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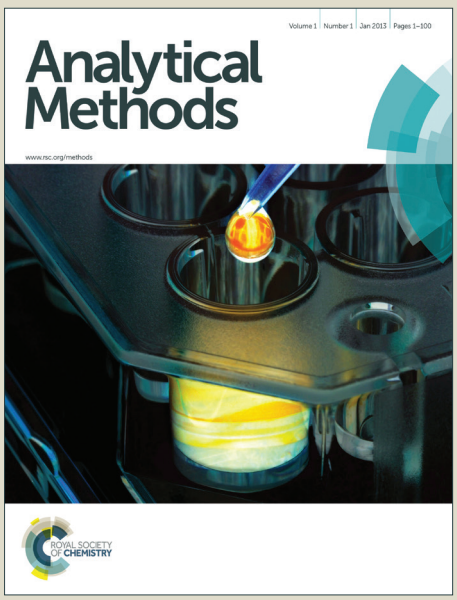

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Preparative separation of isoflavones in plant extract of *Pueraria lobata* by high performance counter-current chromatography

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Five isoflavones, puerarin, daidzin, daidzein, 3′-hydroxy-puerarin and 3′-methoxy-puerarin were isolated and purified simultaneously from *Pueraria lobata* for the first time by high performance counter-current chromatography (HPCCC) using a system consisting of hexane − ethyl acetate − *n*-butanol − ethanol − water (0.5:2:1:0.5:3.5, v/v/v/v/v). A total of 155 mg of puerarin, 41 mg of daidzin, 12 mg of 3′-hydroxy-puerarin, 6 mg of 3′-methoxy-puerarin and 106 mg of daidzein were purified from 5.1 g of the ethyl acetate extract of *P. lobata*, and the purities of the five isoflavones as determined by high performance liquid chromatography (HPLC) were at 98.77%, 96.53%, 97.59%, 90.21% and 98.36%, respectively. The structures of the five compounds were identified by their retention time and the electrospray ionization multi-stage tandem mass spectrometry (ESI-MS^n) in the positive ion mode, and confirmed by ^{1}H-NMR experiments. The characteristic ESI-MS fragmentation patterns of the five compounds were discussed.

Introduction

Pueraria lobata (Chinese name Ge Gen) is the traditional Chinese medicine. It's the major available natural resource of isoflavones such as puerarin, daidzin, daidzein, 3′-hydroxy-puerarin and 3′-methoxy-puerarin,[1-3] which have been investigated widely to treat fever, [4,5] inflammation, [6-8] cancer [9,10] and so on.

Earlier publication have successfully described methods for separation and purification of the main isoflavones in *P. lobata*. He *et al* had purified puerarin in one step from an extract of Radix puerariae by adsorption chromatography on an epichlorohydrin polymerized -cyclodextrin ligand coupled to brominated allyl-group substituted Sepharose HP.[11] Zhou *et al* had found a column packing for sublimating the total flavonoids in Radix puerariae.[12] A novel method using column chromatography on oligo-β-cyclodextrin-Sepharose HP for the preparation of high purity daidzin was proposed by Yang.[13]

High-speed counter-current chromatography (HSCCC) was a support-free liquid–liquid partition method, which has been successfully applied to the preparative separation of some compounds from some medicine herbs.[14–16] Only one paper has reported on the isolation and purification of six isoflavones such as 3′-hydroxypuerarin, puerarin, 3′-methoxy-puerarin, two puerarin-xyloside and daidzin from *P. lobata* extract, respectively, using HSCCC method,[17] however, it had taken 9 hours for the separation of 80 mg *P. lobata* extract. Moreover, compared to HSCCC, high performance counter-current chromatography (HPCCC) had larger separation column, enables higher flow rates and larger sample injection, thus shortening the separation time while maintaining good resolution.[18,19] Up to know, no report has been published on the separation and purification of five isoflavones including puerarin, daidzin, daidzein, 3′-hydroxy-puerarin and 3′-methoxy- puerarin simultaneously from *P. lobata* by HPCCC. Some methods using ultra-performance liquid chromatography–mass spectrometry (UPLC–MS), tandem mass spectrometry (MS) , high-performance thin-layer chromatographic (HPTLC) method and liquid chromatography–mass spectrometry (LC-MS) have been successfully applied to the analysis of isoflavones in *P. lobata*.[20-24] Prasain *et al.* analyzed isoflavone glycosides in *P. lobata* cultures by tandem mass spectrometry method.[20] Five major components including puerarin and daidzein are detected and identified from the methanol extract of *P. lobata* by LC-MS.[21] A liquid chromatography mass spectrometry (LC-MS) method was also developed for the simultaneous quantitation of seven compounds including puerarin and daidzein in rat plasma samples by Yu *et al.*[22] An UPLC–MS method was developed for the simultaneous determination of puerarin, daidzein, baicalin, wogonoside and liquiritin in rat plasma.[23] Chen *et al*. developed a HPTLC method for distinguishing fingerprints of isoflavonoids between P. Lobate and Pueraria Thomsonii.[24] However, the methods discussed above provided only limited structural information of isoflavonoids in *P. Lobate*.

