

## Profiling and Quantification of Isoflavonoids in Kudzu Dietary Supplements by High-Performance Liquid Chromatography and Electrospray Ionization Tandem Mass Spectrometry

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The kudzu vine (*Pueraria* sp.) is a rich source of isoflavones. Dietary supplements based on kudzu have become commercially available. In the present study, liquid chromatography coupled with negative and positive electrospray ionization tandem mass spectrometry (MS/MS) and diode array detection (DAD) has been used for the detection and characterization of isoflavonoids in kudzu dietary supplements (KDS). The MS/MS spectrum of the protonated ion of puerarin showed characteristic product ions of the C-glycoside unit itself, whereas daidzin generated an abundant Y<sub>0</sub><sup>+</sup> aglycon ion in its product ion spectrum. A base peak due to the loss of 120 Da [M + H – 120]<sup>+</sup> is the diagnostic ion for C-glycosides. Neutral loss scans allowed for the detection of other C- and O-glycosides in the methanolic extract of KDS, and their structures have been proposed. The concentration of isoflavonoids in the methanolic extract of commercially available KDS was quantified by using DAD-HPLC. Puerarin, rather than daidzin, was the most abundant component (8.44–30.60 mg/capsule) in commercially available KDS.

**KEYWORDS:** *Pueraria lobota*; kudzu; isoflavonoids; C-glycosides; LC-MS/MS

### INTRODUCTION

Kudzu is a perennial leguminous vine of the genus *Pueraria* native to eastern Asia. It was introduced into the United States in 1876, and in the Depression Era was used to control soil erosion. It now grows throughout the woodlands and forests of the southern United States. *Pueraria radix* (RP), the root of *Pueraria lobota* (Wild.), is an important Chinese traditional medicine used as an antipyretic, antiarrhythmic, diaphoretic, and antiemetic agent (1). Puerarin (daidzein 8-C-glucoside), daidzin (daidzein 7-O-glucoside), and daidzein are the major isoflavonoids in a methanolic extract of RP (2). Isoflavonoids from RP are reported to have important biological activities such as antiproliferative effects on human cancer cell lines (3) and inhibition of aldehyde dehydrogenase and xanthine oxidase (4, 5). In other studies daidzein has been reported to have anti-giardial (6), antioxidant (7), and antidiabetic activities (8). In view of this, the study of RP isoflavonoids is highly relevant to chronic human disease. Because RP has been prepared in

the form of commercially available dietary supplements that are consumed by the public, it is critical to determine their isoflavone content and concentrations. Accordingly, there is an urgent need to develop a method for the rapid identification and quantification of these isoflavonoids in RP supplements. Most reported methods for the analysis of isoflavonoids are based on high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) separation with UV, fluorescence, or electrochemical detection as recently reviewed (2, 9). However, these methods are limited to the detection of a limited number of known compounds and are not applicable for the characterization of unknown isoflavonoids in a crude mixture.

Mass spectrometry (MS) is currently the most sensitive and selective analytical method for the rapid qualitative and quantitative analysis of known compounds as well as the identification of unknown compounds from crude and partially purified samples of natural products (10) and has been applied to soy isoflavones (11). Its unique ability to filter and isolate molecular ions with specific mass-to-charge (*m/z*) ratios from a complex mixture makes MS invaluable for analysis. Generally, electrospray ionization mass spectrometry (ESI-MS) provides a mass spectrum with little or no fragmentation, and this technique is suitable for the characterization of not only a single compound but also complex mixtures of natural products. In this study, an integrated approach consisting of HPLC and LC-

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MS/MS, including neutral loss-scan mass spectrometry, has been used for the quantification and identification of isoflavonoids in kudzu dietary supplements (KDS). To the best of our knowledge, we also for the first time report a series of isoflavone C-glycosides from KDS based on MS/MS analysis.

