

Identification of Isoflavonoids in Several Kudzu Samples by High-Performance Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry

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Abstract

Pueraria lobata is a rich source of isoflavonoids. The detection and identification of isoflavonoid components from *Pueraria radix* (RP), callus and cell cultures, is very important for the safest and most effective use of kudzu as a medicinal plant, and for the studies on quantitative analysis and secondary metabolism of isoflavonoids *in vitro* cultures. Liquid chromatography is coupled with negative and positive electrospray ionization (ESI) tandem mass spectrometry (MS–MS), and photodiode array detection is used to characterize and detect isoflavonoids in root, callus, and cell samples of *P. lobata*. Characteristic product ions of aglycones, *O*-glucosides, and *C*-glucosides were obtained from the full-scan ESI-MS chromatography of the major peaks and the MS–MS spectra of the protonated ions. Five major components of puerarin, daidzin-6"-*O*-acetylyester, genistin-6"-*O*-malonylyester, biochanin A-7-*O*-glucoside-6"-*O*-malonylyester, and daidzein are detected and identified from the methanolic extract of *P. lobata* callus cultures. The major isoflavonoid components of *P. lobata* cell suspension cultures are identified as puerarin, daidzin, daidzin-6"-*O*-acetylyester, genistin-6"-*O*-malonylyester, biochanin A-7-*O*-glucoside-6"-*O*-malonylyester, genistein-8-*C*-glucoside-6"-*O*-malonylyester, and daidzein, on the basis of ESI-MS and MS–MS spectra analysis. Likewise, puerarin, daidzin, genistein-6"-*O*-malonylyester, 3'-methoxypuerarin, and daidzein are detected and identified from RP. Of those isoflavonoid components detected, daidzin-6"-*O*-acetylyester is a new isoflavonoid glucoside and is for the first time detected from *P. lobata* cultures *in vitro*.

Introduction

Kudzu is a perennial leguminous plant of the genus *Pueraria* and widely distributed in tropical, subtropical, and temperate zones of East Asia. The species, which produces large tubers with starch, stem fibers, silage, and hay, is useful as a supplementary material in the food industry, an erosion-controlling soil cover, and a decorative and shading plant (1,2).

Pueraria radix (RP), the root of *Pueraria lobata* (Willd.) Ohwi, is widely used as a drug under the name of Ge Gen or Kakkon in traditional Chinese and Japanese medicines (3). RP is highly valued for its medicinal properties (4,5), and it is one of the most important oriental crude drugs that increases coronary artery blood flow and is used as an antipyretic, antidiarrhetic, diaphoretic, antiemetic, antispasmodic, and antimicrobial remedy. It is also an active agent against angina pectoris, hypertension, deafness, optic nerve atrophy, retinitis, and alcohol abuse (3,5,6).

Kudzu contains phenolic compounds—isoﬂavonoids of different structures (7,8), such as aglycones, *O*-glucosides, and *C*-glucosides—mainly puerarin (daidzein-8-*C*-glucoside), daidzin (daidzein-7-*O*-glucoside), and daidzein (9). Isoﬂavonoids from RP are reported to have many important physiological effects such as combating human cancer cell lines (10) and inhibiting aldehyde dehydrogenase and xanthine oxidase (11,12). In other studies, RP isoﬂavonoids have been reported to possess oestrogenic activities (13) and can be prepared in the form of commercially available dietary supplements (14). Isoﬂavonoids are synthesized predominantly in legumes and show a lot of important physiological functions. They mediate multiple plant–microbial interactions, play key roles in signaling between plants and microbes for defense, and act as antifungal agents and precursors to major phytoalexins (15,16). They also act as screens from harmful UV light and scavengers of reactive oxygen species (17). In view of this, it is of interest to elucidate the distribution of these isoﬂavonoids in different organs because they could play an important role in the kudzu defense mechanism against pathogenic attack and environmental stresses. Furthermore, knowledge of isoﬂavonoid distribution in kudzu is also important for the safest and most effective use of kudzu as a medicinal plant.