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In the present paper, a successful HPCCC method for the first separation of the five isoflavones, including puerarin, daidzin, 3′-hydroxy-puerarin, 3′-methoxy-puerarin and daidzein from *P. lobata* simultaneously was reported. The separation of 5.1 g *P. lobata* extract was achieved in 200 min in a run. The purities of compounds were at 98.77, 96.53, 97.59%, 90.21% and 98.36%, respectively, as determined by high performance liquid chromatography (HPLC). Structural characterization and analysis of the five isoflavones obtained from the HPCCC were accomplished by use of ESI-MS/MS technique, and the interesting losses of neutral molecules have been proposed in the positive ion mode, which provided more detailed structural information. The retention time, molecular weights and the characteristic fragment ions of the five isoflavones are presented and discussed in this paper, and the structures of the five compounds were shown in Fig. 1.

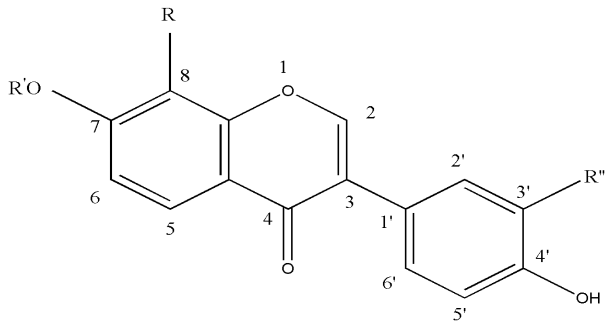

Figure 1. Structures of the five isoflavones identified in this paper: (1) R=glc, R′=H , R″=H, puerarin; (2) R= H, R′= glc , R″=H, daidzin; (3) R=glc, R′=H , R″=OH, 3′-hydroxy- puerarin; (4) R=glc, R′=H , R″=OCH_3, 3′-methoxy-puerarin; (5) R=H, R′=H , R″=H, daidzein.

Experimental

Chemicals and reagents

P. lobata was purchased from Beijing TongRenTang Medicinal Store (Changchun, China); puerarin, daidzin and daidzein were purchased from YuanYe Biological Science and Technology Co., Ltd (Shanghai, China), 3′-hydroxy-puerarin and 3′-methoxy-puerarin were purchased from Oriental HuaZan technology co., LTD(Beijing, China). Acetonitrile and acetic acid were HPLC grade and purchased from Fisher Scientific Company. The other organic solvents used for HPCCC separation were of analytical grade and purchased from Beijing Chemicals (Beijing, China); Water was purified on a Milli-Q water purification system (Millipore, France).

Sample Preparation

P. lobata (500 g) was milled to powder and extracted with 3000 mL of 80% aqueous ethanol for five times at room temperature, each for 12 h. Each time, the extraction mixture was filtered, and the combined filtrates were concentrated to dryness in vacuo at 50 ℃. The extract was re-dissolved in 500 mL of water, which was then defatted two times each with 500 mL petroleum ether. The water layer was then extracted successively five times each with 500 mL of ethyl acetate. The combined ethyl acetate was concentrated to dryness in vacuo at 50 ℃, which yielded 5.1 g ethyl acetate extract. The ethyl acetate extract was stored at -20 ℃ before HPCCC separation.

HPLC Analysis

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler and a photodiode array detector (DAD), was used for the analysis of isoflavones in the ethyl acetate extract of *P. lobata*, the partition coefficient (K) and to monitor the fractions collected from the HPCCC separation. The analysis was carried out with an Agilent Zorbax Extend C18 column (250 × 4.6 mm, 5 μm). The binary mobile phase consisted of acetonitrile (solvent A) and water containing 0.5% acetic acid (solvent B). All solvents were filtered through a 0.45 μm filter prior to use. The flow-rate was kept constant at 0.5 mL/min for a total run time of 25 min. The system was run with a gradient program: 0–18 min: 80% B to 40% B, 18–20 min, 40% B to 80% B; and 20–25 min, 80% B to 80% B. The sample injection volume was 10 μL. Peaks of interest were monitored at 250 nm by a DAD detector.

