Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1032 (2004) 117–124
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination and identification of isoflavonoids in *Radix astragali* by matrix solid-phase dispersion extraction and high-performance liquid chromatography with photodiode array and mass spectrometric detection

H.B. Xiao [a], M. Krucker [a], K. Albert [a,*], X.M. Liang [b]

[a] *Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany*
[b] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 161 Zhongshan Road, 116011 Dalian, China*

Abstract

The isoflavonoids in *Radix astragali* were determined and identified by HPLC–photodiode array detection–MS after extraction employing matrix solid-phase dispersion (MSPD). As a new sample preparation method for *R. astragali*, the MSPD procedure was optimized, validated and compared with conventional methods including ultrasonic and Soxhlet extraction. The amounts of two major components in this herb, formononetin (**6**) and ononin (**2**), were determined based on their authentic standards. Four major isoflavonoids, formononetin (**6**), ononin (**2**), calycosin (**5**) and its glycoside (**1**), and three minor isoflavonoids, (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan (**7**), its glycoside (**3**), and (3*R*)-7,2′-dihydroxy-3′,4′-dimethoxyisoflavone-7-*O*-β-D-glycoside (**4**), were identified based on their characteristic two-band UV spectra and $[M + H]^+$, [aglycone + H]$^+$ and [A1 + H]$^+$ ions, etc. The combined MSPD and HPLC–DAD–MS method was suitable for quantitative and qualitative determination of the isoflavonoids in *R. astragali*.

Keywords: *Radix astragali*; Matrix solid-phase dispersion; Extraction methods; Plant materials; *Astragalus* spp.; Isoflavonoids; Flavonoids

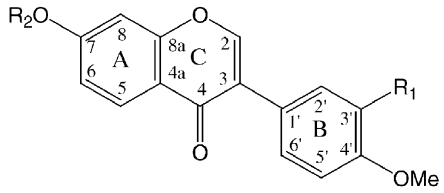
1. Introduction

Radix astragali, the dried root of *Astragalus membranaceus* (*Fisch.*) *Bge.* or *Astragalus membranaceus var. Mongholicus* (*Bge.*) *Hsiao* (family Leguminosae), known as Huangqi in China, is one of the most widely used Chinese herbs prescribed in many Chinese formulas to reinforce "Qi" (vital energy). It possess many biological functions including hepatoprotective, antioxidative, antiviral, antihypertensive, and immunostimulant properties and can strengthen the superficial resistance, discharge of pus and the growth of new tissues [1,2]. It was used specially for treatment of nephritis, diabetes, cancer, etc. as an antiperspirant, a diuretic and an adjunct medicine by Chinese doctor. The known constituents most associated with these properties are isoflavonoids, triterpene saponins, polysacccharides, γ-aminobutyric acid (GABA) and various trace elements [3]. The major isoflavonoids in *R. astragali* are formononetin (**6**), ononin (**2**), calycosin (**5**) and its glycoside (**1**). Besides, (6a*R*,11a*R*)-3-hydroxy-9,10-dimethoxypterocarpan (**7**) and its glycoside (**3**), (3*R*)-7,2′-dihydroxy-3′,4′-dimethoxyisoflavone-7-*O*-β-D-glycoside (**4**) are often detected among different species of *R. astragali* [4,5]. Their structures are listed in Fig. 1.

Isoflavonoids are well-known as one group of beneficial components originated nature and have aromatic structure possessing UV chromophores which are amenable to common HPLC detectors. In the process of chemical evaluation or standardization of *R. astragali* and its products, the inherent isoflavonoids have been chosen as "marker compounds" [5,6]. High-performance liquid chromatography (HPLC) and its coupling to mass spectrometry (HPLC–MS) have been the methods of choice for determination of these compounds. However, in order to quantitatively determine the isoflavonoids, a laborious sample preparation procedure such as extensive Soxhlet extraction followed by repetitive liquid–liquid distribution clean-up is often needed [5]. It is desirable to replace the older methods with protocols which are faster, less expensive and perform better-than classical methods.

* Corresponding author. Tel.: +49-7071-2975335; fax: +49-7071-29-5875.
E-mail address: klaus.albert@uni-tuebingen.de (K. Albert).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.09.032

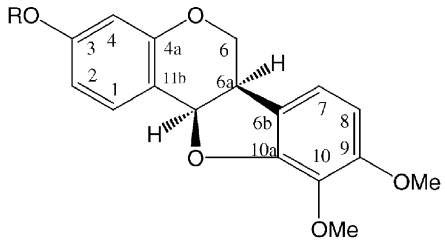


Formononetin (**6**): R_1=H, R_2=H, Mr=268

Ononin(**2**): R_1=H, R_2=glucosyl, Mr=430

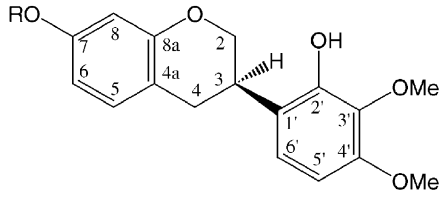
Calycosin (**5**): R_1=OH, R_2=H, Mr=284

Calycosin 7-O-β-D-glycoside (**1**): R_1=OH, R_2=glucosyl, Mr=446



(6aR, 11aR)-3-Hydroxy-9, 10-dimethoxy pterocarpan (**7**): R=H, Mr=300

(6aR, 11aR)-3-Hydroxy-9, 10-dimethoxy pterocarpan-3- O-β-D-glycoside (**3**): R=glucosyl, Mr=462



(3R)-7, 2'-dihydroxy-3', 4'-dimethoxyisoflavone -7-O-β-D-glycoside (**4**): R=glucosyl, Mr=464

Fig. 1. Major isoflavonoids isolated from *Radix astragali*.