## MATERIALS AND METHODS

**Materials.** Puerarin, daidzein, and daidzin were purchased from Indofine (Hillsborough, NJ). Genistein and genistin were previously isolated and purified in our laboratory (12). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO). All other HPLC solvents and reagents were purchased from Fisher (Norcross, GA) and were of HPLC grade. KDS samples (KDS 1–6) were commercially prepared samples and were donated by Dr. Connie Weaver (Purdue University, West Lafayette, IN).

**Extraction of KDS.** Each sample of KDS was extracted in six different quantities (15, 25, 50, 100, 200, and 250 mg) with a constant volume (5 mL) of 80% aqueous MeOH. Fluorescein (50  $\mu$ L of a 20 mg/mL standard solution) was added as an internal standard, and the samples were tumbled for 2 h at 4  $^{\circ}$ C. After extraction, all samples were centrifuged, and aliquots of the supernatant (10  $\mu$ L) were injected on the HPLC for quantitative analyses (11, 12). All samples were analyzed in duplicate.

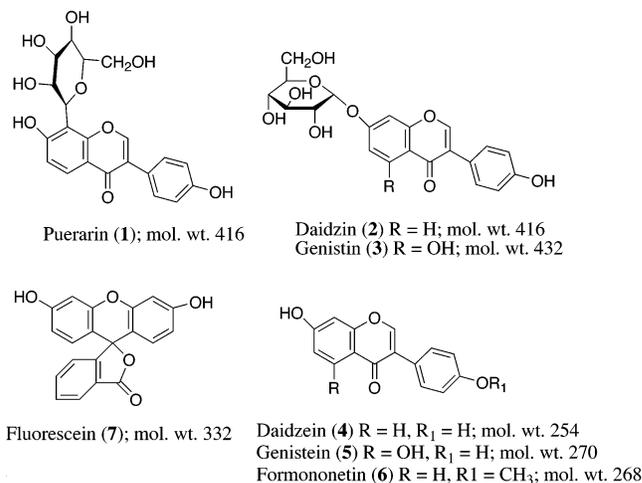
**HPLC Analysis of KDS.** HPLC analyses were carried out on a Brownlee 22 cm  $\times$  4.6 mm i.d., 300  $\text{\AA}$  pore size, Aquapore C<sub>8</sub> reversed-phase column with a 1.5 cm  $\times$  3.2 mm i.d. RP-8 NewGuard guard column (Perkin-Elmer, Norwalk, CT). The mobile phase consisted of solvent A (10% aqueous acetonitrile/0.1% TFA) and solvent B (90% aqueous acetonitrile/0.1% TFA). The samples were eluted from the HPLC column at a flow rate of 1.5 mL/min with a linear gradient increase of solvent B to 70% over 30 min. Over the next 5 min the column was flushed with 100% solvent B. Prior to the next injection, the column was equilibrated for 3 min with solvent A. The column temperature was maintained at 24  $^{\circ}$ C. The 1100 series HPLC (Agilent, Wilmington, DE) system was equipped with a quaternary pump, a refrigerated autosampler, and a diode array detector (DAD). UV spectra were recorded over 225–400 nm with a step of 2 nm and a slit setting of 4 nm. Peak areas were measured at 262 nm. All data were processed using Chemstation software for LC 3D systems (rev. A.08.03, Agilent). The method of quantitation was as previously described (11, 13, 14).

**LC-MS and LC-MS/MS Instrumentation.** Components of the methanolic extract of KDS were separated by HPLC using a 15 cm  $\times$  2.1 mm i.d. C<sub>8</sub> Aquapore reversed-phase column pre-equilibrated with 10 mM ammonium acetate (NH<sub>4</sub>OAc). The mobile phase was initially composed of 10:90 acetonitrile/water (both containing 10 mM NH<sub>4</sub>OAc), followed by a linear gradient to 40% acetonitrile over 30 min at a flow rate of 0.1 mL/min. The column eluate was passed into the Ionspray ionization interface operating in the negative mode of a PE-Sciex (Concord, ON, Canada) API III triple-quadrupole mass spectrometer. The voltage on the Ionspray interface was  $-4900$  V, and the orifice potential was set at  $-50$  V. Negative ion mass spectra were recorded over an  $m/z$  range of 200–800. Selected  $[M + H]^+$  or  $[M - H]^-$  (molecular) ions were analyzed by collision-induced dissociation with 90% argon/10% nitrogen and the daughter ion mass spectra recorded. Neutral loss scanning (a tandem mass spectrometric mode to obtain an array of all parent ions that lose a common neutral fragment) experiments of the methanolic extract of KDS were acquired in the positive ion mode with a dwell time of 5 ms and a step size of 1  $m/z$ .

