh	Ononin (**3**)
6	18.6	463	301	282	(6a*R*,11a*R*)-3-Hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside (**4**)
7	19.3	285	–	250, 290	Calycosin (**5**)
8	20.3	465	303	282	(3*R*)-7,2′-Dihydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside (**6**)
9	24.3	517	269	255, 301sh	Formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (**7**)
10	26.3	549	301	282	Astrapterocarpanglucoside-6″-*O*-malonate (**11**)
11	27.5	551	303	282	Astraisoflavanglucoside-6″-*O*-malonate (**12**)
12	28.0	517	269	255, 301sh	Unknown malonate (isomer of **7**)
13	32.6	269	–	250, 303	Formononetin (**8**)
14	34.3	301	–	283	(6a*R*,11a*R*)-3-Hydroxy-9,10-dimethoxypterocarpan (**9**)
15	36.1	303	–	282	(3*R*)-7,2′-Dihydroxy-3′,4′-dimethoxyisoflavan (**10**)

[a] Peak contains minor impurities.
[b] Could not be measured.

glucoside [6,7,11], did not show detectable signals on both of LC–UV and MS chromatograms (Fig. 3) because the trace amount present in the plant extract does not reach to the limitation for a LC–MS detection [28].

3.3. LC–ESI-MS of the extract of the roots of A. membranaceus

The same study on the roots of *A. membranaceus* found that the flavonoid content of the extract was much lower (around 1/3) than that of *A. mongholicus*. However, both species contain nearly the same flavonoids as described above. It is worth mentioning that the ratio (3.8 to 2.2) of peak area of **4** (or **6**, **9**, **10**, pterocarpans or isoflavans) to **5** (or **3**, **8**, isoflavones) of *A. membranaceus* is much higher than that (2.5 to 8.1) of *A. mongholicus*.

Flavonoid glycoside malonates were also reported in a quite number of other leguminous plants, and the flavonoids of leguminous plants appear likely to form conjugates containing a malonate group [18,19,22–27]. The malonates are of biological interest owing to the ability of plant to utilize this conjugation form to store less soluble flavonoid aglycones. Meanwhile, upon infection of the cell, the aglycones are generated from these precusors by the hydrolysis of the stored form [25–27].

This study also showed that the LC–ESI-MS technique was a suitable and sensitive method to determine different kinds of the flavonoids, such as isoflavones, isoflavans, pterocarpans, at their different existing forms, including aglycone, glycoside and glycoside malonate, in the plant extracts [24–26].

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