HPCCC Separation Procedure

Preparative HPCCC was carried out in a Spectrum HPCCC (DE, England). The HPCCC system was equipped with two check pumps (PREPARATIVE PUMP 1800, KNAUER, Germany). The revolution speed of the apparatus could be regulated between 0 and 1600 rpm. A 2500 sensitive UV detector (KNAUER, Germany) was set at 250 nm. The 980 mL coil was used for the preparative HPCCC, and the apparatus was set in the reversed phase mode. A sample injection valve with a 25 mL sample loop was used for preparative separation. The temperature was held at 30 ℃.

A mixture of hexane–ethyl acetate–*n*-butanol–ethanol–water (0.5:2:1:0.5:3.5, v/v/v/v/v) was used for the HPCCC separation system. This biphasic solvent system was selected based on the partition coefficients (*K*) of the compounds of interest, which were 1.11, 2.37, 7.3, 12.48 and 49 for fractions Ⅰ-Ⅴ, respectively. The *K* value was the ratio of the concentrations in the top and bottom layers of the same compound as determined by HPLC. The entire coiled column was first filled with the upper phase, which serves as the stationary phase. The rotation rate of the apparatus was set at 1200 rpm, and the lower phase (mobile phase) was pumped into the column at a flow rate of 25 mL/min. A 5.1 g of *P. lobata* extract dissolved in 25 mL of the mixture of hexane–ethyl acetate – *n*-butanol – ethanol – water (0.5:2:1:0.5:3.5, v/v/v/v/v) was loaded into the injection valve after the system reached hydrodynamic equilibrium. After the fraction Ⅲ (Fig. 2) was completely eluted, the mobile phase was set at a flow rate of 0 mL/min; then the rotation rate of the apparatus was set at 0 rpm. The stationary phase, which serves as the mobile phase, was pumped into the column at a flow rate of 25 mL/min; the fractions Ⅳ and Ⅴ (compounds 4 and 5) were finally eluted from the column. The effluent from the outlet of the column was continuously monitored by a UV detector at 250 nm and collected into test tubes with a fraction collector set at 2 min for each tube. Fractions from the HPCCC that had the same single peak as determined again by HPLC were combined and freeze-dried. The purities of the five fractions Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅴ corresponding to peaks 1, 2, 3, 4 and 5 as determined by HPLC were 98.77%, 96.53%, 97.59%, 90.21%and 98.36%, respectively. The purified compounds were then stored at -20 °C before ESI-MS^n analyses.

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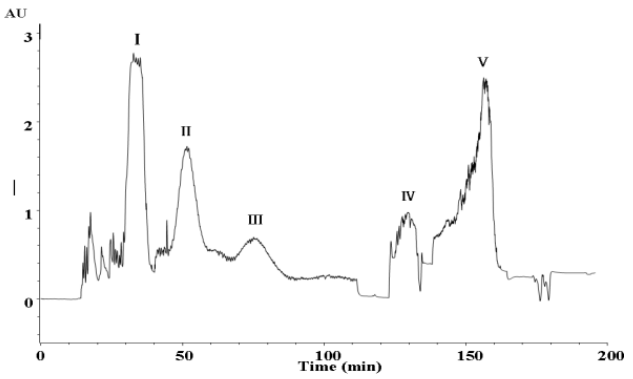

Figure 2. HPCCC chromatograms of the ethyl acetate extract of *P. lobata*: (I) puerarin, (II) daidzin, (III) 3′-hydroxy-puerarin, (IV) 3′-methoxy-puerarin, (V) daidzein.

ESI-MS for Identification

The Thermo Scientific LCQ Fleet mass spectrometer with an electrospray ionization (ESI) interface (ThermoFisher, USA). The operating parameters in the positive ion mode were as follows: the mass range was set at 50~2000; the spray voltage was controlled at 4 kV; the sample was injected through the flow injection pump and the flow rate was set at 5 μL/min; the capillary temperature was set at 200 ℃; the sheath gas was N_2 and the collision gas was He; the collision-induced dissociation energy(CID) was 20%~35%.