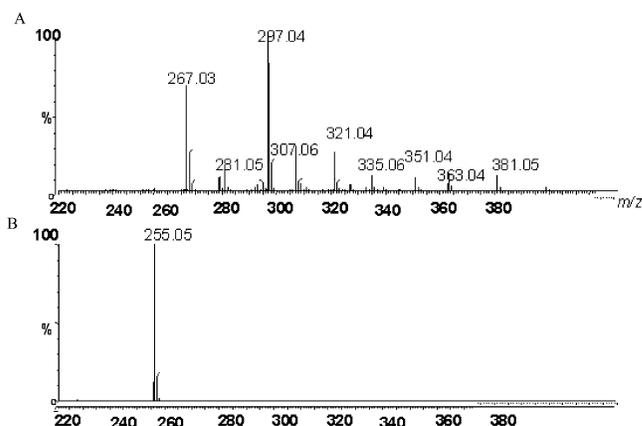
The MS/MS analyses of isoflavonoid glycosides were performed using a Q-TOF mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray ion source. Spectra were obtained in the positive and negative ion modes. Product ion spectra were obtained by selecting the protonated or deprotonated ions for collision (energy = 32 eV), using argon as a collision gas.

## RESULTS AND DISCUSSION

**Fragmentation Analysis of Isoflavonoid Glycosides.** To identify typical product ions of C- and O-glycosides of



**Figure 1.** Structures of standard isoflavonoids (1–6).



**Figure 2.** Comparison of the products ions obtained in ESI-MS/MS experiments of protonated 1 and 2: (A, B) product ions of 1 and 2, respectively.

isoflavonoids, puerarin (1), daidzin (2), and genistin (3) were subjected to LC-MS/MS analysis. The chemical structures of the standards are presented in **Figure 1**. The O-glycosides have sugar substituents bonded to a 7-hydroxy group of the aglycon, whereas C-glycosides have sugar substituents bonded to a carbon of the aglycon, generally at positions C-6 and C-8. Inspection of the product ion spectra of 1 and 2 as shown in **Figure 2A** and **Figure 2B**, respectively, in positive ion mode revealed several diagnostic product ions. Neutral losses of 120 and 150 Da from the protonated 1 gave rise to ions at  $m/z$  297 (base peak) and 267, respectively. The loss of 120 Da is indicative of C-glycosides (15). A series of ions at  $m/z$  399, 381, and 363 were obtained due to the successive neutral losses of water molecules.

Contrary to 1, the product ion spectrum of 2 contains an abundant  $Y_0^+$  aglycon ion at  $m/z$  255 due to the neutral loss of 162 Da (**Figure 2B**). The  $Y_0^+$  ions correspond to rearrangement ions involving loss of the sugar unit and resulting in ions containing only the aglycon moiety. Similarly, MS/MS experiments on 3 also displayed an aglycon ion at  $m/z$  271, indicating the facile loss of the sugar moiety, due to comparatively weaker C–O bond between sugar and aglycon compared with the C–C bond in 1.

The product ion spectrum of 1 in negative ion mode showed prominent ions at  $m/z$  295 and 267 due to the losses of 120 and 148 Da, respectively, but did not produce significant ions due to the loss of water. A possible mechanism of fragmentation of 1 in negative ion mode is shown in **Figure 3**.

