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Capillary liquid chromatography–microcoil ^{1}H nuclear magnetic resonance spectroscopy and liquid chromatography–ion trap mass spectrometry for on-line structure elucidation of isoflavones in *Radix astragali*

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Abstract

Miniaturization and hyphenation of chromatographic separation techniques to nuclear magnetic resonance spectroscopy is being increasingly demanded in the field of biomedical, drug metabolite and natural product analysis. Herein, capillary liquid chromatography was coupled on-line to microcoil ^{1}H nuclear magnetic resonance spectroscopy (capLC–NMR) equipped with a 1.5 μL solenoidal probe for structure elucidation of isoflavones in *Radix astragali*. The extract was screened by HPLC–UV–MS as the preliminary step and four major peaks were identified tentatively by ion trap mass spectrometry molecular weights and characteristic fragments. Then, stopped-flow capLC–UV–NMR was performed using 33 μg extract injected on-column. The four peaks were parked manually in the micro probe one by one and corresponding ^{1}H NMR spectra were recorded with good resolutions under the applied capLC–NMR conditions (120 and 220 ng injected on-column for peaks 2 and 4, respectively). All aromatic regions of ^{1}H NMR spectra correlated well to the characteristic signals of isoflavone aglycone protons. And the signal corresponding to the anomeric proton of the glucopyranoside of isoflavone glycoside was also obtained for peak 1. Therefore, these four peaks are determined as calycosin-7-*O*-β-D-glucopyranoside (**1**), ononin (**2**), calycosin (**3**) and formononetin (**4**) unambiguously. The capLC–NMR results indicate that this hyphenated technique could be used for the determination of a great variety of natural products from small sample amounts, e.g., only 5 g *R. astragali* in this study.

Keywords: Capillary LC–NMR; Natural products; *Radix astragali*; Structure elucidation; HPLC–MS

1. Introduction

Natural products are a major resource for the pharmaceutical industry. Historically, drug discovery from natural products has been a time- and resource-intensive process. Preliminary screening of crude extracts from plant material followed by bioassay-guided-fractionation, isolation and structure elucidation of novel bioactive compounds can last many months. In order to obtain milligrams of pure compounds for structure elucidation and/or bioactivities assay, one has often to extract more than 10 kg plant material especially for minor compounds. For many rare medicinal plants, collection and consumption of more than 10 kg materials each time is becoming increasingly difficult [1]. Additionally, for unstable bioactive compounds like carotenoids, this rather long procedure and exposure to light and oxygen potentially leading to artifact formation or complete destruction makes this traditional approach difficult [2]. In order to solve these problems, on-line rapid structure elucidation investigating only a limited amount of herb has been developed with the aid of diverse high-performance liquid chromatography based hyphenated techniques [3,4].

Compared to traditional approaches, which apply various chromatographic separation techniques, off-line mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy for structure determination, direct on-line hyphenation of a chromatographic separation to structure

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elucidation techniques such as HPLC–DAD, HPLC–MS and, more recently, HPLC–NMR quickly provide a great amount of structural information from less starting material, which lead to a partial or complete structure determination of the natural compounds of interest [5]. Besides de novo structure determination, the integration of these state-of-the-art hyphenation techniques with high throughput sample extraction and pretreatment makes the production and analysis of large natural product libraries more efficient in practical applications [6] and comprehensive metabolite profiling, i.e., metabolomic analysis applicable, in the ongoing field of plant metabolomic research [7].

Among HPLC-hyphenated techniques, one of the most successful is the hyphenation of HPLC to MS using atmospheric pressure ionization interfaces (HPLC–API–MS), which combine the high separation ability of HPLC and the selective and sensitive MS detection as well as its multi-stage fragmentation procedure based structure elucidation function [8,9]. This technique is still developing quickly, particularly in the mass spectrometry area with vastly improved sensitivity and resolution [10].

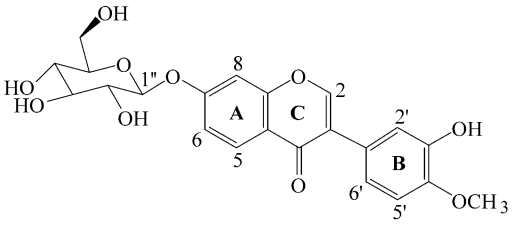
HPLC–NMR combines the HPLC separation power with the superior NMR structural elucidation ability. These two techniques have traditionally been the primary tools used by natural product chemists to isolate and determine the structures for molecules of interest. Recent advances in NMR technology allow the robust practical application of HPLC–NMR, thus providing natural product chemists with a powerful on-line structure elucidation tool in the field of phytochemistry [11–14]. However, a major drawback of this technique is still the inherently rather lower sensitivity of the NMR detector as well as the necessity for elaborate solvent signal suppression. Over the last years, there has been a trend of hyphenating miniaturized separation techniques, such as capillary LC (capLC), CE and CEC employing fully-deuterated solvents, to high field NMR equipped with miniaturized micro/nano liter NMR probes in order to analyze mass/volume limited samples, which is increasingly demanded in the field of biomedicine, metabolite and high-throughput production of natural product libraries etc. [15–21]. Compared to standard HPLC–NMR, this miniaturization reduces the consumption of expensive deuterated solvents and limited biological samples significantly so that the system could use fully-deuterated solvents.