Matrix solid-phase dispersion (MSPD), a process for the simultaneous disruption, extraction of solid or semi-solid samples and clean-up has been almost exclusively applied to the analysis of drugs and pollutants in foods [7,8] since it was introduced in 1989 [9]. This process involves blending a solid or semi-solid sample with a solid support such as silica or its derivative octadecysilyl (C_{18}), packing the unique extraction/clean-up column and eluting the target compounds with a solvent. Thus, MSPD is a distinct analytical process for sample disruption and dispersion, which possesses chromatographic characteristics that may selectively elute a single compound, several classes of compounds or complete fractionation of the sample matrix components. Therefore, it simplifies the extraction and clean-up steps, reduces sample manipulation and is faster than conventional techniques. Recently, this method was combined with on-line liquid chromatography–nuclear magnetic resonance spectroscopy–tandem mass spectrometry (LC–NMR–MS–MS) to rapidly screen the total asterosaponin fraction of the starfish *Asterias rubens* [10].

Natural products are a major resource in pharmaceutical industry. Historically, drug discovery from natural products has been a time- and resource-intensive process. Primary screening of crude extracts of plants or microbial fermentation followed by bioassay guided fractionation, isolation and structure elucidation of novel bioactive compounds can take many months. To speed up the isolation, purification, and characterization of bioactive natural products, a high-throughput method, which integrated many advanced technologies for the production and analysis of

large natural product libraries was reported recently [11]. In this complicated procedure, one of the most successful techniques is the coupling of HPLC with photodiode detection (LC–DAD), atmospheric pressure ionization (API) mass spectrometry (LC–API–MS) and nuclear magnetic resonance spectrometry (LC–NMR) [10,12–15].

The objective of this paper is to develop a MSPD extraction method followed by direct HPLC and HPLC–MS analysis to determine and identify isoflavonoids in *R. astragali*.

2. Experimental

2.1. Materials and reagents

R. astragali was collected in Dongyin, Shandong province of China. It was dried and pulverized to ~60 mesh ASTM. The solid-phase material used for MSPD was silica-based octadecyl (C_{18}), 40–70 μm from IST (Hengoed Mid Glam, UK). The authentic reference compounds of formononetin and ononin were purchased from Extrasynthese (Genay, France). Methanol was purchased from Fisher Scientific (Loughborough, UK) and of HPLC grade. Acetonitrile was obtained from Merck (Darmstadt, Germany) and of gradient HPLC grade. Water was prepared with a Mill-Q purification system from Millipore (Milford, MA, USA).

2.2. Preparation of standards

A 10.25 mg amount of formonontin was weighed and dissolved in a 10 ml volumetric flask with methanol to yield a stock solution (1025 μg/ml). One package of ononin (5 mg) was dissolved directly in the vial with methanol and transferred to a 5 ml volumetric flask to yield a stock solution (1000 μg/ml). A aliquot of 2 ml stock formononetion solution and 0.5 ml ononin stock solution were mixed to give a working solution containing 820 μg/ml formononetion and 200 μg/ml ononin. By serial dilution of this solution with methanol, calibration standards at levels of 820, 410, 82, 41, 8.2 and 4.1 μg/ml for formonetion and 200, 100, 20, 10, 2 and 1 μg/ml for ononin were obtained. All the stock solutions and working solutions were stored at 4 °C and brought to room temperature before use.

2.3. Extraction of isoflavonoids in Radix astragali

2.3.1. Matrix solid-phase dispersion extraction

2.3.1.1. Dispersion, packing and elution. A 0.5 g amount of dried *R. astragali* powder was weighed, macerated with 0.5 ml water and then blended with 1.0 g C_{18} adsorbent by grinding with a pestle for 5 min to produce a homogenous packing material for MSPD column. Before this blend was packed into the MSPD column, a 6 ml solid-phase extraction (SPE) cartridge, 1.0 g C_{18} adsorbent was put on the bottom of the column to form a sequential clean-up layer (2 ml). The blend was introduced to the top of this layer and tamped to form a compact extraction layer (2 ml). Finally, the integral MSPD column (4 ml) consisting of both extraction layer and clean-up layer was packed and eluted sequentially under slight pressure from a syringe plunger. The column was eluted firstly with 10 ml water to wash out ionic and highly polar matrix components. After this clean-up procedure, the target isoflavonoids were eluted out and collected in a vial with a suitable solvent consisting of 10 ml methanol–water (90:10, v/v). The strongly-retained non-polar and polymer matrix components remained in the column and were separated from the target compounds. In order to verify if all the isoflavonoids were eluted out by 10 ml methanol–water (90:10, v/v), a stronger elution solvent, tetrahydro furan (THF), was used to clean-up isoflavonoids. The collected fraction eluted with methanol–water was evaporated to dryness under a stream of nitrogen and redissolved in 1 ml methanol to obtain a solution equivalent to 0.5 g herb per ml solution for direct injection into HPLC column.