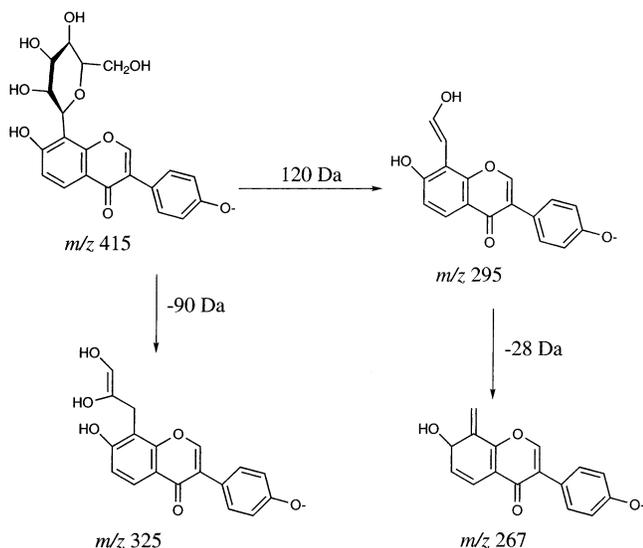


Figure 3. Proposed fragmentation pathways of deprotonated 1 in the MS/MS experiment.

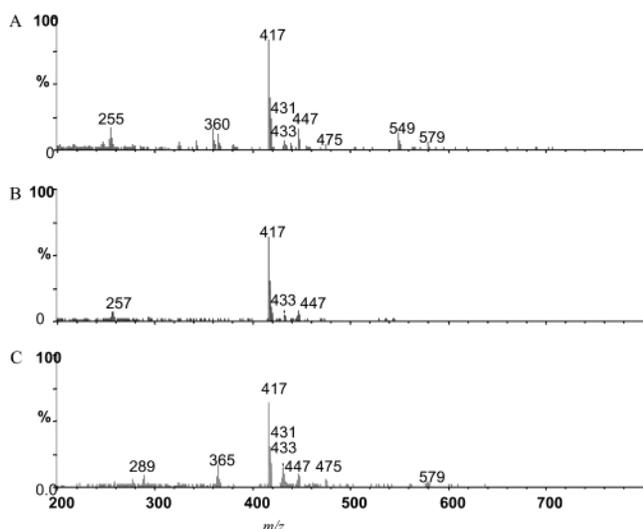


Figure 4. (A) Full-scan ESI-MS of the methanolic extract of KDS, ESI-MS/MS neutral scan to identify the isoflavonoid components in the extract; (B, C) spectra of isoflavonoids with neutral losses of 120 and 162, respectively.

#### Neutral Loss Scan (MS/MS) in the Methanolic Extract.

The ESI-MS spectrum of the methanolic extract of KDS showed several molecular ions of isoflavonoid glycosides clustered in a narrow  $m/z$  range ( $m/z$  400–700). This presented a difficulty for direct analysis. To reduce the complexity of the mass spectrum, the extract was subjected to MS/MS neutral loss scan analysis using typical product ions identified from the fragmentation of standards. As we observed prominent ions in MS/MS experiments of 1 and 2 due to the neutral losses of 120 and 162, respectively, a neutral loss scan was performed to determine which isoflavones are *C*- and *O*-glycosides. Panels A–C of Figure 4 represent the full-scan ESI-MS of the methanolic extract of KDS by direct infusion to ESI-MS and the neutral loss scans for 120 and 162, respectively. The results showed that the ions at  $m/z$  417, 433, and 447 were *C*-glycosides, and  $m/z$  417, 431, 433, 447, 475, and 579 corresponded to *O*-glycosides. Hence, ions at  $m/z$  417, 433, and 447 are both *C*- and *O*-glycosidic isoflavones. To identify these components on the basis of their retention time ( $t_R$ ) and product ions, we performed LC-MS and LC-MS/MS experiments.