Hyphenation of NMR and MS detection in a single setup (HPLC–NMR/MS) has recently been reported, too [22]. Taking into account the much lower sensitivity of NMR relative to MS, a parallel HPLC–NMR/MS setup splitting 95% eluent to the NMR and 5% to the MS seems to be reasonable. However, the mass spectrum might be affected due to D-H exchange. For practical applications, this makes standard HPLC–MS experiments expensive and mass spectral data interpretation complicated. Therefore, a separated HPLC–MS and HPLC–NMR setup with combined data analysis is still preferred by many analytical chemists. Furthermore, preliminary HPLC–MS is often employed as a complementary technique for expensive HPLC–NMR, if the interested peaks are needed to be further confirmed unambiguously by HPLC–NMR. Unfortunately, in many practical cases, the sample amounts prepared for HPLC–MS screening are often below the detection limit of HPLC–NMR so that subsequent HPLC–NMR structure elucidation remains a challenge in comparison with HPLC–MS analysis. However, due to better detection limits and less sample required, capLC–NMR seems to perform complementary structure determination of the same sample for HPLC–MS screening better than standard HPLC–NMR. Therefore, one purpose of this study was to develop the capLC–NMR, instead of standard HPLC–NMR, combined with HPLC–MS for on-line structure elucidation of natural products.

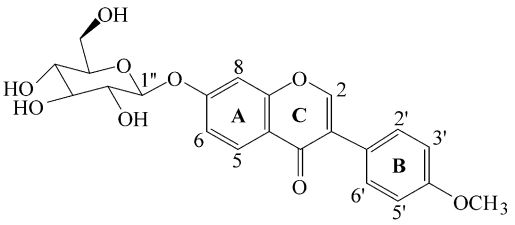
Radix astragali, the dried root of *Astragalus membranaceus (Fisch.) Bge*. or *Astragalus membranaceus var. Mongholicus (Bge.) Hsiao* (family Leguminosae), known as Huangqi in China, is one of the most widely used Chinese herbs prescribed in many Chinese formulas to reinforce “Qi” (vital energy). It possesses many biological functions including hepatoprotective, antioxidative, antiviral, antihypertensive and immunostimulant activities and can strengthen the superficial resistance, discharging of pus and the growth of new tissues [23,24]. It is used for the treatment of nephritis, diabetes, cancer etc., as an antiperspirant, a diuretic and an adjunctive medicine by Chinese doctors. The known constituents mostly associated with these positive effects on human health are isoflavones, triterpene saponins, polysacccharides, γ-aminobutyric acids (GABA) and various trace elements [25].

Flavonoids, a substance class of the most numerous and widespread natural constituents, are of great importance and interest due to a wide variety of physical and biological activities including antioxidative, anti-mutagenic and anti-carcinogenic effects. The flavonoids found in *R. astragali* mainly consist of isoflavones. The structures and molecular weights of four known major components are depicted in Fig. 1, which show characteristic methoxyl and/or hydroxyl substituted aromatic structures suitable for UV, ESI–MS/MS and NMR measurements. From their structures, it can also be deduced that these isoflavones are suited for HPLC separation and therefore could be determined preferably by on-line HPLC–DAD, HPLC–MS and/or HPLC–NMR. Furthermore, the extract of *R. astragali* can be considered as a suitable practical model to investigate the applicability of capLC–NMR in real-life sample analysis especially natural products.

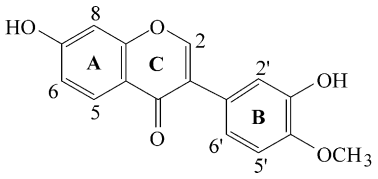
In our previous work, the possibility of hyphenating capillary LC to microcoil ^{1}H NMR was investigated by continuous-flow ^{1}H NMR and stopped-flow 2D ^{1}H, ^{1}H COSY NMR detection of the reference standard mixture of tocopherol homologues [26]. The purpose of this study is to apply this technique to a real-life sample and check its potential to on-line structure determination of natural products. As an example, the on-line structure elucidation of isoflavones in *R. astragali* using capLC–NMR together with HPLC–MS is described in detail.



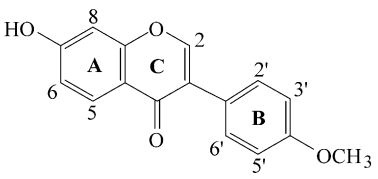
Calycosin-7-O-β-D-glucopyranoside (**1**); Mr=446



Ononin (**2**); Mr=430



Calycosin (**3**); Mr=284



Formononetin (**4**); Mr=268

Fig. 1. Structures of four known major isoflavones in *R. astragali* identified in this study.

2. Experimental

2.1. Reagents

HPLC grade methanol was purchased from Fisher Scientific (Loughborough, UK). Gradient HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). Water was prepared with a Milli-Q purification system from Millipore (Milford, USA). Analytical grade acetic acid (CH_3COOH) and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). Deuterated methanol (CD_3OD, 99.8%), 99.0% deuterated acetonitrile (CD_3CN) and 99.9% deuterium oxide (D_2O) were purchased from Deutero GmbH (Kastellaun, Germany). Deuterated chloroform ($CDCl_3$, 99.95%) was obtained from Merck (Darmstadt, Germany).