Although many secondary metabolites have been isolated from kudzu, previous investigations were limited to the root sample of *P. lobata*, which is used in studies on the separation and quantitative analysis of isoﬂavonoids. Most reported methods in those previous investigations for the analysis of

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isoflavonoids are based on high-performance liquid chromatography (HPLC) or capillary electrophoresis separation with UV, fluorescence, or electrochemical detection (19,20). Those methods are also limited to the detection of a smaller number of known compounds in those kudzu organs, and they are not applicable for the characterization of unknown isoflavonoids in a crude mixture. Isoflavonoids in kudzu dietary supplements (KDS) were detected and characterized by HPLC coupled with electrospray ionization (ESI) tandem mass spectrometry (MS–MS), but KDS samples were commercially prepared and might be mainly from kudzu root (14). Analyzing the extracts of plants involves matrices that are unavoidably complex. Liquid chromatography–MS is best suited for use as a sensitive and selective analytical method for the rapid structural characterization and quantitative analysis of known compounds as well as the identification of unknown compounds from crude and partially purified samples of natural products (21). From this, new medicine sources might be found from the *P. lobata* plant culture in vitro, and new pharmaceuticals can be produced with the culture systems (callus or cell suspension culture) as biological reactors.

In this report, the primary objective was the identification of isoflavonoids in several organs of kudzu by HPLC–MS. To the best of our knowledge, we have also, for the first time, reported a series of isoflavonoids from *P. lobata* plant tissue cultures in vitro based on MS–MS analysis.

Experimental

Materials

The root sample of *P. lobata* was collected in December, 2002 from Shucheng (Anhui, China). The sturdy stems with adventitious roots were transplanted and cultivated in kudzu germplasm resource garden of our campus, and young shoots of *P. lobata* were plucked and used for in vitro cultures. The shoots were washed for 30 s with 70% ethanol solution containing two or three drops of Tween 80. Afterwards, they were subsequently surface-sterilized in 0.1% mercuric chloride solution by shaking for 10 min. After rinsing with sterile water, the shoots were placed on the MS medium supplemented with 0.1 mg/L *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU) and solidified with 0.8% agar, the explants were cultured in the dark at 25°C. Three weeks later, the induced callusi were excised from the section of the kudzu shoots and transferred onto B5 solid medium supplemented with 1.0 mg/L α -naphthalene acetic acid (NAA), 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 mg/L kinetin (KT), and they were cultured in dark or weak light at 25°C. Proliferating callus cultures were subcultured every 20 days, which was decided by the growth curve of kudzu callus established in the preliminary study. Approximately four or five subculture cycles later, the callus cultures loosened and could be used for HPLC–MS analysis of isoflavonoids or cell suspension cultures.

The loose cultures of kudzu callus were transferred to B5 liquid medium supplemented with the same hormone contents previously mentioned at 120 rpm and 25°C. Five or 6 days

later, the dissociated cells were filtered by 50 mesh filter and subcultured every 7 days, which was also determined by the growth curve of cell suspension culture. Approximately two or three cycles later, the cultures were constantly proliferating and could be harvested and used for HPLC–MS analysis of isoflavonoids.

Reagents and solutions

CPPU, NAA, 2,4-D, and KT were from Sigma (Deisenhofen, Germany). The authentic reference compounds of puerarin, 3'-methoxypuerarin, daidzin, and daidzein were purchased from the Institute of Biological Drugs Examination (Beijing, China). Methanol, acetonitrile, and acetic acid were all purchased from TEDIA (Fairfield, OH) and were of HPLC grade. Water was doubly distilled and prepared with a Purelab Classic UF purification system from Pall (East Hills, NY). All other chemicals used were of analytical reagent grade. Stock solutions of puerarin, 3'-methoxypuerarin, daidzin, and daidzein (1 mg/mL) were prepared in 70% (v/v) methanol and diluted to the desired concentrations just prior to use.

Sample preparation

Root samples were dried at 60°C for 6 h in an oven and were then gently pulverized. The powder was dried again just prior to use to the stable weight, and an accurately weighed amount of the powder (2 g) was extracted by sonication for 45 min with 40 mL of 70% methanol at room temperature. This was done instead of refluxing in methanol because prolonged storage of the extracts at an elevated temperature resulted in hydrolysis of isoflavonoid glucosides. Callus and cell cultures were harvested and dried at 60°C to the stable weight, and then both powders were mildly extracted with 70% methanol by ultrasonication and cool-soaked at room temperature. Finally, all of the extracted solutions were reconstituted to the initial weight with 70% methanol. This was done in order to maintain the stable weight of sample and extracting solvent. It was then centrifuged at 10,000 rpm. The supernatant was filtered through a syringe cellulose acetate filter (0.45 μ m) prior to their use and subjected to HPLC–MS analysis. All samples were analyzed in duplicate.