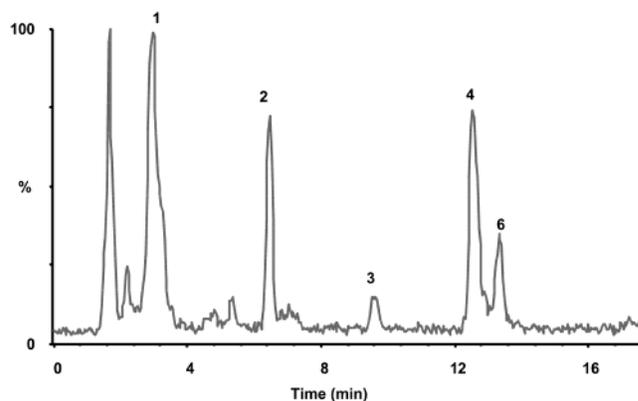


Figure 5. LC-MS total ion chromatogram of the methanolic extract. Peak numbers correspond to the compound numbers in Figure 1.

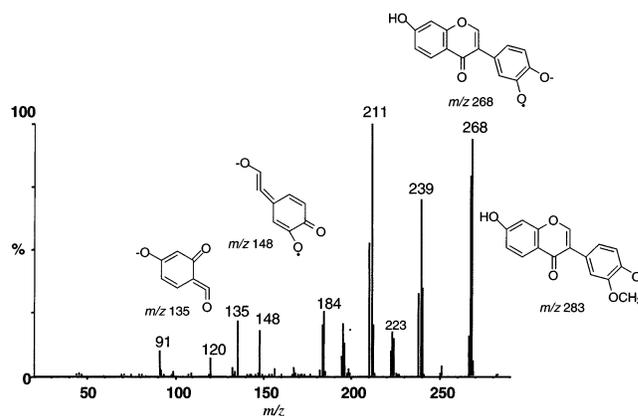
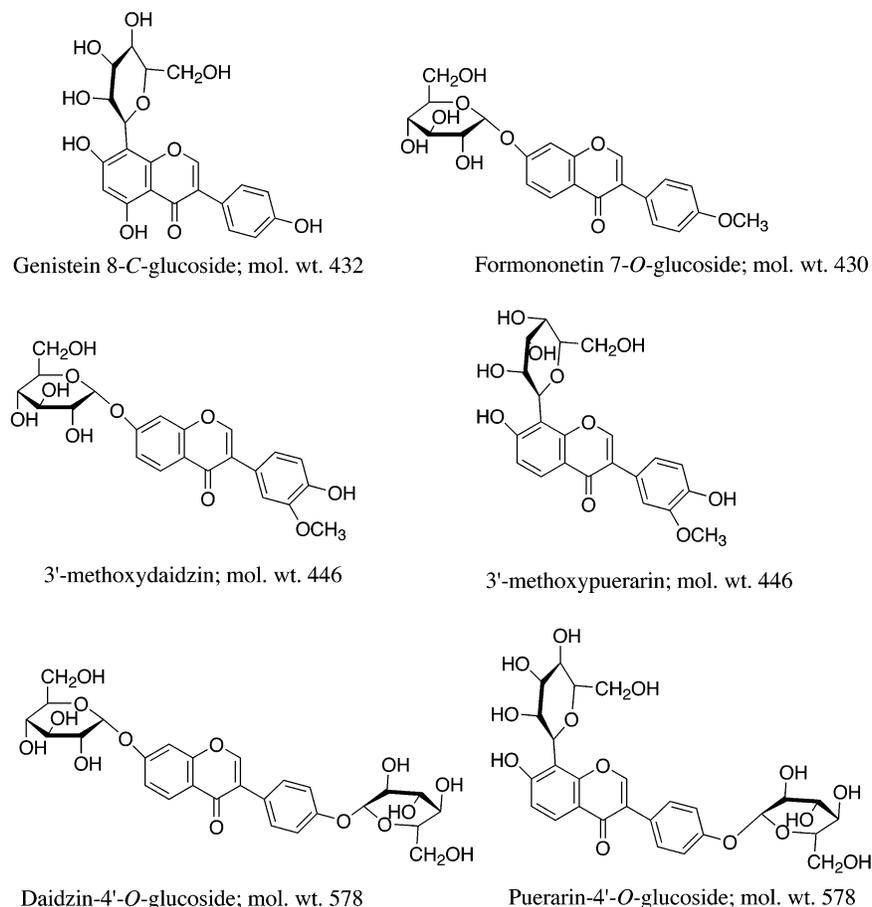


Figure 6. Product ion spectrum of the ion at  $m/z$  283 and its possible structure.