2.2. Preparation of the R. astragali extract

R. astragali was collected in Dongying, Shandong Province of China. It was dried and pulverized to ∼60 ASTM mesh before extraction. The isoflavones in this herb were extracted by the matrix solid-phase dispersion (MSPD) method previously described [27]. A 0.5 g powder was blended with 2.0 g C_{18} adsorbent from I.S.T. Ltd. (Hengoed Mid Glam, UK) in a mortar and then packed in a 6 mL empty SPE cartridge to produce a MSPD column. By sequential elution of this column, the isoflavone extract (∼5.2 mg dry extract per 0.5 g herb), which was used directly for HPLC–MS^n screening, was obtained with 90% methanol in water after the pure water cleanup. For on-line capLC–NMR coupling, 10 extracts (∼41.6 mg total dry weight per 4.0 g herb) used for reproducibility test were accumulated by pooling 80% of each extract, dried and reconstituted in 250 μL of CD_3OD and $CDCl_3$ (4:1, v/v). Finally, a sample of 166.4 mg extract per mL solvent was obtained for capLC–NMR analysis.

2.3. HPLC–MS coupling

The HPLC system consisted of a HP1100 degasser, a binary pump, an autosampler, a column oven and a UV–vis absorbance detector from Agilent Technologies (Waldbronn, Germany). The column used was a (250 mm × 4.6 mm i.d.) Bischoff ProntoSIL Eurobond C_{18} 5 μm column (Leonberg, Germany). The mobile phase consisted of water (A) and acetonitrile (B) each containing a volume fraction of 0.1% acetic acid. The gradient program was adopted as follows: linear from 10 to 90% B (0–25 min), linear from 90 to 100% B (25–28 min), held for 7 min until the separation finished. The temperature of the column oven was set to 25 °C. The flow rate was maintained at 1.0 mL/min. 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 after the eluent was monitored at 260 nm.

The MS system used was a Bruker Daltonik Esquire 3000 plus ion trap mass spectrometer (Bremen, Germany) equipped with an orthogonal 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 psi; drying gas of 10.0 L/min nitrogen at 365 °C. Mass analyzer scanned from 10 to 600 amu. The MS–MS spectra were recorded in auto MS–MS mode. The fragmentation amplitude was set to 2.0 V.

2.4. Capillary LC–NMR coupling

Capillary LC separation was performed with a splitless Waters ternary CapLC gradient pump (Milford, USA) equipped with a Bischoff on-capillary (150 μm) Lamba 1010

UV–vis absorbance detector (Leonberg, Germany) and an 200 nL loop injection valve from Upchurch Scientific (Oak Harbor, WA, USA). Corresponding to this sample size and concentration mentioned above, the amount injected on-column was about 33 μg extract. The column used was a lab-made 150 mm × 250 μm i.d. fused-silica capillary column packed with Bischoff EUROBond C_{18} (120 Å, 5 μm) particles [28]. The mobile phase consisted of D_2O (A) and CD_3CN (B) each containing a volume fraction of 0.1% TFA. A gradient elution program from 10% B to 90% B in 25 min was utilized. The temperature of column oven was set to 25 °C. The flow rate was kept at 5 μL/min. The eluent was monitored at 260 nm.

The capillary HPLC was connected to NMR by a 3.0 m × 50 μm i.d. fused-silica capillary. By using this capillary, the chromatographic peak broadening arising from the connection capillary was negligible. The dwell time from UV cell to NMR micro probe was 3.0 min. The NMR used was a Bruker AMX 600 spectrometer (^{1}H resonance frequency 600.13 MHz, Rheinstetten, Germany) equipped with a ^{1}H-$^{13}C_{inverse}$ solenoidal micro probe with a 1.5 μL active volume from Protasis/MRM (Savoy, IL, USA). ^{1}H NMR spectra were recorded in stopped-flow mode. The flow was stopped manually when the target peak detected in UV cell was transferred exactly to the NMR micro probe according to the known dwell time. The peak was parked in the probe until the end of FIDs accumulation. For the ^{1}H NMR measurements, 30 k, 12 k, 1 k and 1.4 k transients were accumulated for peaks 1–4, respectively, using a time domain of 64 k and a sweep width of 12,195 Hz. Processing was performed with 1D WINNMR software. For all spectra, zero filling of 32 k data points and an exponential multiplication of the FID with a line broadening of 0.5 Hz were performed prior to Fourier transformation. All ^{1}H NMR spectra were referenced to the solvent signal of residual CH_3CN at δ 1.93 ppm.

3. Results and discussion

Sample pretreatment and chromatographic separation play very important roles when on-line structure determination of natural products is carried out using multiple hyphenation technique such as LC–NMR/MS (recently also called hypernation [22]). In order to record high-resolution ^{1}H NMR spectra as well as mass spectra for each peak in HPLC–NMR/MS setup, the target compounds should not only be pretreated and separated well (baseline separation) but also be efficiently extracted, highly enriched and subsequently separated under nearly overloaded conditions for NMR spectrum recording. In this study, a matrix solid-phase dispersion (MSPD) method, which integrates the disruption of solid or semi-solid material, extraction of target compounds and cleanup from matrix interferences in biological or environmental samples analysis [29], was employed to produce the samples for HPLC–MS^n and subsequent capillary HPLC–NMR measurements. According to our previous study [27], this MSPD method exhibited better performance in terms of sample, solvent and time consumption, the extraction efficiency and suitability for HPLC–MS analysis in comparison with conventional Soxhlet and/or ultrasonic methods. By pooling 10 MSPD extracts (they were used for assessing reproducibility of the MSPD method in our previous study [27]), the total amount of target isoflavone compounds reached the detection limit of the micro probe ^{1}H NMR technique (see the following paragraph for the amount injected on-column). The isoflavone fraction obtained in this way was evaporated to dryness with nitrogen, (polysaccharides did not interfere), and then reconstituted in fully deuterated solvents at higher concentration (see the following paragraph for concentrations). It should be noted that the fraction could not be dried completely if too many polysaccharides were present in the fractions as obtained by other methods like Soxhlet extraction with methanol–water. The chromatographic profiles of one MSPD extract using standard HPLC–UV and the sum of 10 extracts using capLC–UV are shown in Fig. 2.