Results and Discussion

HPCCC Separation

The ethyl acetate extract of *P. lobata* and the fractions corresponding to each peak isolated by HPCCC were firstly analyzed by HPLC, the results are given in Fig. 3 (A-F). Tentative identification of the compounds was first achieved by congruent retention times and UV spectra with that of the authentic standards[25], the UV spectrum of compound 1-5 showed the characteristic of isoflavones with absorption peaks at 250, 250, 265, 240, and 250 nm, respectively.

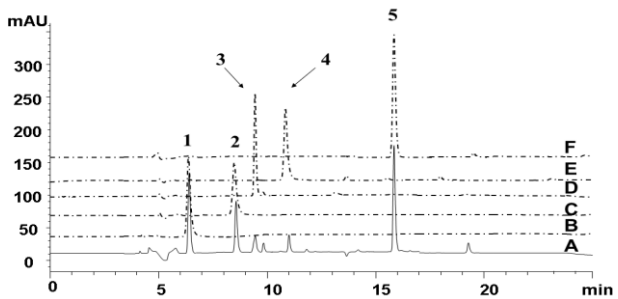

Figure 3. HPLC profiles of ethyl acetate extract of *P. lobata* (A), and purified compounds 1–5 (B–F): puerarin (B), daidzin (C), 3′-hydroxy-puerarin (D), 3′-methoxy-puerarin (E), daidzein (F).

In our experiment, we selected four series of solvent systems according to the solubility of the target compounds. The *K* values of these compounds were calculated from the HPLC data as aforementioned and summarized in Table 1. The first system, containing ethyl acetate/petroleum ether /water (1:0.25:1.25, v/v/v), had very close *K* values for compounds 1 and 2 (K_1=0.13, K_2=0.21), 3 and 4 (K_3=1.38, K_4=1.34), respectively, which caused the peaks 1 and 2, the peaks 3 and 4 to be eluted too close to separate, respectively. When the ratio was adjusted to ethyl acetate/petroleum ether /water (1:0.3:1.3, v/v/v), compounds 1, 2 and 4 still had very low and close *K* values (K_1=0.05, K_2=0.07, K_4=0.41), which would cause the three peaks to be eluted too close to the solvent front, leading to poor performance in separation. A system containing hexane–ethyl acetate–*n*-butanol–ethanol–water, 1:2:1:0.5:4 by volume was found to have a short settling time, but poor separation due to the close *K* values for compounds 1 and 2(K_1=0.44 and 0.90). By further modifying the ratios of the latter system containing hexane–ethyl acetate–*n*-butanol–ethanol–water (0.5:2:1:0.5:3.5) turned out to be the best for separation. The *K* values for compounds 1-5 in this system were at 1.11, 2.37, 7.3, 12.48 and 49, respectively. In our previous paper, we found that *K* values higher than 5 were acceptable for the separation in short time.[15, 16] In this experiment, with a *K* value at 7.3, fraction Ⅲ (compound 3) had a retention time around 80 min. Since high *K* values lead to long separation times, in the case of compounds 4 and 5 whose *K* values were 12.5 and 49

Table 1 The *K* (partition coefficient) values of compounds 1–5 in different solvent systems

Solvent system (volume ration)	1	2	3	4	5
EtOAc/PE/ water (1:0.25:1.25)	0.13	0.21	1.38	1.33	11.3
EtOAc/PE/ water (1:0.3:1.3)	0.05	0.07	1.23	0.41	16.9
Hex/ EtOAc /BuOH/EtOH /water (1:2:1:0.5:4)	0.44	0.90	4.60	8.20	73.0
Hex/ EtOAc /BuOH/EtOH /water (0.5:2:1: 0.5:3.5)	1.11	2.37	7.30	12.5	49.0

(Table 1), they would have taken a long time to be fully eluted. In order to save solvents and time, the corresponding compounds 4 and 5 was collected by flushing the stationary phase out. When this solvent system was applied to the prepared HPCCC separation with a sample load of 5.1g and flow rate at 25 mL/min, as shown in Fig. 2, the fractions Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ were separated within 200 min, and all fractions containing the same compound determined by HPLC were combined, and freeze-dried. The fractions Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ were confirmed as compounds 1, 2, 3, 4 and 5 in Fig. 3. The present preparative HPCCC produced a total of 155 mg, 41 mg, 12 mg, 6 mg and 106 mg of compounds 1, 2, 3, 4 and 5 from 5.1 g of the ethyl acetate extract of *P. lobata*. The purities of the five compounds 1-5 as determined by HPLC were 98.77%, 96.53%, 97.59%, 90.21% and 98.36% respectively.