LC–MS and LC–MS–MS analysis

The HPLC–MS system consisted of an ESI interfaced LCQ Advantage ion trap MS (ThermoFinnigan, San Jose, CA) and a Finnigan Sunveyor HPLC system equipped with an autosampler and a photodiode array absorbance detector. The column used was a (250- \times 4.6-mm i.d.) Hypersil ODS 5- μ m C₁₈ HPLC column (Supelco, Bellefonte, PA). The mobile phase consisted of water containing a volume fraction of 0.5% acetic acid (A) and acetonitrile (B). The composition was programmed was linearly from 0% to 30% B in 40 min, then from 30% to 40% B in the next 20 min. Prior to the next injection, the column was equilibrated for 30 min with 0.5% acetic acid. The chromatographic profile was recorded at 254 nm. In order to adapt to the flow rate requirement of ESI–MS, the flow rate of 0.8 mL/min was reduced to approximately 0.24 mL/min by a split valve at the ratio of 3:10 just before the eluent entered the ESI interface. The ionization parameters were as follows: negative

and positive ion mode; capillary voltage, 15 V; capillary temperature, 350°C; sheath gas flow rate, 35 units; and auxiliary gas flow rate, 10 units. Negative or positive ion mass spectra were recorded over a range of m/z 100–700. The MS–MS analysis of isoflavonoids, aglycones, and glycosides was performed using the same MS equipped with an electrospray ion source, and spectra were obtained in the positive or negative ion modes. Product ion spectra were obtained by selecting the protonated or deprotonated ions for collision (energy = 35 eV), using nitrogen as a collision gas.

Results and Discussion

LC–MS and LC–MS–MS analysis of the methanolic extract from *P. lobata* callus cultures

As shown in Figure 1A, there were five major peaks (1, 2, 3, 4, and 5) of different retention times at 14.08, 19.27, 27.57, 34.85, and 39.16 min, respectively, and their UV spectra were obtained using the photodiode array detector during HPLC analysis of isoflavonoids and its glycosides in *P. lobata* callus samples. The MS spectra of those five peaks were also measured using HPLC–ESI–MS and are shown in Figure 1B–F.

The mass spectrum of **1** contains an abundant Y_0^+ ion at m/z 417 in Figure 1B, and MS–MS experiments on **1** in negative ion mode also showed prominent ions at m/z 295.1 and 267.3 in Figure 2A caused by the losses of 120 and 148 Da, respectively. According to the information from all these fragment structures based on ESI–MS and MS–MS analysis, peak **1** can be identified as puerarin. The mechanism of fragmentation of **1** in negative ion mode is shown in Figure 3 (14).

The ESI–MS spectrum of compound **2** in Figure 1C exhibited an obvious singlet of quasimolecular ion at m/z 474.8 [M–H][−]. The ions at m/z 474.8, 415.1, and 253.3 (in Figure 1C) corresponding to the characteristic losses of 60 and 162 Da, sequentially, were also displayed, confirming the existence of acetyl and glucoside unit, respectively. A possible mechanism of fragmentation of **2** in negative ion mode is shown in Figure 4. Furthermore, by collision induced dissociation in the ion trap, the quasimolecular ion was fragmented in an ion at m/z 416.9 [M+H]⁺, which was further fragmented and transferred completely to another singlet (MS–MS) ion at m/z 255.2 [aglycon+H]⁺ (shown in Figure 2B). All those fragment structures agree with the structure of daidzin-6"-O-acetylcyster, which is different from the result in a previous report (18). To our knowledge, it was a new isoflavonoid glucoside that was first isolated from kudzu cultures in vitro.

Similarly, the ESI–MS spectrum of peak **3** in negative ion mode showed an obvious quasimolecular ion at m/z 516.8, and the characteristic fragment ion derived from the aglycon of isoflavonoids at m/z 253.2 was displayed in Figure 1D. The MS–MS experiment on **3** in the negative ion mode also showed prominent ions at m/z 431.0 and 269.2. The characteristic losses of 86 and 162 Da were also displayed in Figure 2C, confirming the existence of malonyl and glucoside unit, respectively. According to the information from all these fragment structure based on MS–MS analysis, peak **3** can be identified as

genistin-6"-O-malonylcyster (18,22). The possible mechanism of fragmentation of **3** in negative ion mode is also presented in Figure 5.