The MS, MS–MS (MS^2) and triple MS (MS^3) profiles of the four peaks designated in Fig. 2 are shown in Fig. 3, which displays typical successive fragmentation procedures derived from collision-induced dissociation (CID) in the ion trap. The intense singlet molecular ions of the four peaks indicate that they were successfully ionized under suitable ionization conditions. Otherwise adducts and/or fragment ions would appear together with the molecular ions in the MS profiles. Observation of the MS–MS and triple MS profiles indicate that the fragmentation energy for CID was suitable, too. The molecular ions were completely converted into distinctive singlet MS–MS ions for peaks 1 and 2, which were converted into characteristic MS^3 ions after further CID. Or molecular ions were converted completely into characteristic MS–MS ions directly for peaks 3 and 4.

As shown in Fig. 3, the MS and MS–MS profiles of peak 3 match the MS–MS and triple MS profiles of peak 1 after loss of a hexose unit (162 u) from the molecular ion. Therefore, it can be deduced that peak 3 is the aglycone moiety of peak 1 consisting of this aglycone and a hexose. The molecular weights of peaks 1 and 3 are determined to be 446 and 284 Da, respectively, according to their molecular ions $[M+H]^+$ shown in Fig. 3A and C. Similarly peak 4 exhibits the same MS and similar MS–MS profiles as MS–MS and triple MS profiles of peak 2 after the loss of a hexose unit (162 u). Therefore, peak 4 is assigned to the aglycone moiety of peak 2 consisting of this aglycone and a hexose. Their molecular weights are determined as 268 and 430 Da, respectively, according to their corresponding molecular ions $[M+H]^+$ shown in Fig. 3B and D.

As shown in Fig. 3C, the MS–MS profile of peak 3 displays five prominent fragment ions at *m/z* 270, 253, 229, 225 and 136, each of which shows relative abundance >5%. The difference between the aglycone ion at *m/z* 285 and fragment ion at 270 is 15 u, which is indicative of a loss of methyl radical from methoxyl group, i.e., $[M+H-CH_3]^+$. The 17 u difference between fragment ions at *m/z* 270 and

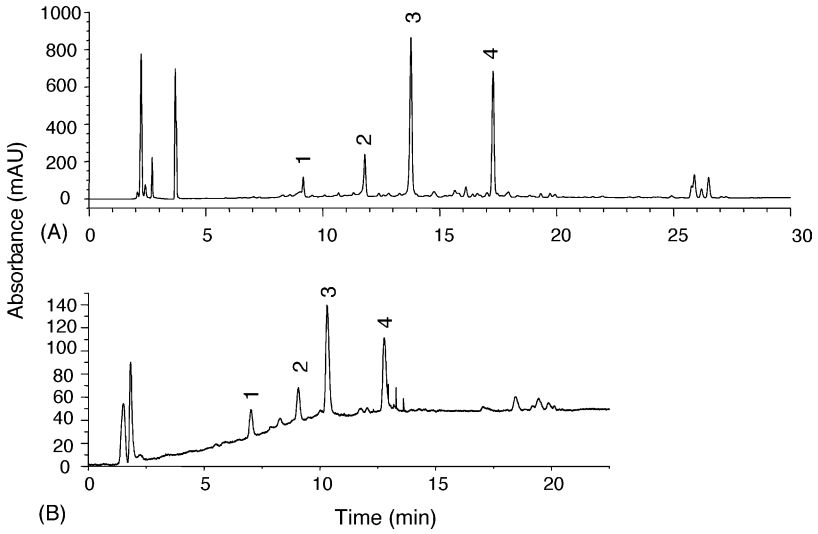


Fig. 2. HPLC–UV traces for the isoflavone fractions of *R. astragali* obtained by matrix solid-phase dispersion method. (A) HPLC separation of one isoflavone fraction on a 250 mm × 4.6 mm i.d. Bischoff ProntoSIL Eurobond C_{18} 5 μm column at 1.0 mL/min, (B) capillary HPLC separation of the sum of 10 fractions on a lab-made 150 mm × 250 μm i.d. fused-silica capillary column packed with Bischoff EUROBond C_{18} (120 Å, 5 μm) particles at 5.0 μL/min. See text for the detailed chromatographic conditions and Table 1 for peak identifications.