2.3.1.2. Reproducibility and recovery. Reproducibility of the MSPD procedure was assessed by evaluating the peak area variation of the major isoflavonoids, formononetin, ononin, calycosin and its glycoside after the herb was extracted and analyzed by HPLC repeatedly. The recovery was assessed by measuring the recovery of 40 μl of formononetin stock solution (equivalent to 41 μg formononetin) after it was added to the mortar and extracted together with 0.5 g herb in the same way as described above.

2.3.2. Soxhlet extraction

A 5 g amount of the herbal powder was extracted in a Soxhlet apparatus with 100 ml methanol–water (80:20, v/v) for 8 h. The extract was concentrated under vacuum and diluted to 25 ml with methanol to yield a final concentration equivalent to 0.2 g/ml of the herb. The solution was filtered before being subjected to HPLC analysis.

2.3.3. Ultrasonic extraction

A 1 g amount of herbal powder was extracted in a centrifugation tube placed in an ultrasonic bath with 10 ml methanol–water (90:10, v/v) at 50 ± 2 °C for 60 min. The extract was separated from the herb powder by centrifugation at 3000 rpm for 3 min. The supernatant volume was adjusted to 10 ml to yield a concentration equivalent to 0.1 g/ml of the herb. The solution was filtered before being subjected to HPLC analysis.

2.4. Chromatography

The HPLC system consisted of a HP1100 degasser, binary pump, photodiode array detector from Agilent Technologies (Waldbronn, Germany) and a Rheodyne 7125 injection valve equipped with 20 μl loop (Cotati, USA). The column used was a (250 mm × 4.6 mm i.d.) Phenomenex Luna 5 μm C_{18}

(2) HPLC column (Aschaffenburg, Germany). The mobile phase consisted of water containing a volume fraction of 0.1% acetic acid (A), and acetonitrile also containing a volume fraction of 0.1% acetic acid (B). Gradient program was adopted as follows: linear from 10 to 90% B (0–25 min), linear from 90 to 100% B (25–28 min), and then held there for 7 min. The flow rate was maintained at 1 ml/min. The photodiode detector scanned from 200 to 400 at 2 nm interval and 2 scans/s. The chromatographic profile was recorded at 260 nm.

2.5. Mass spectrometry

The HPLC–MS system consisted of an electrospray ionization (ESI) interfaced Bruker Daltonik Esquire-LC ion trap mass spectrometer (Bremen, Germany) and an Agilent HP1100 HPLC system equipped with an autosampler and a UV-Vis absorbance detector. The column, mobile phase and elution program were transferred directly from the system described in Section 2.3 to this HPLC system. The eluent was monitored at 260 nm before MS measurement. In order to adapt to the flow rate requirement of ESI–MS, the flow rate of 1.0 ml/min was reduced to 207 μl/min by a split valve at the ratio of 1:3.84 just before the eluent entered the ESI interface.

The ionization parameters were as follows: positive ion mode; capillary voltage 4000 V, end plate voltage −500 V; nebulizing gas of nitrogen at 35.0 p.s.i. (1 p.s.i. = 6894.76 Pa); drying gas of 10.0 l/min nitrogen at 365 °C. Mass analyzer scanned from 10 to 600 u. The MS–MS spectra were recorded in auto-MS–MS mode. The fragmentation amplitude was set to 2.0 V.

3. Results and discussion

3.1. Investigation of MSPD extraction

3.1.1. Optimization of MSPD procedure: column packing and sequential elution

MSPD, as is SPE, is a form of chromatography and the general principles of this technique apply. The performance of MSPD is mainly affected by the column packing technique and the elution procedure. Specially, the analyzed sample (solid or semi-solid) is blended with a suitable adsorbent (e.g. C_{18}) to form a homogenous packing material. After successful packing, the sample/adsorbent column is eluted by a stepwise solvent program similar to SPE. The first step is often to wash out matrix interferences like ionic compounds with water. The target compounds are then eluted out with suitable solvents as the second step and collected in a vial. By using a suitable solvent, the target compounds are eluted out while the matrix components such as polymers and non-polar compounds with lower polarities, which might clog the HPLC column, are still retained in the MSPD column. In this way, the target compounds are extracted, cleaned up and enriched at the same time. The obtained fraction is suitable for HPLC or HPLC–MS analysis.

