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Chemical Profiling and Quantification of Isoflavone Phytoestrogens in Kudzu Using LC/UV/MSD

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Abstract

Method development for determination of isoflavones in kudzu was achieved by HPLC/UV/ESI-MSD. Using three kudzu species of *Pueraria lobata*, *P. thomsonii* and *P. edulis*, and analyzing the isoflavones separately by species and from different plant tissues (roots, stems, leaves, flowers and fruits) in each species, a total of 25 isoflavones were identified by their molecular ions and characteristic fragment ion peaks using LC/MSD under MS and MS/MS mode, and in comparison with standard isoflavones. Two main chemical groups were identified: 1) 8-*C*-glycosyl isoflavone of puerarin and the analogues of 5-OH puerarin, 3'-OH puerarin, 3'-OMe puerarin, and their glycosides; and 2) daidzein, genistein, glycitein and their glycosyl and malonyl derivatives, which are similar to those known in soy. To accurately quantitate total isoflavones, acidic hydrolysis during extraction of kudzu samples was applied to convert the oxygen glycosides into their respective isoflavone aglycones of daidzein, genistein and glycitein, or non-hydrolyzed carbon glycosides of puerarin, 5-OH puerarin, 3'-OH puerarin and 3'-OMe puerarin. Under the multiple optimized conditions, all seven isoflavones in acidic hydrolyzed kudzu extracts were successfully separated within 30 min and quantified individually with calycosin used as internal standard by both UV and MS detectors. For the quantitative study, several standards e.g. 5-OH puerarin, 3'-OH puerarin and 3'-OMe puerarin are not commercially available. Using polyamide, sephdex-LH20 chromatography and Prep-HPLC, we purified these three standards from kudzu extracts and then elucidated their structures by UV, MS and NMR spectrometric methods. This is the first method to simultaneously quantitate all the isoflavones in kudzu.

Keywords: Kudzu, *Pueraria*, Isoflavones, LC/UV/MSD

1. Introduction

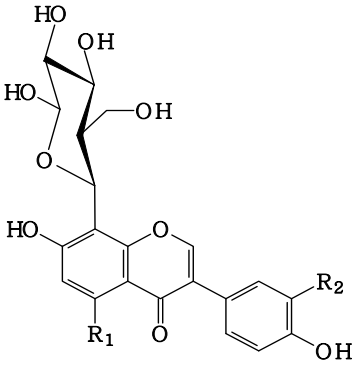
The kudzu root, called "Gegen" in Chinese medicine which is obtained mainly from *Pueraria lobata* (lobed kudzuvine), *P. thomsonii* (Thomson kudzuvine) and *P. edulis* (edible kudzuvine), has been primarily used for the treatment of common cold, influenza, and wrist and shoulder stiffness, or as antidipsotropic agent [1]. Kudzu was reported to contain high amounts of phytoestrogenic isoflavones, such as daidzein, genistein, puerarin and their derivatives [2-4]. These compounds based on the structural similarity to internal estrogen have received much interest for the prevention of menopausal symptoms, osteoporosis, high cholesterol, heart disease and cancer [5-10]. Several laboratories have provided evidence that the major isoflavones isolated from kudzu are effective in reducing alcohol intake [11-15]. Therefore, it is important to better understand the biology of the plant, and the tissues and sites of isoflavone accumulation. Several methods for determining isoflavones in kudzu and the derived products using high performance liquid chromatography combined with ultraviolet and/or mass spectrometric detector have been reported [14-21]. Qualitative studies on kudzu have only led to the identifycation of numerous isoflavones. The quantitative studies focused on the original or acidic hydrolyzed fractions kudzu extracts. However, only part of major components, puerarin, and soy-like isoflavones daidzein, genistein and the glycosides were quantified, and the researchers were not able to determine accurate levels of the total isoflavones in kudzu and the derived products. The purpose of this research was to chemically profile the isoflavones,

accurately quantify the total isoflavones in different parts (root, stem, leaf, flower and fruit) of kudzu and to establish a more robust and inclusive analytical method providing both a qualitative and quantitative method for the isoflavones in kudzu. In this study, we conducted field investigations, collected and authenticated various kudzu samples of *P. lobata*, *P. thomsonii* and *P. edulis* from China. Using HPLC/UV/MSD, different parts (root, stem, leaf, flower and fruit) of the three species were chemically profiled, and led to the identification of 25 isoflavones. The isoflavones can be categorized into two chemical groups: 1) puerarin (8-C-glucoside daidzein) and analogues, e.g. 3'-OMe-puerarin and 5/3'-OH-puerarin (**Figure 1**), and 2) daidzein, genistein, glycitein and their glycosyl and malonyl derivatives, which are similar to those found in soy. Under optimized conditions, the total content of isoflavones in acidic hydrolyzed kudzu was accurately quantified with calycosin used as an internal standard by both UV and MS detectors.