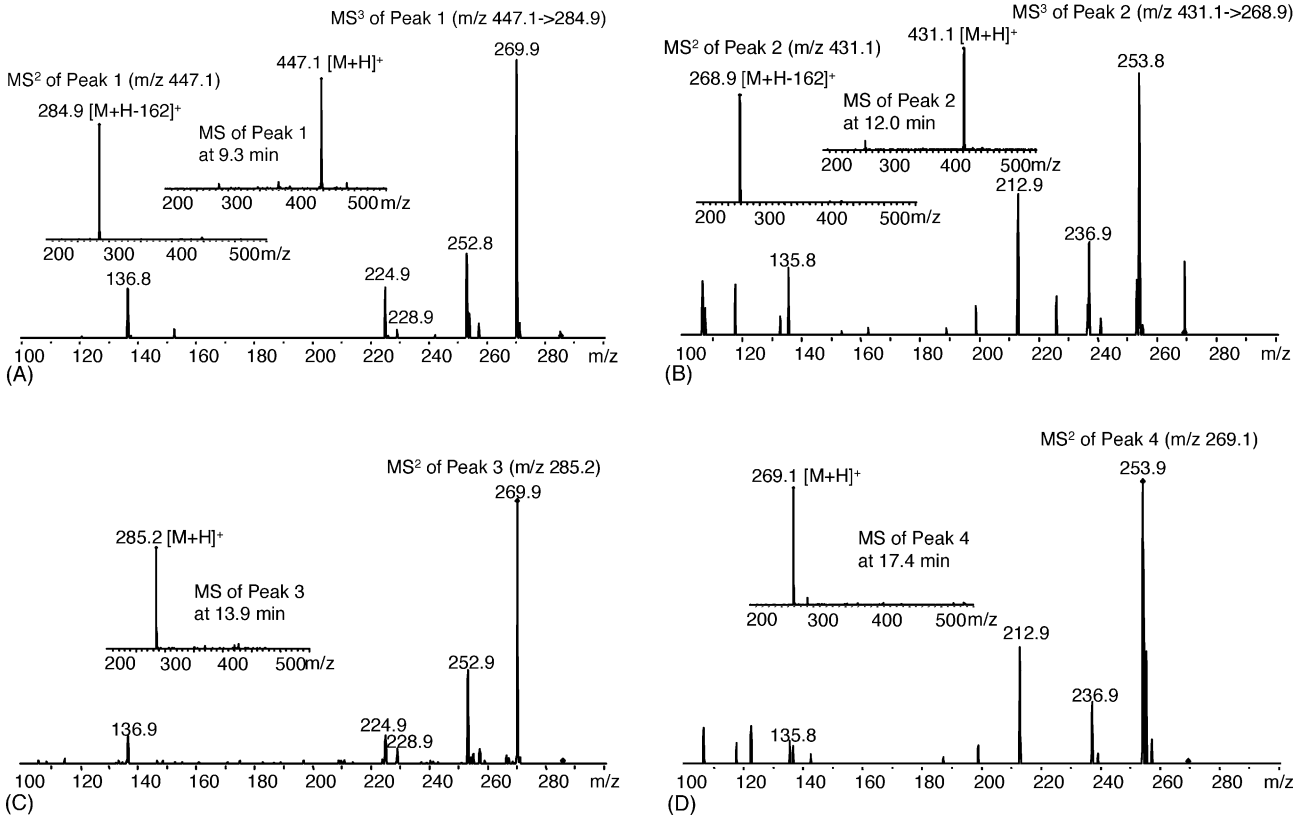


Fig. 3. Multi-stage mass spectrometry of peaks 1–4 in Fig. 2 obtained by HPLC–MS^n. (A) ESI–MS, MS–MS (MS^2) and triple MS (MS^3) of peak 1. (B) ESI–MS and MS–MS (MS^2) of peak 2. (C) ESI–MS, MS–MS (MS^2) and triple MS (MS^3) of peak 3. (D) ESI–MS and MS–MS (MS^2) of peak 4. The peaks were ionized using positive electrospray ionization (ESI) and the MS–MS and triple MS were obtained by collision-induced dissociation (CID) in ion trap. See text for the detailed mass spectrometric conditions and deductions about fragmentation procedures.

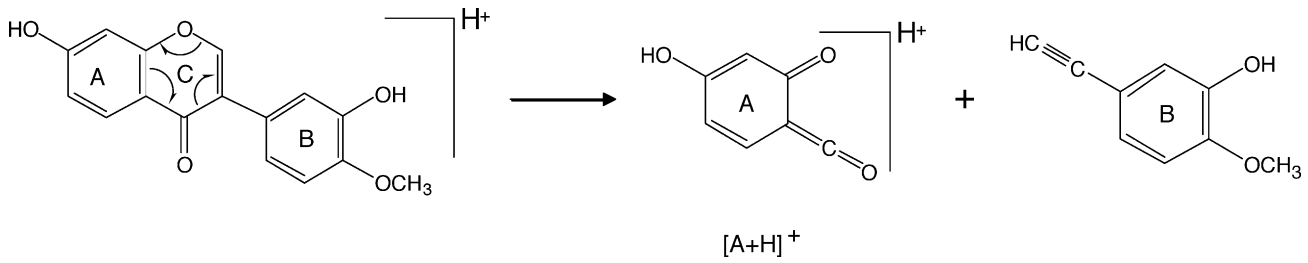


Fig. 4. Retro-Diels-Alder (RDA) fragmentation procedure (*m/z* 285 → 137) of peak 3 (calycosin (**3**)).

253 is in accordance with a further loss of hydroxyl radical, i.e., $[M + H - CH_3 - OH]^+$. The 28 u difference between fragment ions at *m/z* 253 and 225 is in accordance with a subsequent loss of CO, i.e., $[M + H - CH_3 - OH - CO]^+$. The fragment ion at *m/z* 229 corresponding to $[M + H - 56]$ is diagnostic of isoflavone aglycone [30], which resulted from the double neutral loss of CO of the aglycone ion at *m/z* 285. The fragment ion at *m/z* 137 represents the indicative ion $[A + H]^+$ of flavonoids with one hydroxyl substitution derived from Retro-Diels-Alder (RDA) fragmentation procedure [31]. This RDA fragmentation is shown in Fig. 4. By matching molecular weight and structure information derived from fragmentation procedure with compounds found in *R. astragali* [32,33], this compound is assigned to calycosin, tentatively. Correspondingly peak 1 is tentatively assigned to the glycoside of calycosin.