**LC-MS and LC-MS/MS Analyses of the Methanolic Extract of KDS.** An LC-MS chromatogram (total ion current) in negative ion mode of the methanolic extract of KDS is shown in Figure 5. The molecular mass of each component was obtained at its  $t_R$ . An interesting feature of the LC-MS chromatogram is that the ions at  $m/z$  415 ( $t_R = 2.9$  and 6.6 min) and 431 ( $t_R = 4.6$  and 9.4 min) appeared at different  $t_R$  values, indicating that they could be isomeric compounds.

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 the MS/MS data with those of standards, the ions at  $m/z$  253 and 269 (in negative ion mode) were identified as daidzein (4) and genistein (5), respectively. Likewise, the product ions of  $m/z$  267 were found to be identical with those of standard formononetin (6). Formononetin has previously been reported from RP (1).

The MS/MS experiment of the ion at  $m/z$  283 displayed product ions due to the losses of 15 ( $\text{CH}_3$ ), 29 ( $\text{CHO}$ ), and 28 ( $\text{CO}$ ), representing a methylated flavonoid (Figure 6). Attempts were made to elucidate the structure of the ion at  $m/z$  283 on the basis of its product ions. For this, we compared the MS/MS data of standard methylated isoflavonoids (biochanin A, glycitein, and prunetin;  $[\text{M} - \text{H}]^-$  283 Da) with those of the ion at  $m/z$  283 in the methanolic extract of KDS (data not shown). Careful examination of these product ions indicated that an ion at  $m/z$  132 is a diagnostic product ion derived from the ring B (with one OH or  $\text{CH}_3\text{O}$  group). This ion was absent in the product ion spectrum of the ion at  $m/z$  283 in the extract of KDS. Instead, prominent product ions at  $m/z$  135 and 148

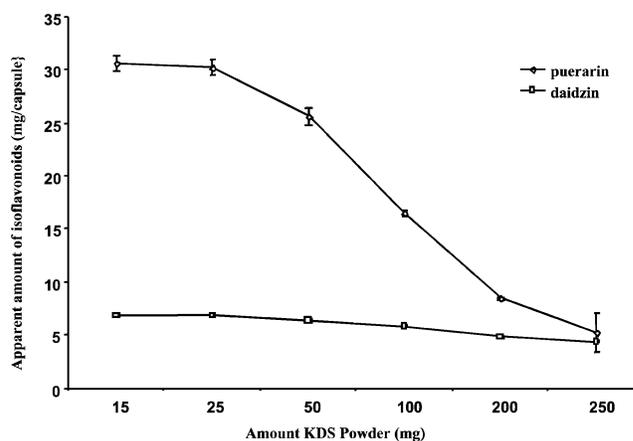


**Figure 7.** Proposed structures of isoflavonoid glycosides based on their MS/MS analysis.

were observed (**Figure 6**). These product ions could be A- and B-ring-derived ions, respectively (16). These fragmentation patterns are in agreement with a feature of 3-OMe and 4-OH groups in ring B. On the basis of these pieces of evidence, the structure of this compound is proposed to be 3'-methoxydaidzein.