2. Experimental

2.1. Materials

Standard compounds, genistein and glycitein were purchased from Indofine Chemical Company, Inc. (Somerville, NJ) and daidzein from Sigma Chemical Co. (St. Louis, MO). The internal standard calycosin was purified from hydrolyzed extracts of the above ground biomass from red clover and the standards puerarin, 5-OH puerarin, 3'-OH puerarin and 3'-OMe puerarin were purified from kudzu extract in this laboratory. HPLC-grade methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), aqueous ammonia, and concentrated hydrochloric acid (HCl) were procured from Fisher Scientific Co. (Fair Lawn, NJ); formic acid was purchased from Acros Organics (NJ); and polyamide 6 and sephdex-LH20 were purchased from Sigma-Aldrich Co. (St. Louis, MO). HPLC-grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA), and was used to prepare all solutions. All the kudzu samples including each of the different plant parts from the roots, stems, leaves, flowers and fruits from *P. lobata*, *P. thomsonii* and *P. edulis* were collected in Yunnan Province, China. All botanical samples were identified and authenticated by species in Key laboratory, Department of Plant Pathology, Yunnan Agricultural University, Kunmin, Yunnan, China.



Puerarin: R1 = R2 =H
3'-OH-puerarin: R1 = H, R2 = OH
5-OH-puerarin: R1 = OH, R2 = H

Figure 1. The structures of puerarin and its analogues.

2.2. Apparatus

HPLC separation was performed on a Phenomenex Prodisyl ODS (3) column, 5 μm, 150 × 3.2 mm I.D. (Phenomenex Inc., Torrace, CA). For LC/ESI-MS and LC/MS/MS experiments, an Agilent 1100 Series LC/MSD trap (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump, photodiode array and multiple wavelength detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI) and software of HP ChemStation, Bruker Daltonics 4.1 and DataAnalysis 4.1 was used. Waters Prep-HPLC with a Phenonex Luna Phenyl-Hexyl column, 10 μm, 250 × 30 mm I.D. (Phenomenex Inc., Torrace, CA), Delta 600 pump, 2487 Dual λ absorbance detector, 600 controller, 717 autosampler, fraction collector II, in-line degasser AF and software of Millennium 32 was used for standard purification.

2.3. Purification of Internal Standard of Calycosin from the Aerial Part of Red Clover and Standards of Puerarin, 5-OH Puerarin, 3'-OH Puerarin and 3'-OMe Puerarin from Kudzu Roots

The internal standard of calycosin was purified from the above ground biomass of red clover as described in our previous paper [22]. For purification of the standards puerarin and it's analogues, approximately 100 g of dried roots of *P. lobata* was used as the starting material. The kudzu material was first refluxed in 500 mL 80% methanol for 2 hours for 2 times. The filtrations were combined and the solvent was evaporated under reduced pressure to obtained methanol extract (~12 g). The extract was then chromatographed on polyamide column using a step-gradient MeOH-H_2O (10% - 50% MeOH) and 250 ml fraction was collected. Those fractions containing the four target components as judged by LC/MS were collected and then re-chromatographied on sephdex-LH20 prior to further purification using Prep-HPLC. Prep-HPLC was performed using the mobile phase of

MeCN-H_2O (7% MeCN) at a flow rate of 7 mL/min to get puerarin (40 mg), 5-OH puerarin(12 mg), 3'-OH puerarin (14 mg) and 3'-OMe puerarin (8 mg). The structures of these four compounds were then determined and verified by UV, MS and NMR spectrometric methods.

2.4. Preparation of Stock Solutions and Calibration Standards

Individual stock solutions of 7 standards were prepared by dissolving the appropriate amounts of ~5.0 mg in 15.0 mL of diluent (water and MeOH, 3:7). The final volume of each solution was then diluted to 25 mL with diluent. Calibration standards were prepared by diluting the stock solutions with diluent and spiked with same amount of internal standard of calycosin. The calibration curve ranges for UV and MS methods show excellent linearity (**Table 1**). In the calibration plots, 8 and 6 different concentration levels were used for UV and MS detection, respectively.

2.5. Plant Sample Preparation

For qualitative study, ~200 mg of finely ground material was extracted with 10 mL 80% methanol using sonication for 1 hour at room temperature. The extracts were filtered through 0.45 μm filter and 20 μL extract was injected for each analysis. The extraction procedure for quantitative analysis was adopted from our prior studies [22,23]. Approximately 1000 mg of powdered kudzu material was placed into a 250 mL flask along with 50 mL of ethanol, 20 mL of DI water, and 8 mL of concentrated HCl. The mixture was refluxed for 2 hours protected by N_2. The solution was filtered and diluted to volume of 100 mL. Each hydrolyzed sample of 5 μL filtered over 0.45 μm filter was analyzed by triplicate injections.

2.6. Liquid Chromatographic and Mass Spectrometric Conditions for Identification of Isoflavones

HPLC separation was performed with the mobile phase consisting of solvent A and B in gradient, where A was 0.1% formic acid (v/v) in water and B was 0.1% formic acid (v/v) in acetonitrile. The linear gradient profile was from 10% to 40% B in 40min. The wavelength of UV detection was 254 nm. Column compartment was set at 25˚C. The flow rate was 1.0 mL/min. The electrospray ion mass spectrometer (ESI-MS) was operated under positive ion and auto MS/MS mode (Threshold, 30,000) and optimized collision energy level of 80%, scanned from *m/z* 100 to 700. ESI was conducted using a needle voltage of 3.5 kV. High-purity nitrogen (99.999%) was used as dry gas and nebulizer at a flow rate of 12 L/Min, and capillary temperature at 350˚C. Helium was used as collision at 60 psi. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. The auto MS/MS total ion chromatogram was processed by extracting the molecular ions of each isoflavone for identification.