Identification by HPLC and ESI-MS^n

The HPLC chromatogram of the ethyl acetate extract of *P. lobata* is given in Fig.3. A good separation was achieved within 25 min. Five major peaks 1-5 were separated and detected with retention times at 6.5, 8.6, 9.4, 11.0 and 15.8 min, respectively. Compounds 1-5 were tentatively identified as puerarin, daidzin, 3′-hydroxy-puerarin, 3′-methoxy-puerarin and daidzein by congruence in its retention time with those of authentic standards, and all gave rise to very big peaks on the HPLC-DAD chromatogram at 250 nm.

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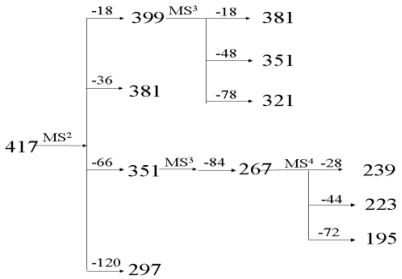
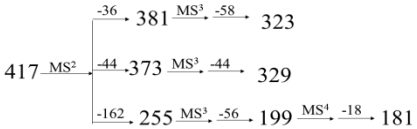
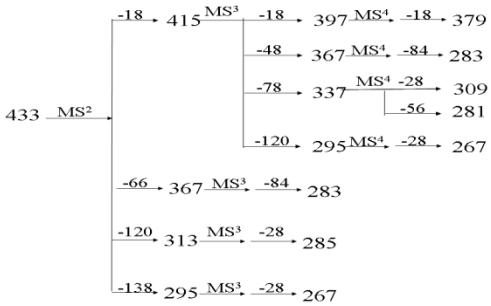
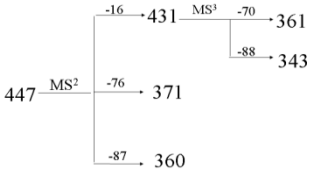
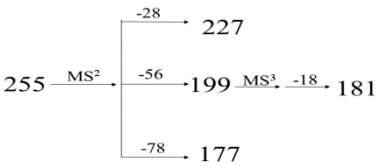
Table 2 The MS^n data in the positive mode (m/z values) of peaks 1–5 in LC profile

Peak identification	M [a]	$[M+H]^+$ [b]	MS^n (*m/z*)[c]
1 puerarin	416	417	
2 daidzin	416	417	
3 3′-hydroxy-puerarin	432	433	
4 3′-methoxy-puerarin	446	447	
5 daidzein	254	255	

a) Neutral molecules. b) Deprotonated molecular ion. c) MS^n fragment ions

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To further investigate the structures of these five peaks, ESI-MS^n experiments were attempted and the results are shown in Fig. 4 and Table 2. Our study proved that positive ion mode is more sensitive, and provided more information on isoflavone structures than those obtained in the negative ion mode, which were in agreement with the results by Zhang *et al* from the study on *P. lobata*.[4] Under ESI-MS conditions, compounds related to peaks 1–5 in Fig. 3 exhibited intense molecular ions $[M+H]^+$ at *m/z* 417, 417, 433, 447 and 255 in the positive mode, from which the molecular weights of peaks 1–5 are confirmed to be 416, 416, 432, 446 and 254, respectively. By comparing the molecular weight information with those found in the literature,[21,26,27] the identities of compounds 1-5 were confirmed as puerarin, daidzin, 3′-hydroxy-puerarin, 3′-methoxy-puerarin and daidzein, respectively. As shown in Table 2, the ESI-MS^n data display some common features, such as the neutral lose of a molecular of water (18), the neutral lose of CO (28) and CO_2 (44), the cleavage of hexose(120), which are very useful for determining the presence of specific functional groups in the structures.

The nomenclature of the product ions containing intact A and B rings of flavone skeleton was adapted from the literatures,[28-30] and the $^{i,j}A^+$ and $^{i,j}B^+$ represent product ions, the superscripts *i* and *j* indicate the C-ring bonds of flavones skeleton that have been broken.