Protonated ions at  $m/z$  417, 431, 433, and 447 appeared to be isoflavonoid monoglucosides. Product ions of  $m/z$  417 eluting at  $t_R = 2.9$  and 6.6 min corresponded to puerarin and daidzin, respectively. Similarly, the product ion spectrum of  $m/z$  433 showed ions due to the neutral losses of 18, 120, and 150 Da, characteristic of C-glycosides; it was therefore proposed to be genistein 8-C-glucoside. The characteristic loss of 162 Da together with the appearance of the  $Y_0^+$  ion ( $m/z$  267) was observed in the product ion spectrum of the ion at  $m/z$  431. It is probable that this compound is formononetin 7-O-glucoside. MS/MS experiments on the ion at  $m/z$  447 generated ions characteristic of C- and O-glycosides (data not shown). Thus, they were proposed to be 3'-methoxypuerarin and 3'-methoxydaidzin, respectively (**Figure 7**).

We also detected a series of protonated ions at  $m/z$  549, 563, 565, and 579, corresponding to isoflavonoid diglycosides. These ions showed  $Y_1^+$  and  $Y_0^+$  product ions due to cleavage at the glycosidic linkage, allowing one to assign the glycan sequence, that is, hexose or pentose-hexose-isoflavonoid. MS/MS experiments on the ions at  $m/z$  549, 563, and 565 produced base peaks at  $m/z$  417, 431, and 433, respectively, after the loss of a pentose sugar unit (neutral loss of 132 Da). These product ions ( $m/z$  417, 431, and 433) further produced ions due to the neutral losses of 18, 120, and 150, indicating the linkages of pentose and hexose sugars to their aglycons are O- and C-glycosidic, respectively. We could not elucidate the structure



**Figure 8.** Extractability of puerarin and daidzin with various quantities of KDS powder in 5 mL of 80% aqueous MeOH.

of the pentose sugar merely based on MS/MS data. The product ion spectrum of the ion at  $m/z$  579 showed ions at  $m/z$  417 ( $Y_1^+$ ) and 255 ( $Y_0^+$ ) with comparable intensities due to the successive losses of two hexose units ( $2 \times 162$  Da). At the same time, it also produced ions due to the losses of 120 and 18 Da, indicating the C-glycosidic linkage. Because the relative abundance of the ions ( $Y_1^+$  and  $Y_0^+$ ) is not similar to those reported for isomeric flavonoid O-diglycosides with 1-2 and 1-6 linkage types (17), it seems to have no interglycosidic linkage. Probably, it could be a mixture of daidzin and puerarin 4'-O-glucosides (**Figure 7**). A previous report of daidzein 4',7-diglucoside from RP further supported the proposed structure (1).