The product ion spectrum of **4** in negative ion mode contains a quasimolecular ion at m/z 532.7 [M–H][−], characteristic fragment ion at m/z 269.2, and other fragment ions at m/z 473.0 and 374.9, etc., in Figure 1E. From all those fragment structures based on ESI–MS analysis, compound **4** can be identified as biochanin A-7-O-glucoside-6"-O-malonylcyster.

The characteristic fragment ion at m/z 253.1 [M–H][−] displayed in Figure 1F is an obvious singlet of the quasimolecular ion from the mass spectrum of **5** in the negative ion mode. Combining the retention time (t_R) of HPLC–MS analysis, and

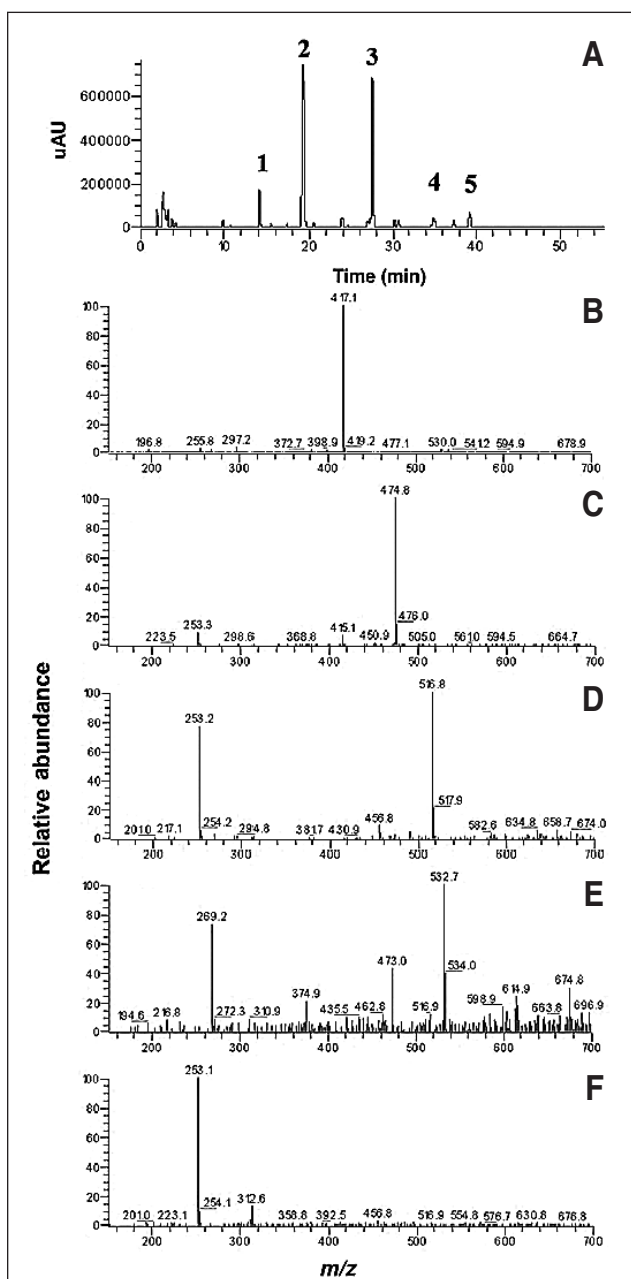
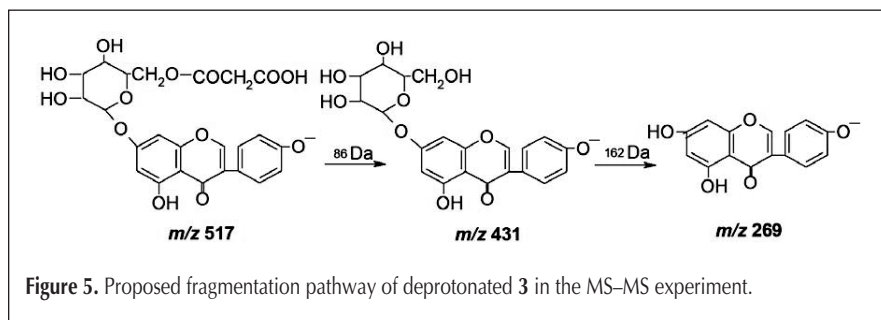
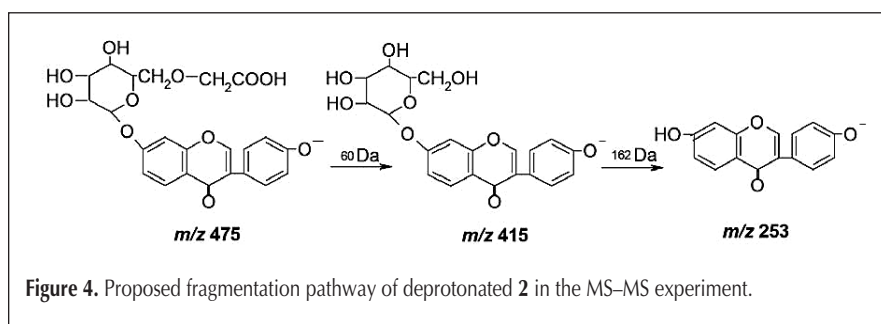
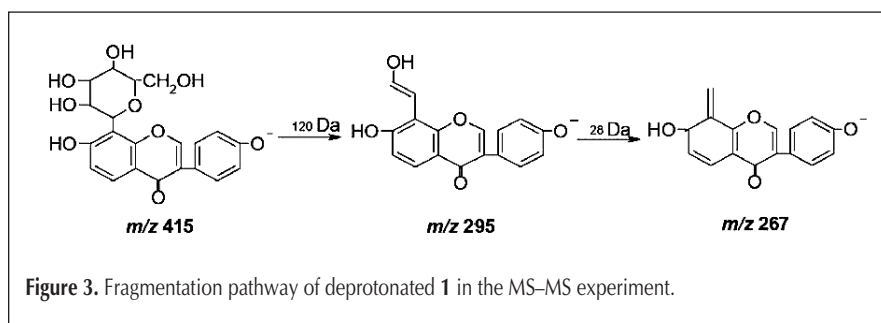
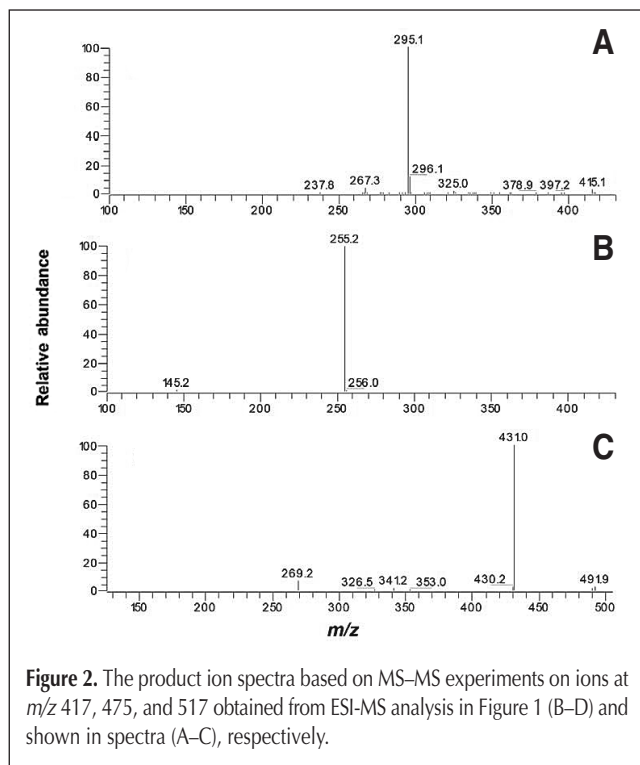


Figure 1. LC–MS chromatogram of the methanolic extract from *P. lobata* callus cultures (A) and full-scan ESI–MS of the major compounds shown in spectra (B–F).



the specific fragmentation profile of each standard, the ion of m/z 253.1 was found to be identical with standard daidzein.