2.7. Liquid Chromatographic and Mass Spectrometric Conditions for Quantification of Isoflavones

MS detection was conducted under collision energy level of 80% and scanned from *m/z* 100 to 600. Other MS parameter and LC conditions were the same as described above. Under SIM mode (selected ion monitoring), protonated $[M^+ + H]$ ions were isolated for each isoflavone. The mass spectrometer was set into two time segments: 1) from 0 to 16.5 min for 3'-OH puerarin; puerarin; 3'-OMe puerarin and 5-OH puerarin with isolation of *m/z* 417, 433 and 447; 2) from 16.5 to 30 min for daidzein, glycitein, genistein and calycosin of *m/z* 255, 271 and 285. The isolation width was set as 1.0 *m/z*. The calibration curves were plotted using a 1/x-weighted quadratic model for the regression of peak area acquired from UV and MS detector versus analyte concentration.

3. Results and Discussion

3.1. Characterization of Isoflavones

Simultaneous UV and processed auto MS/MS chromatograms of 80% methanol extract of *P. lobata* root are illustrated in **Figure 2**. The identities, retention time, protonated $[M+H]^+$ and characteristic fragment ions for individual peaks are listed in **Table 2**. In kudzu, the carbon glucoside of daidzein, puerarin (**7**, peak 6 in **Figure 2**) is well known to dominate the isoflavone constituents. Therefore, some minor analogues of puerarin e.g. 5-OH puerarin, 3'-OH puerarin, 3'-OMe puerarin and their glycosides are also detected on the basis of MS and MS/MS spectral interpretation and some of them by comparison to the authenticated standards. The MS spectra of puerarin and its analogues showing the protonated molecular ions $[M+H]^+$ are illustrated in **Figure 3**. Based on the above analysis, a total of 10 puerarin analogues were determined in kudzu (Compounds **1-9** and **12** in **Table 2**), with some of the structures of puerarin and its analogues shown in **Figure 1**. Daidzein, genistein, glycitein and their derivatives of glycoside and glycoside malonate, which are well-known to be present in soy, were also detected in kudzu (Compounds **10**, **11** and

13-25 in **Table 2**). Their structures were identified by analysis of MS spectral data, and by comparison to those in soy as well. Some of them e.g. daidzein, genistein, glycitein and the glucosides were also verified by comparison to the authenticated standards. Comparative evaluation of the different plant tissues indicated that the chemical profile of kudzu root and stem is very similar, among which the puerarin is the major peak. In contrast, the content of puerarin in kudzu leaf is relatively lower than daidzein and its malonyl derivative. In the flower, the major isoflovones are daidzein, genistein, glycitein and their glycoside derivatives, while only trace levels of isoflavones were detected in kudzu fruit. The chemical profile of kudzu root from different plant sources of *P. lobata*, *P. thomsonii* and *P. edulis* are very similar (**Figure 4**).

3.2. Quantification of Isoflavones

A qualitative study revealed that kudzu contains large mount of isoflavones, and most of these are available as glycoside and malonate conjugates, e.g. glycoside of daidzein, genistein and puerarin; and glycoside malonate of daidzein and genistein. Therefore, in this study to facilitate the quantification and accurately evaluate the total isoflavones in kudzu, the kudzu samples were hydrolyzed during extraction. Under optimized conditions, all three isoflavone aglycones of daidzein, genistein and glycitein, and four non-hydrolyzed carbon glycosides of puerarin, 5-OH puerarin, 3'-OH puerarin and 3'-OMe puerarin were successfully quantified by using HPLC/UV/ESI-MSD. This is the first method to quantify all the isoflavones in kudzu, and therefore to provide an accu-