In the MSPD procedure, it is an important thing to select a suitable adsorbent because it not only acts as adsorption separation material but also as a blending solid support to disrupt and disperse the herb. In this paper, silica-based C_{18} was employee due to its good mechanical strength, its suitable adsorption of isoflavonoids and its reproducibility. In the MSPD procedure, the adsorbent was normally mixed with herbs at the ratio of 4:1 to disperse the sample and produce a homogenous packing material with suitable absorbtion capacity. In this paper, the packing procedure was adjusted to make full use of the expensive adsorbent. The MSPD column was packed in an extraction layer and a separation layer to increase the chromatographic separation. In this way, half of adsorbent was packed at the bottom of the cartridge as a separation layer. The other half of adsorbent was mixed with the herb and packed on the top of the separation layer as an extraction layer. Although the adsorbent used for blending herb decrease at half the herb was well dispersed with the adsorbent and the mixture could be packed well. The results for the fraction collected from two layer columns indicated that the two-layer method was better than the homogenous packing method.

The Elution profile is another important factor in the MSPD procedure, because it also plays two roles, one for separation acting as a general mobile phase and one for dissolution/extraction of target compounds. Primarily, the selected MSPD solvents should have a good solubility for the target compounds. According to this principle, several different solvents with good solubility for isoflavonoids, including acetone, ethylacetate, methanol–water, acetronitrile–water were investigated. As expected, the isoflavonoids were extracted by all these solvents. But when using ethylacetate and acetone, too many matrix components were also extracted, which might arise from these solvent's strong dissolution and elution properties for a wide range of compounds. The elution profile using methanol–water and acetronitrile–water showed better results due to their selective dissolution/elution for isoflavonoids. By further comparison of the results obtained using methanol–water with that using acetronitrile–water, it was concluded that the best result was obtained with 90% aqueous methanol elution compared to different concentration of methanol–water and acetronitrile–water such as methanol–water (50:50, v/v) and acetronitrile–water (50:50, v/v). The results are shown in Fig. 2b.

In order to verify if all the isoflavonoids (peaks 1–7) were contained in the fraction eluted with 90% aqueous methanol, three fractions before (water fraction in Fig. 2a) and after (THF fraction in Fig. 2c) this step were analyzed and compared. By comparing the chromatograms of these three fractions shown in Fig. 2, it can be seen that the isoflavonoids were extracted and eluted exclusively by the 90% aqueous methanol while no more could be eluted by THF.

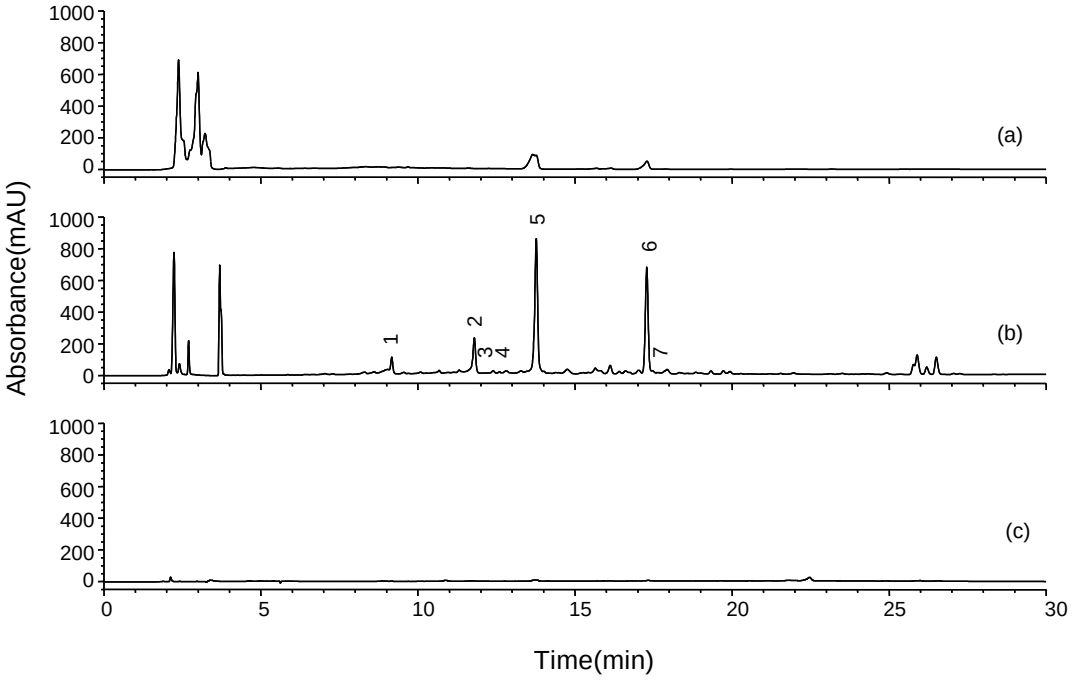


Fig. 2. HPLC analysis of the fractions obtained by sequential elution of the MSPD column. Column, Phenomenex Luna C_{18}(2) column (250 mm × 4.6 mm, i.d., 5 μm); mobile phase, liner gradient elution from acetonitrile (ACN)–water (10:90, v/v) containing 0.5% acetic acid (A) to ACN–water (90 10, v/v) containing 0.5% acetic acid (B) within 25 min. DAD and the chromatogram obtained at 260 nm. (a) Water fraction; (b) 90% MeOH fraction; (c) THF fraction.