LC–MS and LC–MS–MS analysis of the methanolic extract from *P. lobata* cell suspension cultures

An LC–MS chromatogram in negative ion mode of the methanolic extract from kudzu cell suspension cultures are shown in Figure 6A. There were seven major peaks in the retention range from 14 to 40 min, and the molecular mass of each component was obtained at its t_R . Following the LC–MS analysis, the sample was subjected to LC–MS–MS, during which the product ions of each component were obtained at its retention time, providing a specific fragmentation profile of each component. On the basis of comparison of MS–MS data with those of standards, the peaks of t_R at 14.83, 20.36, and 39.76 min shown in Figures 6B, 7, and 8A were identified as puerarin, daidzin, and daidzein, respectively.

An interesting feature of the LC–MS chromatogram is that the ions at m/z 416 ($t_R = 14.83$ and 20.36 min) appeared at different t_R values. The MS–MS experiment of the ion at m/z 415 with different retention times indicated that the fragmentation profiles of MS–MS data shown in Figures 6B and 7 are quite different from each other. As shown in Figure 6B, neutral losses of 120 and 150 Da from the protonated component of t_R at

14.83 min gave rise to ions at m/z 295.1 (base peak) and 253.2 in negative ion mode, respectively. The loss of 120 Da is indicative of C-glycoside. On the basis of comparison of MS–MS data with those of standard puerarin, the component of t_R at 14.83 min was successively identified as puerarin. On the contrary, the product ion spectrum of the compound of t_R at 20.36 min contains an abundant Y_0^+ aglycon ion at m/z 255 in positive ion mode caused by the neutral loss of 162 Da (Figure 7). The Y_0^+ ions correspond to rearrangement ions involving loss of the sugar unit and resulting in ions containing only the aglycon moiety (14). The product ions based on the MS–MS experiment were found to be identical with those of standard daidzin.

Likewise, the product ions of m/z 417 at 474.8, 516.8, and 532.8 in the negative ion mode from the peaks of t_R at 19.49, 28.76, and 35.61 min shown in Figures 6C, 8B, and 8C, respectively, were found to be very similar to those compounds (**2–4**) in kudzu callus cultures described previously. They were identified as daidzin-6''-O-acetyler, genistin-6''-O-malonyler, and biochanin A-7-O-glucoside-6''-O-malonyl-ester, respectively.

The ESI–MS spectra of compound at t_R of 27.96 min in Figure 8D contained the ions at m/z 516.8, 490.8, 431, and 269.3 in negative ion mode. The characteristic

losses of 86 and 162 Da, sequentially, were also exhibited confirming the existence of malonyl and glucoside unit, respectively, and the characteristic fragment ion at m/z 269.3 min in negative ion mode is indicative of genistein. All those fragment structures agree with the structure of genistein-8-C-glucoside-6"-O-malonylester, which has previously been reported from kudzu cell suspension cultures (22).

LC-MS and LC-MS-MS analysis of the methanolic extract from RP

As shown in Figure 9A, there were six major peaks of different t_R in the range from 13 to 40 min. Also following the comparison of ESI-MS and MS-MS data with those of standards, the peaks of t_R at 14.77 and 39.05 min were determined as puerarin (Figure 9C) and daidzein (Figure 10C), respectively. Similarly, on the basis of comparison of ESI-MS and MS-MS data with those of peaks at t_R 20.36 and 28.76 min shown in Figures 7 and 8B, the peaks of t_R at 20.26 and 27.75 min were identified as daidzin (Figure 10A) and genistin-6"-O-

malonylester (Figure 10B), respectively. An interesting feature of MS spectra shown in Figures 9B and 9C is that the ions at m/z 417 ($t_R = 13.60$ and 14.77) appeared at different t_R values, indicating that they could be isomeric compounds of puerarin. The product ion spectrum of the peak at t_R 15.73 min shown in Figure 9D contained ion at m/z 447 [M+H]⁺ and other ions at m/z 416.9, 240.9, and 160.1. From those fragment structures based on ESI-MS analysis and the t_R of standard