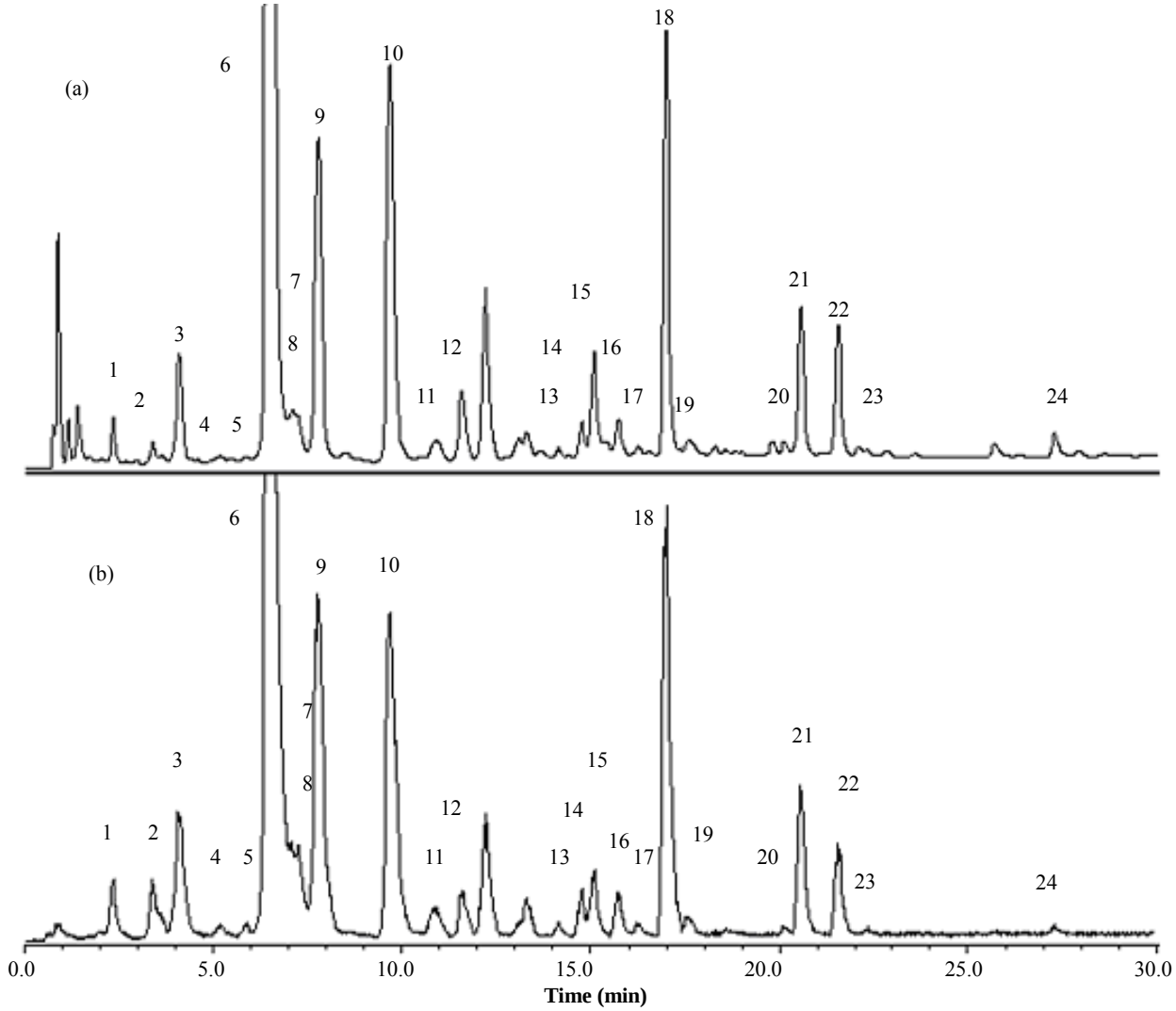


Figure 2. Simultaneous LC-UV (a) and processed auto MS/MS (b) chromatograms of *P. lobata* root extract. The identities, t_R value and MS of each peak are listed in Table 2.

Table 1. Calibration curve ranges and regressions (r2) of 7 analytes by UV and MS detection.

Analyte	UV (ng/mL)	r^2	MS (ng/mL)	r^2
Daidzein	95.70-12250	1	23.93-765.63	0.9977
Glycitein	105.47-13500	0.9998	26.37-843.75	0.9931
Genistein	105.47-13500	1	26.37-843.75	0.9968
Puerarin	103.52-13250	0.9997	25.88-828.13	0.9959
3'-OMe-puerarin	99.61-12750	0.9994	24.90-796.88	0.9969
3'-OH-puerarin	105.47-13500	0.9999	26.37-843.75	0.9987
5-OH-puerarin	109.38-14000	0.9999	27.34-875.00	0.9923

Eight and six concentration levels were used for calibration plots under UV and MS detection, respectively.

Table 2. Peak assignments and the presence of isoflavones in kudzu from different plant sources.

Peak	t_R (min)	$[M+H]^+$ (*m/z*)	MS fragment ion (*m/z*)	Identities	PL-R	PL-S	PL-L	PL-Fl	PL-Fr	PT-R	PT-S	PT-L	PE-R	Compound Code
1	2.3	579	417	Puerarin-G	+	-	-	-	-	+	+	-	-	**1**
1a	2.3	579	433	5/3'-OH-puerarin-Rha	+	-	-	-	-	-	+	-	-	**2**
2	3.4	579	417	Puerarin-G	+	+	-	-	-	+	+	+	+	**3**
3	4.1	433		3'-OH-puerarin*	+	+	T	-	-	+	+	+	+	**4**
4	5.2	565	433	5/3'-OH-puerarin-Api/Xyl	+	-	-	-	-	-	+	T	-	**5**
5	5.9	595	433	5/3'-OH-puerarin-G	+	T	-	-	-	T	+	+	T	**6**
6	6.5	417		Puerarin*	+	+	+	T	T	+	+	+	+	**7**
7	7.1	549	417	Puerarin-Api/Xyl	+	+		-	-	+	+	+	+	**8**
8	7.2	447		3'-OMe-puerarin*	+	+	T	-	-	+	+	+	+	**9**
9	7.8	549	417	Puerarin-Api/Xyl	+	+	+	-	-	+	+	+	+	**10**
10	9.7	417	255	Daidzein-G*	+	+	T	-	T	+	+	+	+	**11**
11	10.9	447	285	Glycitein-G*	+	+	+	+	T	+	+	+	+	**12**
12	11.6	433		5-OH-puerarin*	+	+	T	-	-	+	+	+	+	**13**
13	14.1	519	271	Genistein-G-M	+	T	T		-	T	+	T	+	**14**
14	14.8	565	433, 271	Genistein-G-Api/Xyl	+	+	T	-	-	+	+	T	-	**15**
15	15.1	433	271	Genistein-G*	+	+	+	-	T	+	+	+	+	**16**
16	15.7	503	255	Daidzein-G-M	+	+	-	T	T	+	+	T	+	**17**
17	16.2	503	255	Daidzein-G-M	+	T	T	-	-	+	+	T	+	**18**
18	17.0	503	255	Daidzein-G-M	+	+	+	+	-	+	+	+	+	**19**
19	17.6	533	285	Glycitein-G-M	+	T		-	T	+	+	-	-	**20**
20	20.1	519	271	Genistein-G-M	+	T	T	-	-	+	+	T	T	**21**
21	20.5	519	271	Genistein-G-M	+	+	+	-	-	+	+	+	+	**22**
22	21.5	255		Daidzein*	+	+	+	-	-	+	+	+	+	**23**
23	22.3	285		Glycitein*	+	T	T	+	-		T		+	**24**
24	27.2	271		Genistein*	+	+	+	+	-	+	+	+	+	**25**