3.1.2. Validation: reproducibility and recovery

Reproducibility of the MSPD procedure was assessed by the peak area variation of the four major peaks in five replicate analyses. The peak areas and relative standard deviation (R.S.D.) values of calycosin-3-*O*-β-D-glycoside (**1**), ononin (**2**), calycosin (**5**), formononetin (**6**) are shown in Table 1. From Table 1, it can be seen that the R.S.D. values for the larger peaks 5 and 6 were less than 5%, which is quite suitable for quantification. For the relatively smaller peaks 1 and 2, the R.S.D. values were larger than 20%. The relatively larger variation might partly arise from HPLC analysis because these two peaks were not separated to baseline. Therefore, it can be concluded the reproducibility of MSPD procedure was acceptable even though it integrated complex extraction and chromatographic separation factors together.

Table 1
MSPD reproducibility and recovery indicated by the four major peaks in Fig. 2b

Peak	Peak area	R.S.D. (%)[a]	Recovery (%)[b]
Calycosin-3-*O*-β-D-glycoside (**1**)	265.1	22.0	–
Ononin (**2**)	358.5	21.5	–
Calycosin (**5**)	5690	4.06	–
Formononetin (**6**)	5573	4.81	83.0

[a] Calculated by dividing the standard deviation obtained from the five replicate analyses by the mean and converting to percent.

[b] Calculated as {[(mean total amount after spiking − mean amount in herb) − spiked amount]/spiked amount} × 100.

By spiking the standard solution of formononetin (**4**), the recovery of this component was measured. The amount of the spiked formononetin was calculated by subtracting the total amount of formononetin after spiking from the amount in the herb before spiking. The spiking experiments were repeated three times to get the mean total amount of formononetin after spiking. The mean amount of formonoentin in herb was determined by the analysis of the five replicates mentioned above. The recovery of formononetin was calculated as 83.0% and is shown in Table 1.

3.1.3. Comparison of MSPD extraction with ultrasonic and Soxhlet methods

The MSPD extraction was compared with two conventional extraction methods, ultrasonic and Soxhlet methods, and the results are shown in Table 2. From these data, it can be seen that the best extraction efficiency for formononetin and calycosin was obtained using MSPD. Whereas for their glycosides, the conventional Soxhlet method gave better results. In addition to the differences in extraction way, this might partly arise from the different solvent compositions. The ultrasonic extraction efficiency is relatively lower, especially for the major components of formononetin and calycosin, much lower than that using MSPD in our experiments. Compared with ultrasonic method, the Soxhlet method exhibited better extraction efficiency. But it is very clear in Table 2 that this method consumes much more sample, time and solvent than the MSPD method and the ultrasonic method. Taking into account the extraction efficiency

Table 2
Comparison of MSPD extraction with ultrasonic and Soxhlet methods

Extraction method	MSPD[a]	Ultrasonic[b]	Soxhlet[c]
Extraction efficiency (peak area/mg herb)			
Calycosin-3-*O*-β-D-glycoside (**1**)	55.2	147.4	1323
Ononin(**2**)	146.8	166.6	634.6
Calycosin(**5**)	582.3	131.5	413.2
Formononetin(**6**)	425.6	113.1	320.4
Consumption			
Sample (g)	0.5	1.0	5.0
Solvent (ml)	10	10	100
Time (h)[d]	2.5	1.5	12.5

[a] The MSPD extract was dried using N_2 and then redissolved in 1 ml methanol.
[b] The ultrasonic extract was centrifuged and then filtered for HPLC analysis.
[c] The Soxhlet extract was concentrated to 25 ml and then filtered for HPLC analysis.
[d] The total sample preparation time needed for HPLC analysis including extraction, concentration, filtering, etc.

and consumption, MSPD extraction is a comparatively better method.

3.2. Quantitative determination of major isoflavonoids in Radix astragali by HPLC

With the aid of authentic reference compounds of formononetin and ononin, the amount of these two compounds in the herb were determined. The calibration curve of the peak area (y) versus the concentration (x, μg/ml) was linear; $y = -288.6 \pm 138.3x$, $R = 0.998$ ($n = 5$) from 4.1 to 410 μg/ml for formononetin; $y = -144.9 \pm 113.6x$, $R = 0.9999$ ($n = 6$) from 1.0 to 200 μg/ml for ononin. The concentrations of these two compounds in the analyzed solution were 34.42 and 18.84 μg/ml, respectively, which were located in the middle of the linear range. Correspondingly, the amount of formononetin and ononin in the herb were 68.8 and 37.6 μg/g herb, respectively.

As for the other two isoflavonoids, calycosin and its glycoside, the accurate quantification like above mentioned was not possible due to the lack of authentic reference standards. To some extent, the semi-quantitaive determination by comparison their peak areas to formononetin and ononin might be the only choice because they have similar UV spectra. However, we are planning to create some reference standards by ourselves in the future.