Likewise, the MS–MS profile in Fig. 3D shows structurally characteristic fragment ions at *m/z* 253.8 $[M + H - 162 - 15]^+$, 236.9 $[M + H - 162 - 15 - 17]^+$, 213 $[M + H - 162 - 56]^+$, 135.9 $[A]^+$. By matching molecular weight and structure information with compounds found in *R. astragali* [32,33], peak 4 is assigned to formononetin. Correspondingly peak 2 is assigned to the glycoside of formononetin (ononin). It is interesting to note that the RDA fragment of peak 4 (formononetin) is $[A]^+$, which is different from that of peak 3 (calycosin), i.e., $[A + H]^+$ mentioned above. This phenomenon might be ascribed to the fact that formononetin contains only one methoxyl group, no hydroxyl group, in the B-ring so that hydrogen transfer from the aromatic hydroxyl in the B-ring to the A-ring during RDA rearrangement procedure does not happen here.

Although the mass spectra of the four peaks are in good agreement with four isoflavones in *R. astragali* in terms of molecular weight and characteristic fragments these data are not enough to unambiguously determine the structures of these four peaks. In order to confirm the assignment above, supplementary capLC–NMR experiments using a 1.5 μL solenoidal NMR probe were performed. To fulfill this task, the standard HPLC separation was transferred to capillary LC separation with high loading capacity as the first step. As shown in Fig. 2A, four major peaks with strong UV absorbance intensities between 5 and 25 min were separated to baseline. Similarly, the sum of 10 extracts was separated well on the lab-packed capillary column as shown in Fig. 2B. The pattern of four major peaks in Fig. 2B is similar to that in Fig. 2A (same elution order except for shorter retention times), which indicates that the separation method was successfully transferred from standard HPLC to capillary LC.

According to reference [27], the concentrations of peak 2 (ononin) and peak 4 (formononetin) in Fig. 2A are 18.8 and 34.4 μg/mL, respectively. The concentration of peak 2 in Fig. 2B is 37.6 × 4 / 0.25 = ca. 600 μg/mL [27]. Similarly, the concentration of peak 4 in Fig. 2B is 68.8 × 4 / 0.25 = ca. 1100 μg/mL [27]. So the peak concentrations increase about 32-fold from Fig. 2A to B although the UV absorbance intensity in Fig. 2B seems lower than that shown in Fig. 2A. However, the good peak shapes and baseline separation in Fig. 2B indicate that they were well separated with narrow peak width at higher concentrations, which is important for capLC–NMR analysis. Corresponding to 200 nL injection size, the amounts injected on-column are 601.6 × 0.2 = 120 ng and 1101 × 0.2 = 220 ng for peaks 2 and 4, respectively.

The stopped-flow ^{1}H NMR spectrum of peak 1 is shown in Fig. 5 while the aromatic regions of the ^{1}H NMR spectra of peaks 1–4 are shown together in Fig. 6. As shown in Fig. 5, the ^{1}H NMR spectrum of peak 1 (the smallest peak among the four peaks according to the UV trace shown in Fig. 2) resolves well even for the glycosyl protons in a reasonable recording time (overnight, 30 k transients). As shown in Fig. 6, the characteristic aromatic regions of the flavonoids are well resolved in suitable recording time (1–2 k transients for peaks 2–4), too. Each of the spectra shows a distinguished singlet peak at a chemical shift from 8.09 to 8.19 ppm corresponding to the H-2 from the C-ring, which indicates that these four peaks belong to isoflavone class.

The aromatic region of ^{1}H NMR spectrum of peak 1 (Fig. 5) clearly shows signals for three ortho/meta-coupled protons from an aromatic ring at δ 6.97 ppm (1H, d, J = 2.0 Hz), 6.99 ppm (1H, dd, J = 9.2, 2.0 Hz) and 7.02 ppm (1H, d, J = 9.2 Hz) corresponding to an ABC-spin system, which matches H-2′, H-6′ and H-5′, respectively, in the B-ring of flavonoid skeleton. Similarly, the signals at δ 7.19 ppm (1H, dd, J = 9.3, 2.2 Hz), 7.22 ppm (1H, d, J = 2.2 Hz) and 8.09 ppm (1H, d, J = 9.3 Hz) are ascribed to three ortho/meta-protons from another aromatic ring corresponding to an ABX-spin system, which match H-6, H-8 and H-5, respectively, in the A-ring of flavonoid skeleton. The