*Identity based on MS spectral and retention data using authentic standards. G: Glucosyl/Galactosyl; Rha: Rhamnosyl; Api: Apiosyl; Xyl: Xylosyl; M: Malonyl. +: Present; -: Not detectable; T: Trace. PL, *P. lobata*; PT, *P. thomsonii*; PE, *P. edulis*; L, Leaf; S, Stem; R, Root; Fl, Flower; Fr, Fruit.

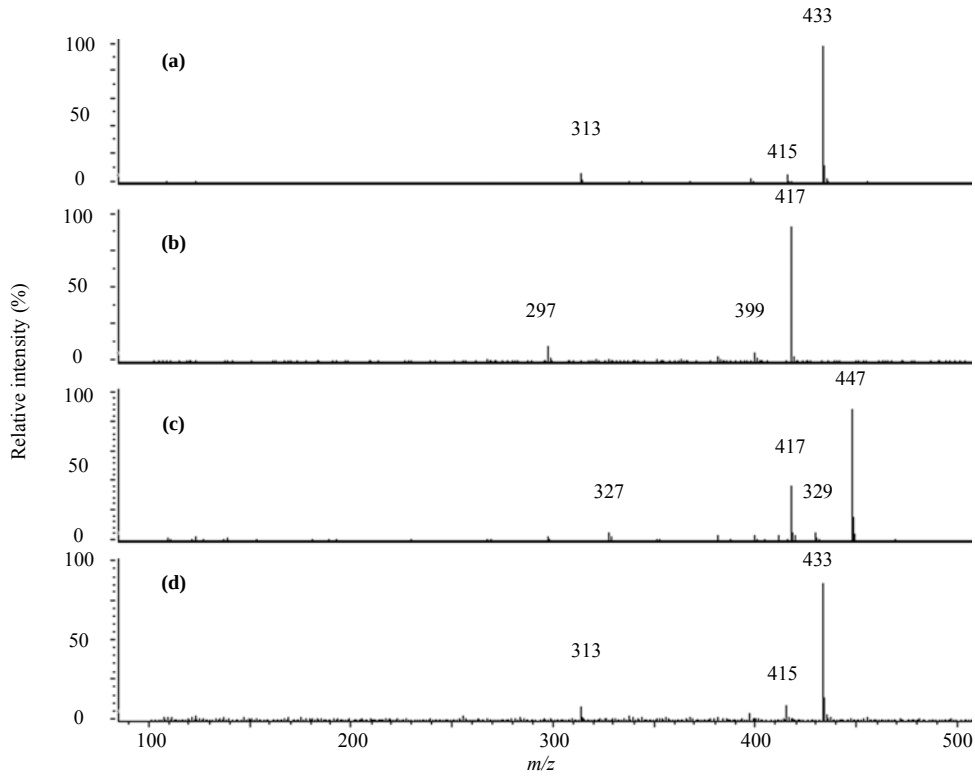


Figure 3. Representative MS spectra of 8-C-glycosyl isoflavones of 3'-OH puerarin (a), puerarin (b), 3'-OMe puerarin (c) and 5-OH puerarin (d).