3.3. Identification of isoflavonoids in Radix astragali by HPLC–DAD–MS

As shown in Fig. 2b, there were four major peaks (1, 2, 5 and 6) in the retention range from 5 to 25 min and their UV spectra were obtained using the photodiode array detector during HPLC analysis. These four peaks could be correlated to isoflavonoids from the UV spectra by two absorbance bands around 258 and 288 nm [16,17]. However, due to the

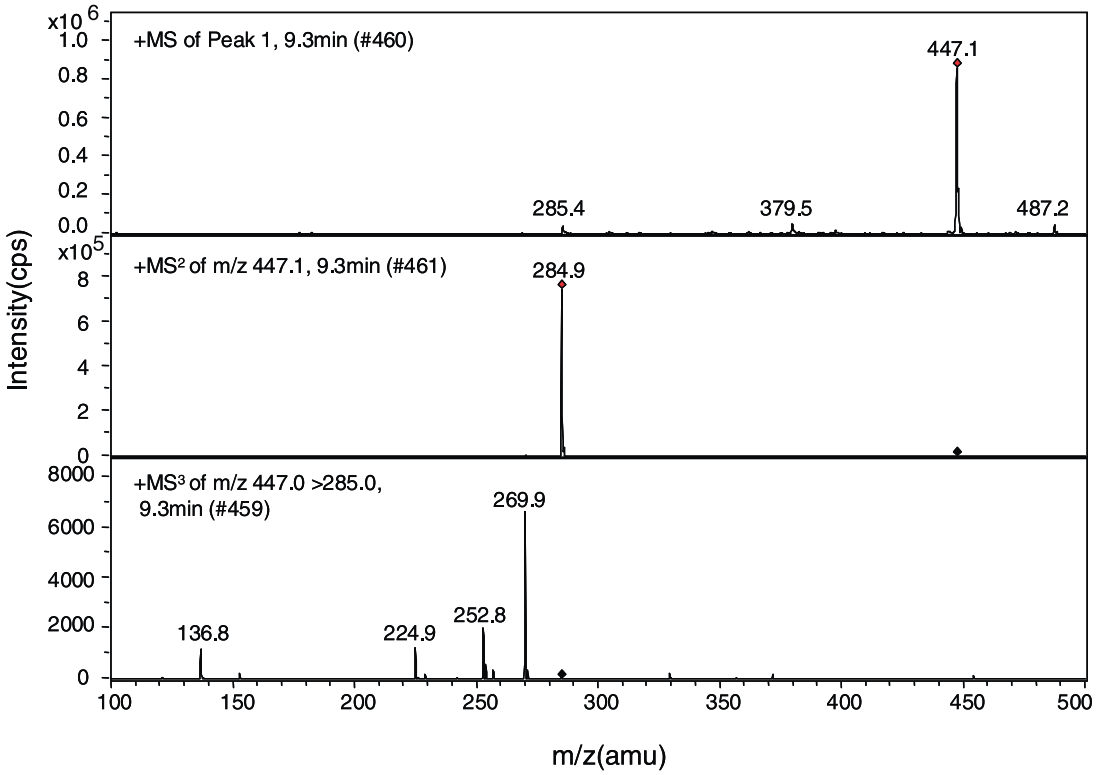


Fig. 3. HPLC–ESI–MS (MS), MS–MS (MS^2) and triple MS (MS^3) of peak 1 (calycosin-3-*O*-β-D-glycoside). Mass range, 10–600 u, positive ion mode; capillary voltage, 4 kV; nebulizing gas, N_2 at 35.0 p.s.i.; drying gas, 10.01 l/min of N_2 at 365 °C; auto-MS–MS; fragmentation amplitude, 2.0 V.

Table 3
Identification of isoflavonoids in the MSPD extract of *Radix astragali*

Peak	t_R (min)	MS (*m/z*)	MS–MS (*m/z*)	MS^3 (*m/z*)	λ_{max} (nm)	Identification
1	9.3	447.0	285.0	269.9[a], 252.8, 224.9, 136.8	258, 288 (sh)	Calycosion-3-*O*-β-D-glycoside (**1**)
2	11.9	431.1	268.9	253.8[a], 236.9, 212.9, 135.8	258, 302 (sh)	Ononin (**2**)
3	12.6	462.9, 301.2[a]	268.9[a], 190.9, 166.9[a]	133.9	280	9,10-Dimethoxy-pterocarpane-3-*O*-β-D-glycoside (**3**)
4	12.8	465.0, 303.4[a]	180.9,166.9[a], 148.9, 123.0	161.5, 151.9, 134.0, 107.1[a]	282, 322 (sh)	2′-Hydroxy-3′,4′-dimethoxy isoflavone-*O*-β-D-glycoside (**4**)
5	13.8	285.4	269.9[a], 252.9, 224.9, 136.9	252.8, 213.9, 136.9[a]	248, 290 (sh)	Calycosin (**5**)
6	17.4	269.2	253.9[a], 237.0, 212.8, 135.9	236.8[a], 135.9, 118.0	248,300 (sh)	Formononetin (**6**)
7	18.0	301.4	268.9, 190.9, 166.9[a]	151.7	280	L-3-Hydoxy-9,10-dimethoxyl-pterocarpane (**7**)

[a] Base peak.

low selectivity of assignment by UV spectra, the MS spectra of the four peaks were also measured using HPLC–ESI–MS and shown together with retention time and UV λ_{max} in Table 3.