The ESI-MS data of compound 1 is shown in Table 2. In the full scan positive ion mode, peak 1 exhibited as $[M+H]^+$ ion at *m/z* 417, indicating the molecular weights of 416, the same as puerarin.[4, 21, 26] In the MS^2 experiment, the ion at *m/z* 417 yielded four fragment ions at *m/z* 399, 381, 351 and 297. The daughter ion at *m/z* 399 and 381 were produced directly from the parent ion of *m/z* 417 due to the neutral loss of one and two molecular of water. The fragment ion observed at *m/z* 351 in the MS^2 spectrum came from the loss of a CH_2O and one molecular of water together from the parent ion. The fragment ion at *m/z* 297（$[M+H-120]^+$）in the MS^2 spectrum was formed from the cleavage of hexose $^{0,2}{}_6X$, which is consistent with the literature data on puerarin in LC-ESI-MS^2 experiments[4,21,26] The MS^3 spectrum of the ion at *m/z* 399 gave three major ions at *m/z* 381, 351 and 321, respectively. The ion at *m/z* 351 was produced by the loss of a CH_2OH and an OH group at hexose. The ion at *m/z* 321 was produced by the cleavage of B ring $^{1,3}{}_6X$ and together with the loss of a water. And the MS^3 spectra of *m/z* 351 gave one major ion at *m/z* 267, which was formed from the cleavage of hexose $^{0,1}{}_6X$. The further ESI-MS^4 experiment of *m/z* 267 gave three major ions at *m/z* 239, 223 and 195, respectively, which were exhibited from the loss of a CO molecule, a CO_2 molecule, and a CO and a CO_2 together. Based on the ESI-MS^n data of the standards, and the matching retention time, peak 1 was confirmed to be puerarin.

Although the HPLC profile showed that peaks 1 and 2 were eluted at different times, they had the same molecular weight ($[M+H]^+$, *m/z* 417) in the positive ion mode. As shown in Fig.1, peaks 1 and 2 having the same backbone, and the only difference is the position of glycosidic moieties were at R and R', respectively, however, they have different ESI-MS pattern. Three major fragment ions at *m/z* 381, 373 and 255 were observed in the MS^2 experiment for peak 2. The fragment ion at *m/z* 381 followed the same fragment pattern as peak 1. The fragment ion at *m/z* 373 was produced by the loss of one molecular of CO_2, and the fragment ion at *m/z* 255 came from the parent ion by the loss of one molecular of hexose which is consistent with the result of the LC–MS-MS^2 analysis in positive ion mode [21]. The MS^3 spectra of *m/z* 381 gave one major ion at *m/z* 323 from the cleavage of hexose $^{1,4}{}_6X$. The MS^3 spectra of the ion at *m/z* 373 produced one major ion at *m/z* 329 from the loss of one molecular of CO_2. The MS^3 spectra of the ion at *m/z* 255 gave one major ion at *m/z* 199. The further ESI-MS^4 experiment of *m/z* 199 gave one major ion at *m/z* 181, which was exhibited from the loss of a molecular of water. The peak 2 was confirmed as daidzin by compared with the retention time and MS^n data of the standard daidzin.

As shown in Fig. 4(A) and Table 2, peak 3 eluted at 9.4 min in Fig.3, and the $[M+H]^+$ ion at *m/z* 433 with high abundance and a small $[M+Na]^+$ at *m/z* 456 were observed in the positive mode in Fig.4(A), suggesting the molecular weight to be 432, the same as that for 3'-hydroxy-puerarin. Further investigation in the MS^2 experiment of the *m/z* 433 ion yielded four fragment ions at *m/z* 415, 367, 313 and 295, respectively (Fig. 4B). The notable daughter ion at *m/z* 415 was produced directly due to the neutral loss of a molecular of water. The ion at *m/z* 367 followed the same fragmentation pathway as described in peak 1. The fragment ion observed at *m/z* 313 in the MS^2 spectrum came from the cleavage of hexose $^{0,2}{}_6X$. The ion at *m/z* 259 is resulted from the cleavage of hexose $^{0,2}{}_6X$ and one molecular of water together from the parent ion. The MS^3 spectrum of the ion at *m/z* 415 exhibited four base peak ion at *m/z* 397, 367, 337 and 295 (Fig. 4C, Table 2). The ion at *m/z* 397 was produced by the loss of a water molecule, the ion at *m/z* 367 is shown in scheme 1, the ion at *m/z* 337 is resulted from the cleavage of hexose $^{2,4}{}_6X$ and one molecular of water together, and the ion $[M+H-120]^+$ at *m/z* 295 followed the same fragmentation pathway as described in peak 1. The MS^3 spectrum of the ion at *m/z* 367 exhibited one base peak ion at *m/z* 283 which was formed from the cleavage of B ring [M+H – H_2O-CH_2-OH-84] $^{0,4}{}_6X$. And the MS^3 spectrum of the ion at *m/z* 313 and 295 exhibited one base peak ion at *m/z* 285 and 267 respectively, which were both formed from the loss of CO molecular. The MS^4 spectra of *m/z* 397, 367 and 295 all exhibited one base peak ion at *m/z* 379, 283, and 267 respectively. The MS^4 spectra of *m/z* 337 exhibited two major ions at *m/z* 309 and 281 which were formed from the loss of one and two CO molecular. Scheme 1 shows the fragments of the peak 3 observed in this study. Based on the ESI-MS^n data of the standard, and the matching retention time, peak 3 was confirmed to be 3′-hydroxy-puerarin.