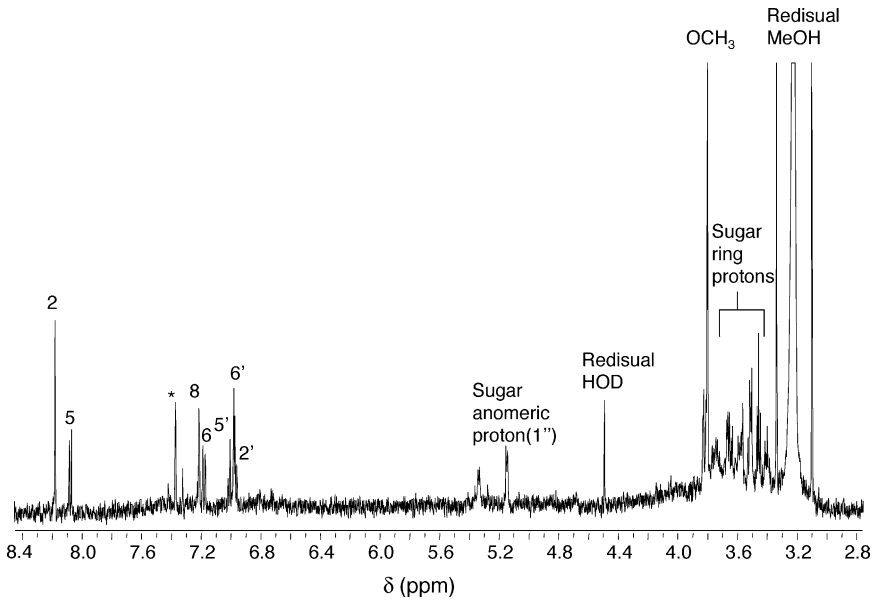


Fig. 5. Stopped-flow ^{1}H NMR spectrum of peak 1 (calycosin-7-*O*-β-D-glucopyranoside (**1**)) in Fig. 2 obtained with capillary HPLC–NMR equipped with a 1.5 μL solenoidal NMR probe. The spectrum was recorded with a 30 k transients corresponding to an overnight acquisition time, a time domain of 64 k and a sweep width of 12,195 Hz and referenced to the solvent signal of residual CH_3CN at δ 1.93 ppm. See Table 1 for peak assignment. *Ghost signal from system.

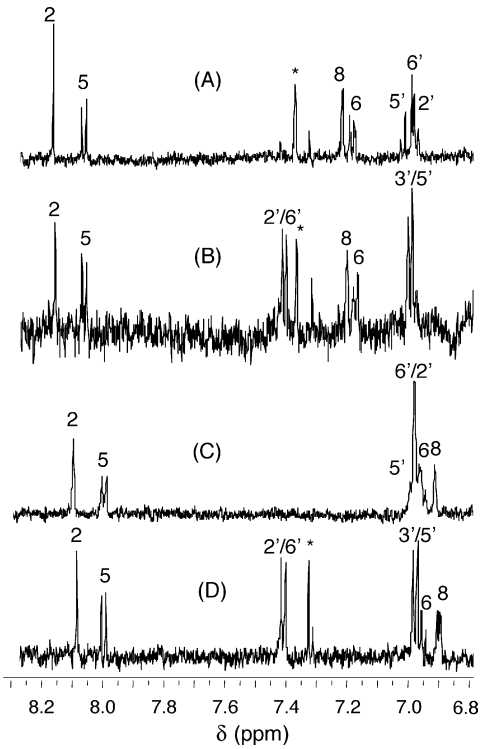


Fig. 6. The aromatic regions of the ^{1}H NMR spectra of peaks 1–4 in Fig. 2 obtained by direct stopped-flow capillary HPLC–NMR. (A) 30 k transients for peak 1. (B) 12 k transients for peak 2. (C) 1 k transients for peak 3. (D) 1.4 k transients for peak 4. All spectra were recorded with a time domain of 64 k and sweep width of 12,195 Hz and referenced to the solvent signal of residual CH_3CN at δ 1.93 ppm. See Table 1 for peak assignment. *Ghost signal from system.

intensive singlet single at δ 3.81 ppm corresponds to three protons from the methoxyl group, which is in agreement with the loss of methyl radical in mass spectrometry. The signal at δ 5.16 ppm (1H, d, J = 5.7 Hz) corresponds to the anomeric proton (H-1″) of the glucopyranoside unit with β-configuration. Taking into account the assignment given by mass spectrometry and comparing these data with literature values (see Table 1) [32], this compound is assigned to calycosin-7-*O*-β-D-glucopyranoside (**1**) unambiguously. The chemical shift differences of 0.20 ppm for the H-2 proton and less than 0.1 ppm for the aromatic protons shown in Table 1 arises from the change of solvent necessary for capLC–NMR. It should be noted that the signals of protons H-2′, H-6′ and H-5′ in this study were better resolved compared to classic tube NMR measurement using DMSO-d_6 as solvent [32]. This might be ascribed to the higher field magnet (600 MHz) as well as to solvent effects, i.e., a mixture of CD_3CN and D_2O containing 0.1% TFA used in this study. However, the signal of hydroxyl protons was not detected in this protic mobile phase used for the capillary separation.

The aromatic region of the ^{1}H NMR spectrum of peak 2 (Fig. 6B) shows two ortho-coupled protons for the B-ring with a pair of *para*-substituted groups corresponding to an $(AB)_2$-spin system, as well as a similar three ortho/meta-protons ABX-spin system to that of peak 1 for the A-ring. This similarity of A-ring resonance implies that these two compounds contain the same A-ring because the different substitution in the B-ring has little effect on the A-ring ^{1}H NMR signals [34]. The structure information obtained above is in agreement with the assignment given by mass spectrometry, and the values of chemical shifts and coupling constants are shown in detail in Table 1. By further comparison of the

Table 1
^{1}H NMR data (chemical shifts and coupling constants) and comparison with literature values for peaks 1–4 shown in Fig. 2