As an example, the MS, MS–MS and triple MS (MS^3) spectra of peak 1 are shown in Fig. 3. The ESI–MS spectra (MS) exhibited an obvious singlet of quasi-molecular ion at *m/z* 447.1 $[M+H]^+$ confirming the molecular weight of this compound (446 u). By collision induced dissociation (CID) in the ion trap, the quasi-molecular ion was fragmented and transferred completely to another singlet (MS–MS) confirming the product ion at *m/z* 284.9 [aglycone + H]$^+$ of quasi-molecular ion by loss of the glucosidic unit (162 u). By further fragmentation of this aglycone ion (MS^3), the characteristic fragment ion derived from A ring of isoflavonoids at *m/z* 136.8 [A1 + H]$^+$ was displayed [18]. And the ions

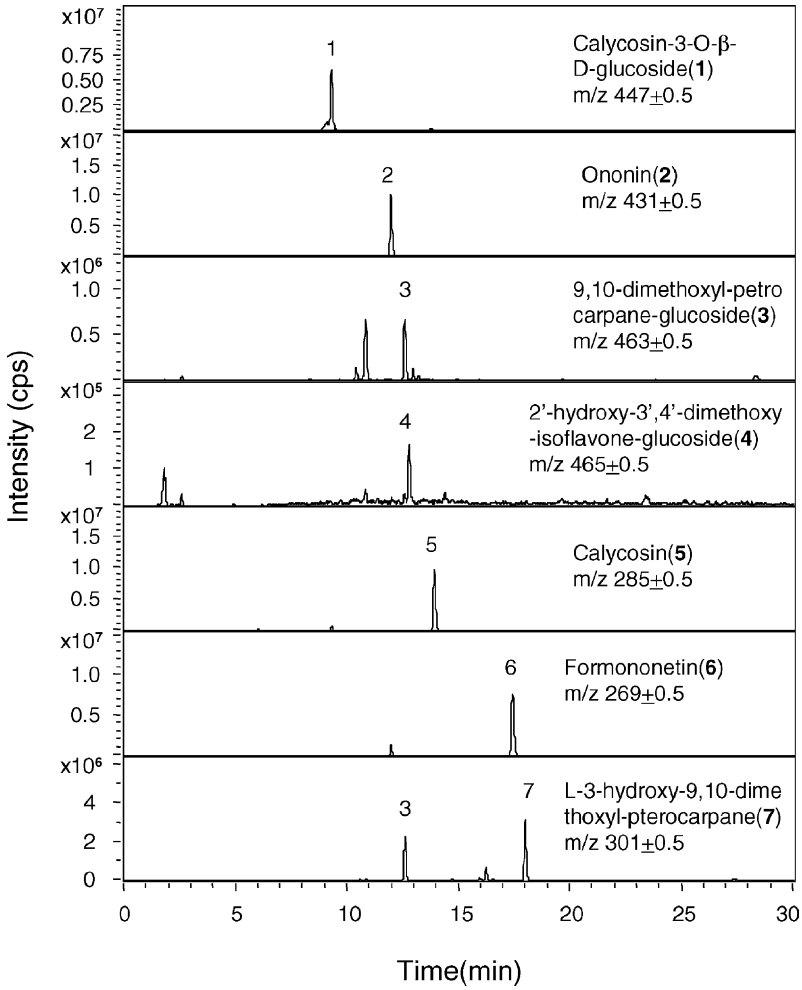


Fig. 4. Extracted ion chromatogram (EIC) of isoflavonoids in the MSPD extract of *Radix astragali*.

at *m/z* 269.9, 252.8 and 224.9 corresponding to the characteristic losses of 15 u (–CH_3), 17 u (–H_2O) and 28 u (–CO) sequentially were also displayed conforming the existence of CH_3, OH and C=O group in B ring and C ring. All these fragment structures agree with the structure of calycosin. Peak 1 is assigned to calycosin-3-*O*-β-D-glycoside by correlating the UV spectrum, molecular mass, and all MS data to the reported structure of isoflavonoids from *R. astragali* [4,5]. Similarly, peak 2 showed the singlet quasi-molecular ion at *m/z* 431.1 $[M + H]^+$, singlet fragment ions at *m/z* 268.9 $[M + H–Glu]^+$ from MS–MS and structurally informative fragment ions at *m/z* 253.8 $[M + H–Glu–CH_3]^+$, 236.9 $[M + H–Glu–CH_3–OH]^+$, 135.9 $[A1]^+$ from MS^3. So it is assigned to ononin. The MS spectrum of peak 5 was the same as the aglycone of peak 1 and is assigned to calycosin. Similarly, the MS spectrum of peak 6 was the same as the aglycone of peak 2 and is assigned to formononetin.