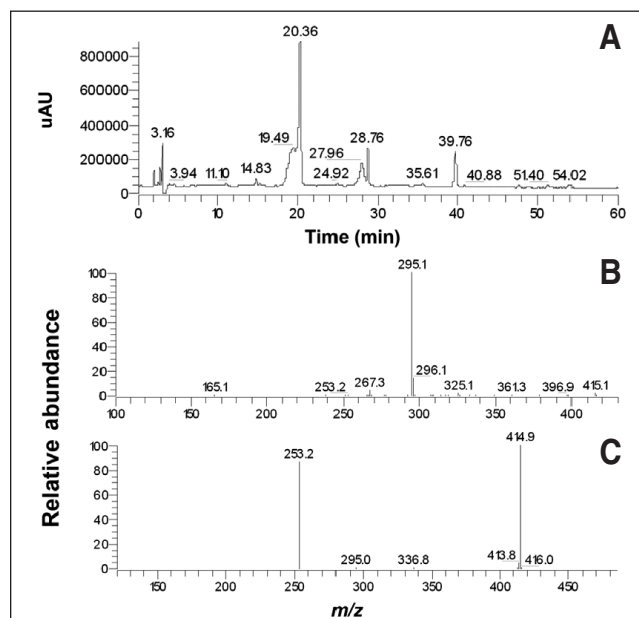


Figure 6. LC-MS chromatogram of the methanolic extract from *P. lobata* cell suspension cultures (A), and the product ion spectra based on MS-MS experiments on ions at m/z 415 and 475 obtained from ESI-MS analysis of the major compounds shown in spectra (B and C), respectively.

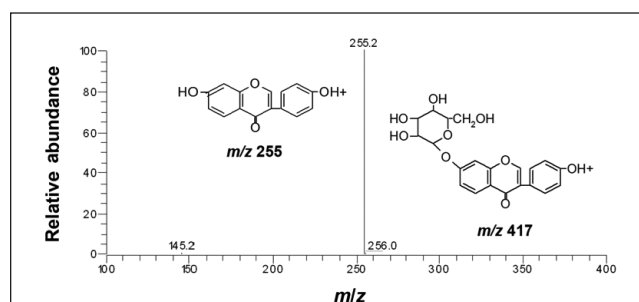


Figure 7. Product ion spectrum of the ion at m/z 417 and its possible structure.

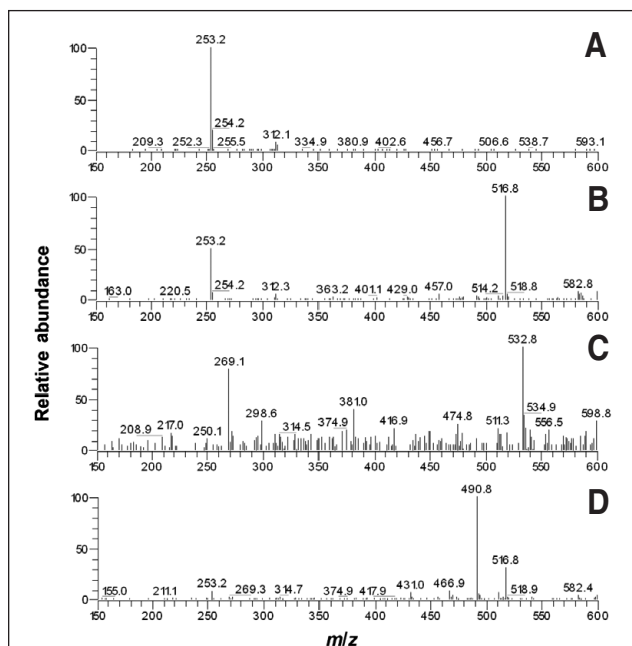


Figure 8. Full-scan ESI-MS of the other major compounds shown in spectra (A-D).