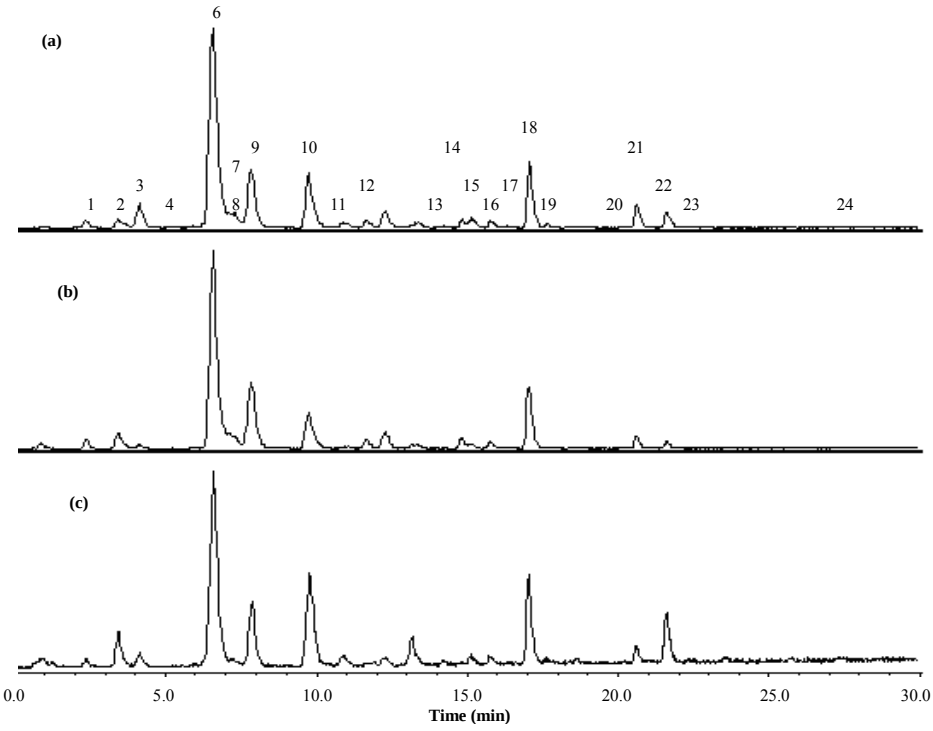


Figure 4. Processed auto MS/MS total ion chromatograms of extracts of root of *P. lobata* (a), *P. thomsonii* (b) and *P. edulis* (c). Total ion chromatograms are extracted with molecular ions of each isoflavones. Peak assignment in A (*P. lobata* root) is listed in Table 2. The presence of chemical profiles for each sample is listed in Table 2.

rate method for its quality control.

3.2.1. UV Method

Using the conditions optimized under multiple preliminary assays, this system enables separation of 7 target isoflavones within 30 min. The chromatograms of a standard mixture with internal standard calycosin, and hydrolyzed extract of *P. lobata* under UV detection at wavelength of 254 nm are illustrated in **Figure 5**. Peak assignments were made with single compound injections and MS spectral data. Baseline separation was successfully achieved for all analytes.

3.2.2. MS Method

In hydrolyzed kudzu extracts, compared to puerarin, 5-OH puerarin, daidzein and genistein, the other 3 analytes of 3'-OH puerarin, 3'-OMe puerarin and glycitein were all found in much lower concentrations, requiring an MS method with higher sensitivity and selectivity. Under SIM mode, protonated $[M^+H]$ ion was isolated for individual target compounds of daidzein at *m/z* 255, genistein at *m/z* 271, glycitein and calycosin (IS) at *m/z* 285, puerarin at *m/z* 417, 5-OH puerarin and 3'-OH puerarin at *m/z* 433, and 3'-OMe puerarin at *m/z* 447. **Figure 6** illustrates the MS chromatogram of hydrolyzed red *P. lobata root* extract with selected ion monitoring (SIM) that demonstrates baseline separation of the 7 components in complex plant matrices within 30 min.

The content of total isoflavones detected in root of lobed kudzuvine (*P. lobata*) and Thomson kudzuvine (*P. thomsonii*), which are both collected as the official medicinal plant species in China pharmacopoeia (The