By HPLC–MS detection, not only the four major peaks can be identified but also the minor components. Peaks 3, 4 and 7, which were hardly detected by UV absorbance detection in Fig. 2b, were detected and identified. Fig. 4 showed the extracted ion chromatograms (EIC) of isoflavonoids in *R. astragli*. It can be seen from Fig. 4 that by selective detection of the quasi-molecular ion of isoflavonoids in *R. astragli*, three additional isoflavonoids were detected in the herb besides the four major components. According to the UV data and MS spectra shown in Table 3, peaks 3 and 4 are assigned to 9,10-dimethoxyl-pterocarpane-3-*O*-β-D-glycoside and 2′-hydroxy-3′,4′-dimethoxyisoflavone 7-*O*-β-D-glycoside, respectively. Peak 7 is the 9,10-dimethoxyl-pterocarpane, the aglycone of peak 3.

4. Conclusions

The isoflavonoids were extracted effectively by MSPD and then determined and identified by HPLC–DAD–MS. The MSPD method exhibited acceptable reproducibility, recovery, extraction efficiency and consumption (sample, solvent and time) relative to conventional extraction techniques such as ultrasonic and Soxhlet methods. In combination with HPLC–DAD–MS, the isoflavonoids were quantified based on reference standards and identified based on their UV spectra, characteristic molecular ions and abundant fragment ions obtained by “soft” ionization and multi-stage fragment techniques used in ion trap mass spectrometry.

Acknowledgements

The authors are very grateful to the Max-Plank Society for providing a scholarship and assistance to Dr. Xiao. The authors thank the Chinese Academy of Sciences for support of this study. Special thanks also go to Mr. Q. Xu and Mrs. X. Zhang from the Dalian Institute of Chemical Physics, Chinese Academy of Sciences for providing the herb of *Radix astragli*. The authors wish to thank Dr. Gregory G. Dolnikowski from Tufts University for help in editing the manuscript.

References

[1] Z.F. Xie, Z.C. Lou, X.K. Huang, Classified Dictionary of Traditional Chinese Medicine, New World Press, Beijing, 1994, p. 374.
[2] J.L. Rios, P.G. Waterman, Phytother. Res. 11 (1997) 411.
[3] H.Z. Zheng, Z.H. Dong, Q. She, Modern Study of Traditional Chinese Medicine, vol. 4, Xue Yuan Press, Beijing, 1998, p. 3886.
[4] L.Z. Lin, X.G. He, M. Lindermaier, G. Nolan, J. Yang, M. Cleary, S.X. Qiu, G.A. Cordell, J. Chromatogr. A 876 (2000) 87.
[5] X.Q. Ma, Q. Shi, J.A. Duan, T.T.X. Dong, K.W.K. Tsim, J. Agric. Food Chem. 50 (2002) 4861.
[6] R. Upton, C. Petrone, American Herbal Pharmacopoeia and Therapeutic Compendium, Astragalus Root, *Astragalus membranaceus* & *Astragalus membranaceus var. mongholicus*, Analytical, Quality Control, and Therapeutic Monograph. American Herbal Pharmacopoeia, Santa Cruz, 1999.
[7] S.A. Barker, J. Chromatogr. A 880 (2000) 63.
[8] S.A. Barker, J. Chromatogr. A 885 (2000) 115.
[9] S.A. Barker, J. Chromatogr. 475 (1989) 353.
[10] M. Sandvoss, A. Weltring, A. Preiss, K. Levsen, G. Wuensch, J. Chromatogr. A 917 (2001) 75.
[11] G.A. Eldridge, H.C. Vervoort, C.M. Lee, P.A. Cremin, C.T. Williams, S.M. Hart, M.G. Goering, M. O’Neil-Johnson, L. Zeng, et al., Anal. Chem. 74 (2002) 3963.
[12] S.C. Bobzin, S. Yang, T.P. Kasten, J. Chromatogr. B 748 (2000) 259.
[13] F.D.P. Andrade, L.C. Santos, M. Datchler, K. Albert, W. Vilegas, J. Chromatogr. A 953 (2002) 287.
[14] K. Albert (Ed.), On-line LC–NMR and Related Techniques, Wiley, Chichester, 2002.
[15] J.L. Wolfender, K. Ndjoko, K. Hostettmann, J. Chromatogr. A 1000 (2003) 437.
[16] C.Q. Song, Z.R. Zhen, D. Liu, Z.B. Hu, Acta Botanica Sinica 39 (1997) 764.
[17] C.Q. Song, Z.R. Zhen, D. Liu, Z.B. Hu, W.Y. Sheng, Acta Botanica Sinica 39 (1997) 1169.
[18] T.J. Mabry, K.R. Markham, in: J.B. Harborne, T.J. Marby, H. Msbry (Eds.), The Flavonoids, Chapman and Hall, London, 1975, p. 78.