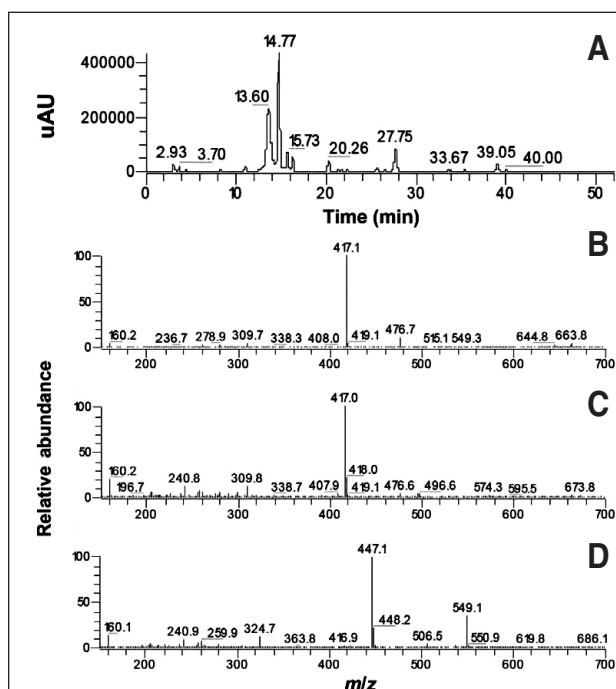


Figure 9. LC-MS chromatogram of the methanolic extract from RP (A) and full-scan ESI-MS of the major compounds shown in the spectra (B-D).

component at the LC–MS chromatography (total ion current), this compound was proposed to be 3'-methoxypuerarin.

Conclusion

Several aglycones, *O*-glucosides, and *C*-glucosides of isoflavonoids in RP, callus, and cell cultures were identified with characteristic product ions in ESI–MS–MS experiments. Structure identification of unknown isoflavonoids in the extract was confirmed by comparison with product ion spectra of known compounds. Puerarin, daidzin, 3'-methoxypuerarin, daidzein, and a series of other isoflavonoid glucosides were detected in the present study. To the best of our knowledge, daidzin-6"-*O*-acetyester is a new isoflavonoid glucoside that was detected for the first time from *P. lobata* cultures in vitro based on MS–MS analysis. The detection and identification of all of isoflavonoids is very important for the safest and most effective use of kudzu as a medicinal plant, and for the studies on quantitative analysis and secondary metabolism of isoflavonoids in in vitro cultures.

On the basis of HPLC–MS analysis, the major isoflavonoid components of *P. lobata* cell suspension cultures are very similar to those of callus cultures, but different from those of RP. However, puerarin, daidzin, and daidzein, etc., are all separated and detected in those three samples, but their concentrations showed great differences from each other by comparison of their peak areas or peak heights in each LC chromatogram.

Puerarin is the major component in root samples of *P. lobata*, and there are also a great deal of daidzin, 3'-methoxypuerarin and daidzein in RP, but the main components of isoflavonoids in callus and cell cultures are rather different from puerarin, daidzin, 3'-methoxypuerarin, and daidzein. The separation and quantitation of isoflavonoids in RP has been investigated in several reports (23–25), but as for the isoflavonoids in callus and cell cultures of kudzu, the accurate quantitation seems a little difficult because of the lack of

authentic reference standards. To some extent, the semiquantitative determination by comparison of their peak areas to puerarin might be the only choice because they have similar UV spectra. For the quantitative analysis and biosynthesis of isoflavonoids, and comprehensive utilization of kudzu sources, we are planning to examine the concentration of the main isoflavonoid constituents in callus and cell cultures in vitro after creating some of our own reference standards in the future.

Acknowledgments

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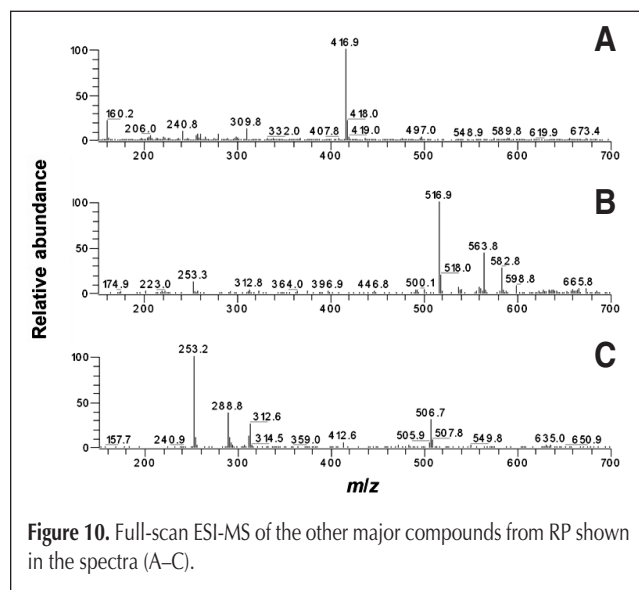


Figure 10. Full-scan ESI-MS of the other major compounds from RP shown in the spectra (A–C).

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