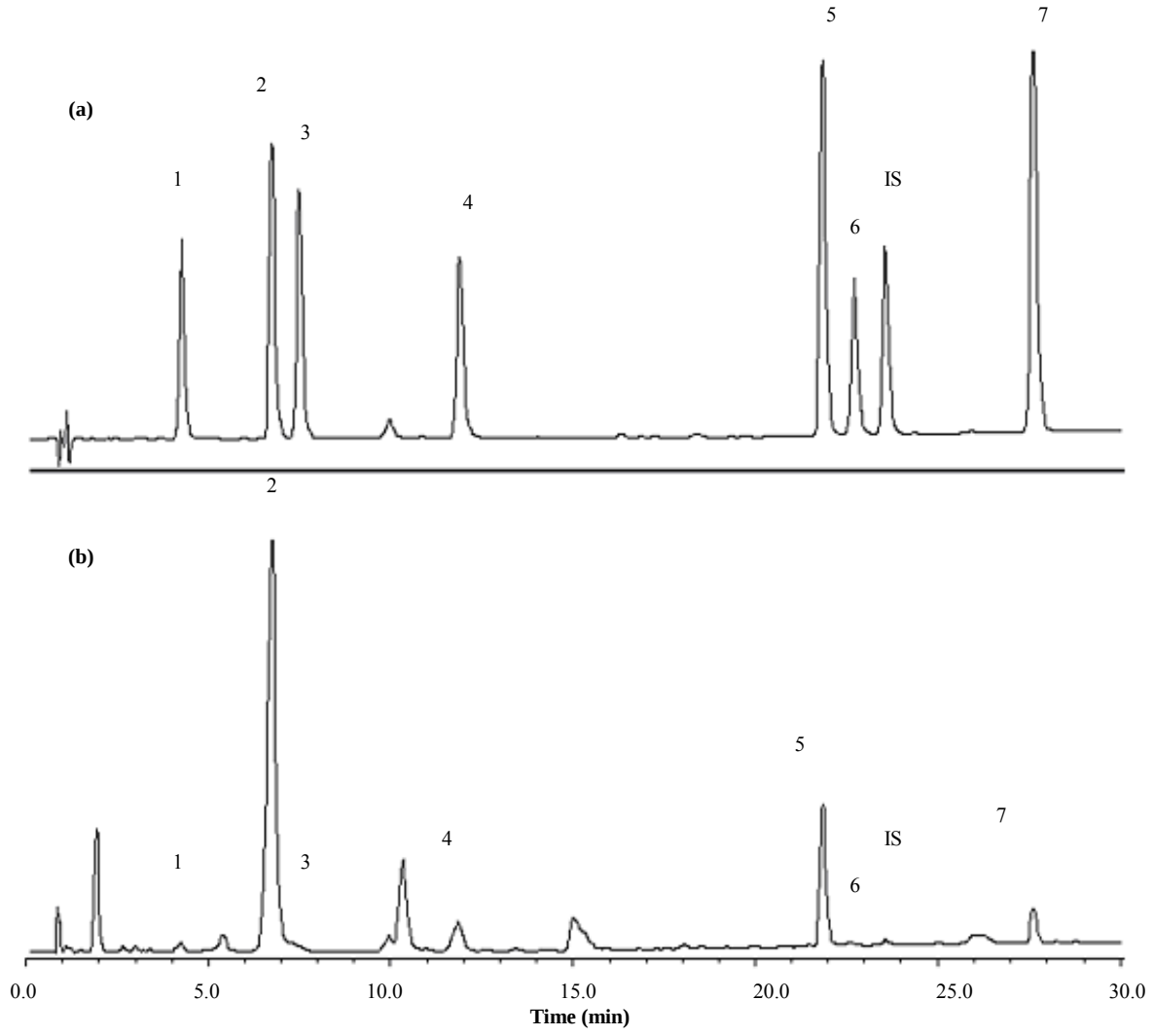


Figure 5. HPLC/UV chromatograms of mixture of 7 standards with internal standard calycosin (a) and acidic hydrolyzed *P. lobata* extract. 1 = 3'-OH puerarin; 2 = puerarin; 3 = 3'-OMe puerarin; 4 = 5-OH puerarin; 5 = daidzein; 6 = glycitein; 7 = genistein; IS = internal standard, calycosin.