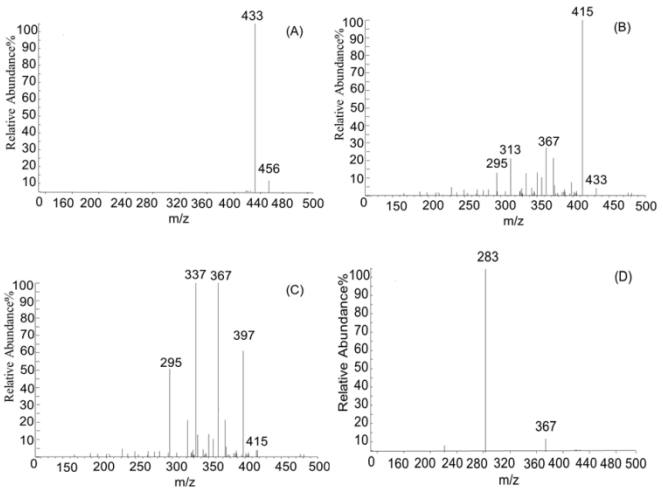

Figure 4. (A) LC-ESI-MS spectrum of m/z 433 of compound 3 in the positive mode; (B) LC-ESI-MS^2 spectrum of the parent ion at *m/z* 433; (C)LC-ESI-MS^3 spectrum of the fragment ion at *m/z* 415 of compound 3; (D) LC-ESI-MS^4 spectrum of the fragment ion m/z 367 of compound 3.

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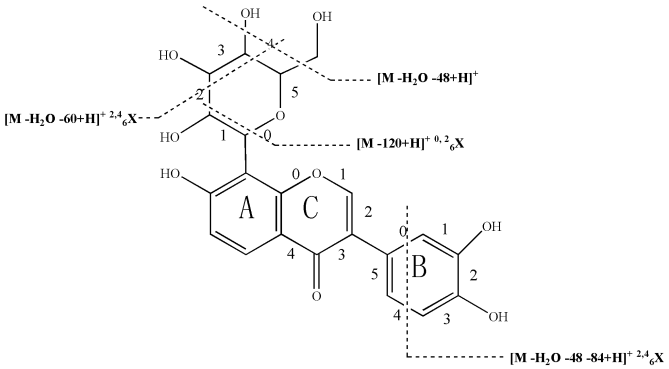

Scheme 1. Proposed fragmentation pathway for the deprotonated molecular ion $[M+H]^+$ of 3′-hydroxy-puerarin.

Peak 4 in Fig.3 was further investigated by ESI-MS^n, and the data were summarized at Table 2. In full scan positive ion mode, peaks 4 exhibited as $[M+H]^+$ ion at *m/z* 447. The MS^2 spectra of the ion at *m/z* 447 showed three major ions at *m/z* 431, 371, and *m/z* 360. The fragment ion observed at *m/z* 371 came from the neutral loss of $[M+H -CH_2OH -CO]^+$ from the parent ion. The ion ($[M+H -87]^+$) observed at *m/z* 360 in the MS^2 spectrum was formed from the cleavage of C ring $^{1,2}{}_6X$ and the loss of a molecule of water together, which indicates that there was one OCH_3 in the structure. The fragment ion in the ESI-MS^3 of the ion at *m/z* 431 is due to the loss of two CO_2 molecular. Based on the ESI-MS^n data described above, peak 4 followed the similar fragmentation pathway as 3′-methoxy-puerarin.