Proton	Peak 1; calycosin-7-*O*-β-D-glucopyranoside (**1**)[a]		Peak 2; ononin (**2**)[a]		Peak 3; calycosin (**3**)[a]		Peak 4; formononetin (**4**)[a]	
	δ ppm (multiple, *J* Hz)	$\Delta\delta$[b] ppm	δ ppm (multiple, *J* Hz)	$\Delta\delta$[b] ppm	δ ppm (multiple, *J* Hz)	$\Delta\delta$[b] ppm	δ ppm (multiple, *J* Hz)	$\Delta\delta$[c] ppm
2	8.19 (s)	−0.20	8.18 (s)	−0.24	8.11 (s)	−0.17	8.09 (s)	−0.24
5	8.09 (d, 9.3)	+0.03	8.09 (d, 9.3)	+0.03	8.01 (d, 9.3)	+0.01	8.01 (d, 9.3)	+0.02
6	7.19 (dd, 9.3, 2.2)	+0.04	7.19 (dd, 9.3, 2.2)	+0.04	6.96 (dd, 9.3, 2.4)	+0.01	6.96 (dd, 9.3, 2.0)	+0.02
7	OGlu-1″ 5.16 (d, 5.7)		OGlu		OH		OH	
8	7.22 (d, 2.2)	−0.02	7.21 (d, 2.2)	−0.03	6.92 (br)	+0.04	6.90 (br)	+0.03
2′	6.97 (d, 2.0)	0	7.42 (d, 9.2)	−0.12	6.99 (br)	+0.03	7.42 (d, 9.2)	−0.09
3′	OH		7.00 (d, 9.2)	0	OH		6.98 (d, 9.2)	−0.02
4′	OCH_3 3.81 (s)	+0.01	OCH_3 3.78 (s)	0	OCH_3 3.81 (s)	0	OCH_3 3.79 (s)	−0.01
5′	7.02 (d, 9.2)	+0.05	7.00 (d, 9.2)	0	7.00 (br)	+0.04	6.98 (d, 9.2)	−0.02
6′	6.99 (dd, 9.2, 2.0)	−0.09	7.42 (d, 9.2)	−0.12	6.99 (br)	−0.09	7.42 (d, 9.2)	−0.09

[a] Identified structure.
[b] Chemical shift differences calculated by subtracting the chemical shifts measured in this study from the values reported in reference [32].
[c] Chemical shift differences calculated by subtracting the chemical shifts measured in this study from the values reported in reference [33].

chemical shifts with the values published for ononin [32], which is assigned by mass spectrometry, the assignment of ononin (**2**) for peak 2 is confirmed.

As shown in Fig. 6C, the aromatic proton signals of the A- and B-ring severely overlap except for H-5 at δ 8.01 ppm (1H, d, J = 9.3 Hz). However, these data fully correlate to those reported in literature [32] for calycosin (**3**), which is assigned to peak 3 by mass spectrometry in this study. The poor resolution might be ascribed to the fewer transients (1 k) as well as their inherent undistinguishable chemical shifts under normal NMR conditions. By comparing Fig. 6C with A, it can be seen that the signals of H-8 and H-6 shifted upfield across the signal region of H-5′, H-6′ and H-2′ and changed their order due to the substitution of the glycosyl unit to the hydroxyl group so that the signal of H-6 overlaps with the poorly resolved signals of H-5′, H-6′ and H-2′ as shown in Fig. 6C.

The aromatic region of the ^{1}H NMR spectrum of peak 4 (Fig. 6D) shows well-resolved signals corresponding to an $(AB)_2$-spin system for the B-ring as well as an ABX-spin system similar to that in Fig. 6C for the A-ring, which indicates that this compound contains the same A-ring as peak 3 (calycosin). By comparing their chemical shifts and coupling constants with those reported for formononetin [33], which is assigned to peak 4 by mass spectrometry in this study, the assignment of formononetin (**4**) is confirmed. The details about chemical shifts, coupling constants, proton assignment and chemical shifts difference compared to published values are given in Table 1. By comparing Fig. 6D with B, it can be seen that the signals of H-2′/6′ and H-3′/5′ shifted slightly downfield due to the substitution of the glycosyl unit to the hydroxyl group.

MS preliminary structure investigation followed by supplementary NMR determination is a typical structure elucidation procedure in natural product research. Due to the wide application of HPLC–MS for identifying natural products, further confirmation of MS peak assignments by following HPLC–NMR attracts a lot of interest. However, as a subsequent supplementary confirmation, this direct HPLC–NMR analysis of the HPLC–MS sample for unambiguous structure determination is often hindered by the relatively low sensitivity of NMR, although HPLC–MS following HPLC–NMR and/or parallel HPLC–NMR–MS could be performed. Tedious repetitive preparation of large amount of sample for HPLC–NMR analysis is unavoidably the only choice in most cases. However, capLC–NMR reduces the gap arising from sensitivities between HPLC–MS and HPLC–NMR due to its better detection limit and decreased sample amount requirement. In this study, the extracts for HPLC–MS^n screening were analyzed successfully using capLC–NMR by pooling only 10 extracts (5 g herb used in total) and solvent reconstitution. From this example, the applicability of capLC–NMR in natural products research especially as a subsequent tool for HPLC–MS screening could be evaluated, at least partially.

4. Conclusions

The hyphenation of capillary liquid chromatography to microcoil ^{1}H nuclear magnetic resonance spectroscopy was successfully used for on-line structure determination of four major components of *R. astragali*. The obtained good ^{1}H NMR resolution and informative structure data in this study showed the practicability of this technique for natural product research (the ^{1}H NMR resolution achieved for calycosion-7-*O*-β-D-glucopyranoside (**1**) in this study was even better than that in classic tube NMR measurement using DMSO-d_6 as solvent). The improved sensitivity and

reduced sample/solvents requirement of capLC–NMR make this technique more applicable than standard HPLC–NMR, especially as subsequent supplementary and unambiguous determination of HPLC–MS peaks. By combining this technique with routine HPLC–MS^n, rapid on-line structure elucidation investigating small sample amounts (5 g *R. astragali* in this study) is reasonable compared to traditional step-by-step separation and structure elucidation procedures.

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