**Quantitation of Isoflavonoids in KDS.** To quantitate the amount of isoflavonoids in a capsule of each KDS, we first

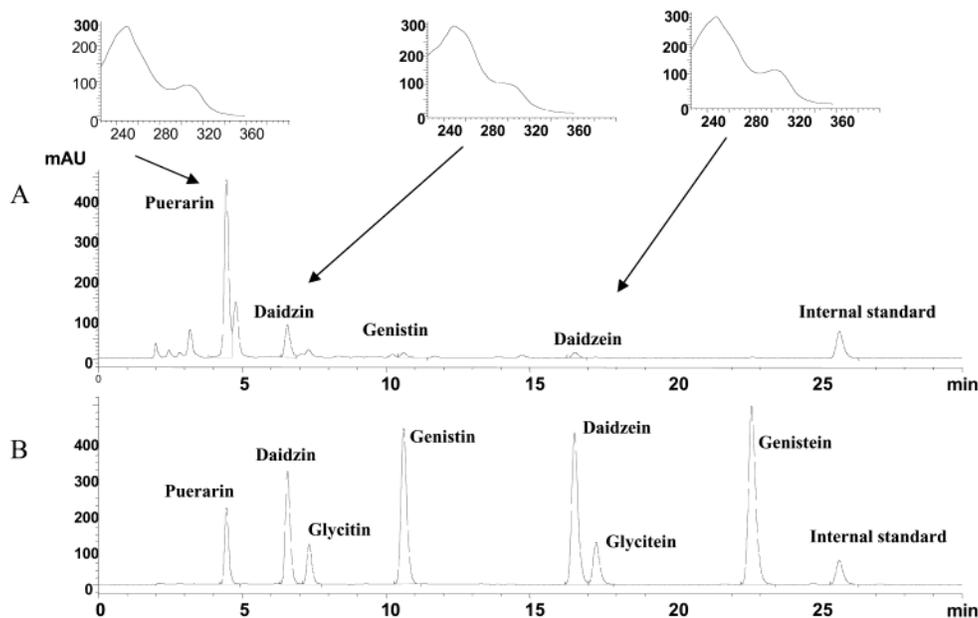


Figure 9. DAD-HPLC chromatogram of (A) a kudzu dietary supplement versus (B) authentic isoflavone standards.

Table 1. Isoflavonoid Content in Some Commercially Available Brands of KDS (KDS 1–6)<sup>a</sup>

KDS	capsule content, mg	puerarin	daidzin	genistin	daidzein	total isoflavonoid
1	249	18.71 ± 1.29	4.71 ± 0.24	0.79 ± 0.04	1.29 ± 0.07	25.51 ± 1.65
2	249	30.60 ± 0.66	6.86 ± 0.15	0.72 ± 0.01	1.23 ± 0.03	39.41 ± 0.86
3	558	8.44 ± 0.37	1.59 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	10.03 ± 3.65
4	320	20.27 ± 1.57	4.11 ± 0.31	0.43 ± 0.03	0.16 ± 0.01	24.98 ± 1.92
5	632	16.11 ± 0.19	3.77 ± 0.15	0.60 ± 0.00	0.32 ± 0.01	20.81 ± 0.34
6	194	23.93 ± 1.88	5.44 ± 0.21	0.67 ± 0.09	0.32 ± 0.01	30.36 ± 2.01

<sup>a</sup> KDS were assayed as described under Materials and Methods. Results are reported as milligrams of isoflavonoid per capsule. Samples were assayed in duplicate, and the results represent the mean ± standard deviations.

evaluated the extractability of the major isoflavonoids puerarin and daidzin. For that, various quantities of KDS (15, 25, 50, 100, 200, and 250 mg) were extracted in a fixed volume (5 mL) of 80% aqueous MeOH. As can be seen in Figure 8, 15 mg of KDS powder yielded the highest amount of puerarin and daidzin. Because puerarin is more hydrophilic than daidzin and sparingly soluble in 80% aqueous MeOH, the ratio of KDS powder to extracting solvent seemed to be critical for efficient extraction of puerarin. A ratio of 15 mg of KDS powder/capsule in 5 mL of 80% aqueous MeOH was used for the quantitation of isoflavonoids in six KDS samples.

The isoflavonoids in KDS were identified in the HPLC by comparison of their  $t_R$  values with those of standards. A representative chromatogram is shown in Figure 9. The puerarin amount in KDS ranged from  $8.44 \pm 0.037$  to  $30.60 \pm 0.66$  mg/capsule (Table 1). Puerarin was the most abundant in KDS followed by daidzin. Free daidzein appeared in very small amounts (0.16–1.29 mg/capsule). Interestingly, manufacturers have labeled KDS as having 1–3 mg of daidzin. Our laboratory has shown that the compound, which was assumed to be daidzin, is in fact puerarin. The manufacturers' labels did not state puerarin as one of the compounds in KDS.

**Conclusion.** Deprotonated and protonated C- and O-glucosidic isoflavonoids produce distinct product ions in ESI-MS/MS that can be used in the discrimination of the isomers. Neutral loss scanning of the methanolic extract of KDS using the typical

losses of 120 and 162 was able to provide a detailed profile of the isoflavone glucosides. Daidzein, genistein, formononetin, puerarin, daidzin, and a series of other glucosidic isoflavones were detected in KDS. Structure identification of unknown isoflavonoids in the extract was addressed by comparison with product ion spectra of known compounds. The isoflavones of KDS were quantitatively determined using HPLC, and puerarin was found to be the most abundant component. This finding is important for preclinical studies and clinical trials of the health claims associated with KDS.

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