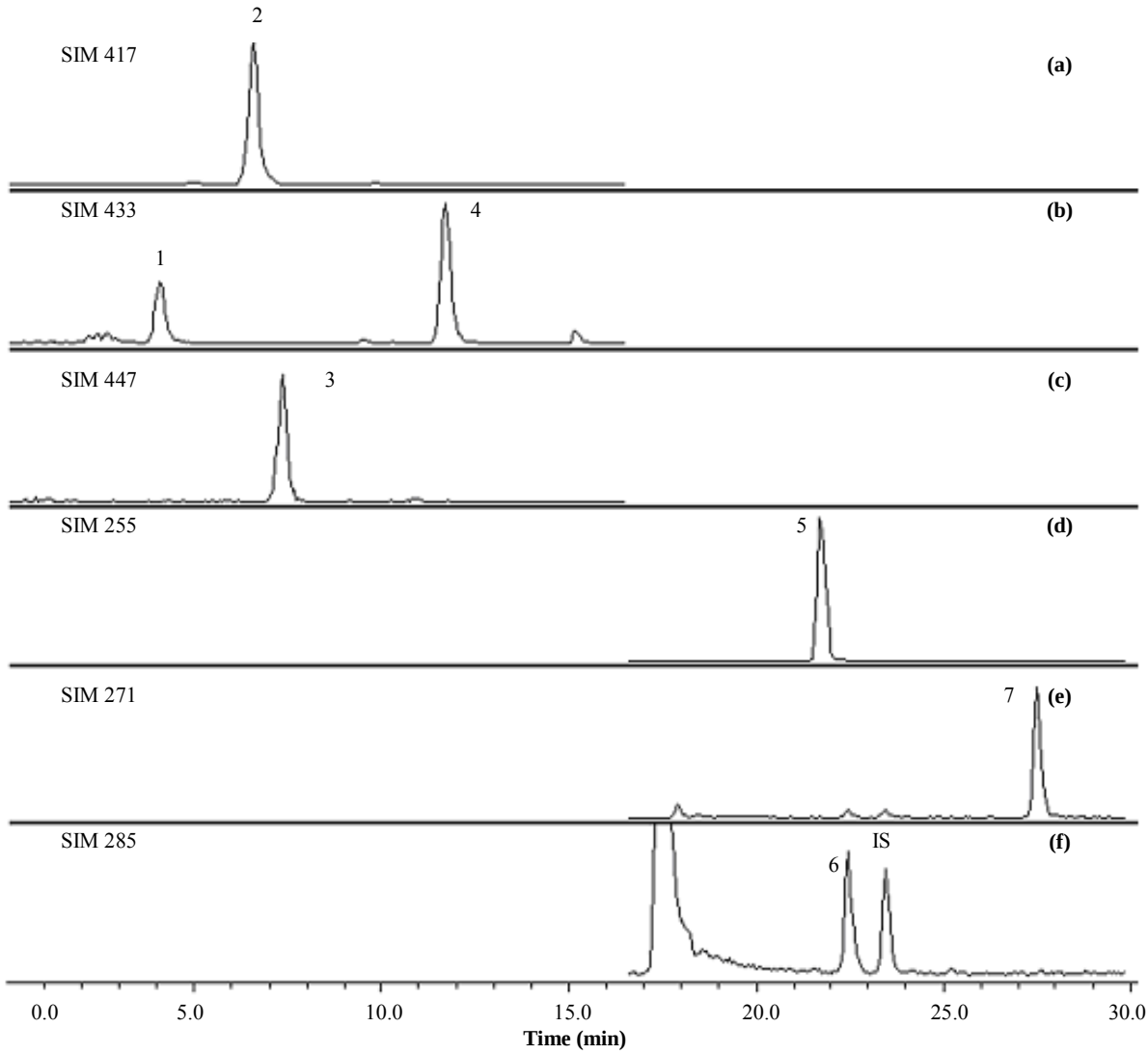


Figure 6. Processed MS chromatograms of hydrolyzed root extract of *P. lobata*. Reconstructed ion chromatogram for *m/z* 417 (a), 433 (b), 447 (c), 255 (d), 271 (e) and 285 (f). 1 = 3'-OH puerarin; 2 = puerarin; 3 = 3'-OMe puerarin; 4 = 5-OH puerarin; 5 = daidzein; 6 = glycitein; 7 = genistein; IS = internal standard, calycosin.

Pharmacopoeia Commision of P. R. China, 2005) was much higher than that found in the edible kudzuvine (*P. edulis*) (**Table 3**). It is interesting that the total isoflavones in *P. thomsonii* stem was very high (**Figure 7**). Additionally, in the kudzu flower, the soy like isoflavones of daizein, genistein and glycitein are the major isoflavones.

4. Conclusions

High performance liquid chromatography combined with ultraviolet and electrospray ionization mass spectrometric detector (HPLC/UV/ESI-MSD) has been applied to the study of isoflavones in plant matrices of kudzu from various *Pueraria* species of *P. lobata* (lobed kudzuvine), *P. thomsonii* (thomson kudzuvine) and *P. edulis* (edible kudzuvine). Under the multiple optimized HPLC and MSD conditions, 25 isoflavones including 10 puerarin analogues (isoflavone C-glycosides) and 15 soy-like isoflavones (daidzein, genistein, glycitein and the glycosides and glycoside malonates) have been identified based on analysis of the UV and MS data and by comparison to the authenticated standards. A simple method was developed for quantification of total isoflavones in hydrolyzed kudzu extracts by HPLC with UV and MS detectors. Within 30 min, all the 7 isoflavones were totally separated and eluted individually. This is the first method to quantify all the 7 isoflavones in kudzu organs. For this study, some standards e.g. puerarin, 5-OH puerarin, 3'-OH puerarin and 3'-OMe puerarin are not com-

Table 3. Isoflavone content in kudzu from different plant sources.

Sample Code	Content (%)							
	Daidzein	Glycitein	Genistein	Puerarin	3'-OMe-puerarin	3'-OH-puerarin	5-OH-puerarin	Total
PL-R	0.2436	0.0166	0.0739	1.9670	0.0240	0.0365	0.1619	2.5235
PL-S	0.0373	0.0016	0.0231	0.0664	0.0028	T	0.0273	0.1586
PL-L	0.0127	0.0011	0.0246	0.0162	T	T	T	0.0546
PL-Fl	0.0067	0.0963	0.0285	T	T	T	T	0.1315
PL-Fr	T	T	T	T	T	T	T	T
PT-R	0.1522	0.0034	0.0201	1.4572	0.0112	0.0009	0.1382	1.7831
PT-S	0.2298	0.0157	0.0500	0.7831	0.0117	0.0016	0.0421	1.1340
PT-L	0.0185	0.0024	0.0503	0.0236	T	T	0.0192	0.1141
PE-S	0.0810	0.0059	0.0160	0.1625	0.0011	0.0016	0.0196	0.2876

PL, *P. lobata*; PT, *P. thomsonii*; PE, *P. edulis*; L, Leaf; S, Stem; R, Root; Fl, Flower; Fr, Fruit. T, trace level. The content of isoflavones of daidzein, genistein, puerarin and 5-OH-puerarin was calculated using UV detection and the content of minor isoflavone of Glycitein, 3'-OMe-puerarin and 3'-OH-puerarin calculated using MS detection.

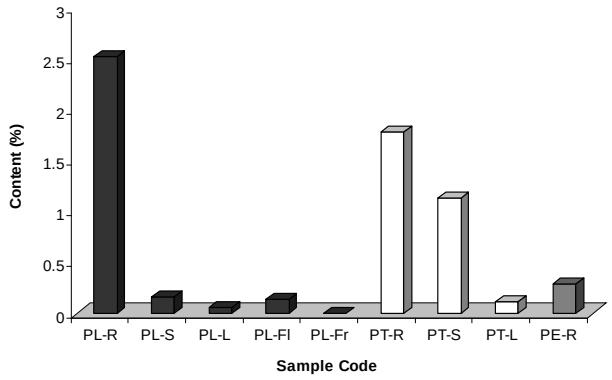


Figure 7. Content of total isoflavones in kudzu from different plant sources PL, *P. lobata*; PT, *P. thomsonii*; PE, *P. edulis*; L, Leaf; S, Stem; R, Root; Fl, Flower; Fr, Fruit.

mercial available. Using polyamide, sephdex-LH20 chromatography and Prep-HPLC, we purified these four standards from kudzu extracts and elucidated their structures by UV, MS and NMR spectrometric methods.

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