Peaks 5 exhibited as $[M+H]^+$ ion at *m/z* 255 (Table 2). The MS^2 spectra of the ion at *m/z* 255 showed three major ions at *m/z* 227, 199, and *m/z* 177 respectively. The ion $[M+H -78]^+$ at *m/z* 177 followed the same fragmentation pathway of *m/z* 321 as described in peak 1. The MS^3 spectra of *m/z* 199 exhibited only one major ion at *m/z* 181 which was formed from the loss of one molecular of water. Comparing the ESI-MS^n data of the standard with literatures,[4, 26] peak 5 at *m/z* 255 can be identified as daidzein. however, the exact structure of these compounds need to be confirmed by NMR spectroscopy.

Identification by ^{1}H-NMR

Further studies in ^{1}H-NMR experiments of these five compounds showed that all ^{1}H-NMR data of compounds 1–5 matched with the authentic standards, and the reported data for puerarin, daidzin, 3′-hydroxy-puerarin, 3′-methoxy-puerarin and daidzein.[31,32] Based on all available data, we therefore conclude that compounds 1, 2, 3, 4 and 5 are puerarin, daidzin, 3′-hydroxy-puerarin, 3′-methoxy-puerarin and daidzein, respectively.

Compound 1: Puerarin, ^{1}H-NMR (600 MHz, CD_3OD) δ: 8.28(1H, s, H-2)、8.14 (1H, d, J=8.8 Hz, H-5) 、6.95(1H, d, J=8.8 Hz, H-6) 、7.45 (2H, d, J=8.5 Hz, H-2′, 6′)、6.80(2H, d, J=8.5 Hz, H-3′, 5′)、5.18 (1H, d, J=9.3 Hz)、3.97~ 3.99 (4H, m)、3.57~ 3.62(6H, m).

Compound 2: Daidzin, ^{1}H-NMR (600 MHz, CD_3OD) δ: 8.23 (1H, s, H-2)、8.04 (1H, d, J=8.8 Hz, H-5) 、7.47 (2H, d, J=8.5 Hz, H-2c, 6c) 、7.30 (1H, d, J=2.3 Hz, H-8)、6.95 (1H, dd, J=8.8, 2.3 Hz, H-6) 、6.93 (2H, d, J=8.5 Hz, H-3′, 5′)、5.19 (1H, d, J=7.5 Hz)、3.49~3.66(6H, m).

Compound 3: 3′-hydroxy-puerarin, ^{1}H-NMR (600 MHz, CD_3OD) δ: 8.25 (1H, s, H-2)、8.14(1H, d, J=8.8 Hz, H-5) 、7.12 (1H, d, J=1.9 Hz, H-2′)、7.07 (1H, d, J=8.8 Hz, H-6)、6.91 (1H, dd, J=8.1, 1.9 Hz, H-6′)、6.76 (1H, d, J=8.1 Hz, H-5′)、5.18 (1H, d, J=9.6 Hz)、4.20~5.20(4H, m)、3.56~4.2(6H, m).

Compound 4: 3′-methoxy-puerarin, ^{1}H-NMR (600 MHz, CD_3OD) δ: 8.32 (1H, s, H-2) 、8.15 (1H, d, J=8.8 Hz, H-5) 、7.26 (1H, d, J=1.6 Hz, H-2c) 、7.09 (1H, d, J=8.8 Hz, H-6) 、6.99 (1H, dd, J=8.1, 1.6 Hz, H-6′) 、6.94 (1H, d, J=8.1 Hz, H-5c) .

Compound 5: Daidzein, ^{1}H-NMR (600 MHz, CD_3OD) δ: 8.22 (1H, s, H-2)、8.14 (1H, d, J=8.7 Hz, H-5) 、7.45 (2H, d, J=8.4 Hz,H-2′, 6′)、6.92 (1H, dd, J=8.7, 2.3Hz, H-6)、6.87 (1H, d, J=2.3 Hz, H-8) 、6.81 (2H, d, J=8.4 Hz, H-3′, 5′) .

Conclusions

In this paper, the preparative HPCCC was developed for the isolation and purification of puerarin, daidzin, daidzein, 3′-hydroxy-puerarin and 3′-melhoxy-puerarin from *P. lobata* for the first time. The solvent system containing hexane–ethylacetate–*n*-butanol–ethanol–water(0.5:2:1:0.5:3.5, v/v/v/v/v) provided fast and efficient separation, and good purity for the five major compounds in *P. lobata*. Meanwhile, the structures of the five isoflavones in *P. lobata* have been investigated by means of ESI-MS^n, and confirmed by ^{1}H-NMR experiment. Some characteristic features of isoflavones were found, which allowed us to determine the functional groups in the structures.

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