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LC/UV/ESI-MS Analysis of Isoflavones in Edamame and Tofu Soybeans

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High-performance liquid chromatography coupled with ultraviolet and electrospray ionization mass spectrometry (HPLC/UV/ESI-MSD) was applied to the study of isoflavones in both Edamame and Tofu soy varieties, from which the immature fresh soybeans or the mature soybean seeds are consumed, respectively. Positive atmospheric pressure interface (API) MS and MS/MS were used to provide molecular mass information and led to the identification of a total 16 isoflavones, including three aglycones, three glycosides, two glycoside acetates, and eight glycoside malonates. The major isoflavones in soybean seeds were daidzein and genistein glycoside and their malonate conjugates. Trace levels of daidzein and genistein acetyl glycosides were found only in the mature dry soybean seeds. To facilitate quantitative analysis, acid hydrolysis during extraction of soy samples was selected to convert the various phytoestrogen conjugates into their respective isoflavone aglycones, allowing accurate quantitation of total phytoestrogens as aglycones. On the basis of HPLC combined with UV and MS detection, all three targeted soy isoflavone aglycones, daidzein, genistein and glycitein in hydrolyzed extracts were successfully quantified within 25 min with formononetin used as the internal standard. The standard curves of UV detection were fitted in the range of 14.16−29000 ng/mL for daidzein, 15.38−31500 ng/mL for genistein, and 11.72−24000 ng/mL for glycitein. For MS detection, the standard curves were established in the range of 3.54−1812.5 ng/mL for daidzein, 3.85−1968.75 ng/mL for genistein, and 2.93−1500 ng/mL for glycitein. Good linearities ($r^2 > 0.999$ for UV and $r^2 > 0.99$ for MS) for standard curves were achieved for each isoflavone. The accuracy and precision (RSD) were within 10% for UV detection and 15% for MS detection ($n = 10$). Using this method, the phytoestrogen levels of total isoflavone aglycones from 30 soybean seed varieties were then evaluated for confirmation of the technique. Total isoflavones ranged across the varieties from 0.02 to 0.12% in the Edamame varieties, which are harvested while the seeds are still immature, and from 0.16 to 0.25% in Tofu varieties, harvested when the seeds are physiologically mature. While the literature has focused on the isoflavone content of soy products and processing soy, this report provides a reliable analytical technique for screening of authenticated fresh immature Edamame soybeans and Tofu soybeans.

KEYWORDS: Isoflavones; *Glycine max* (L.); LC/UV/MSD; daidzein; genistein; glycitein

INTRODUCTION

Isoflavones are major phytoestrogens from natural resources including soybeans, one of the world's most valuable crops (*1*). On the basis of the structural resemblance to the endogenous estrogen, 17-β-estrodiol (**Figure 1**), isoflavones have been implicated in potential health benefits related to age-related and hormone dependent diseases, including cancer, menopausal symptoms, cardiovascular disease, and osteoporosis (*1*−*7*). Due to the purported beneficial effects of soy isoflavones, the use of soy products has become popular as dietary supplements. The isoflavones, daidzein, genistein and glycitein, and their glycosides and malonate conjugates, are the main phenolic compounds in soy (*8*, *9*). Prior analytical research using HPLC with UV and/or MS detection have focused on a variety of processed soy products (*10*−*19*). Further work has also examined the chemical forms in a myriad of soy extracts and processed products during processing (*20*−*24*). Few analytical studies, however, have characterized the isoflavones in the actual

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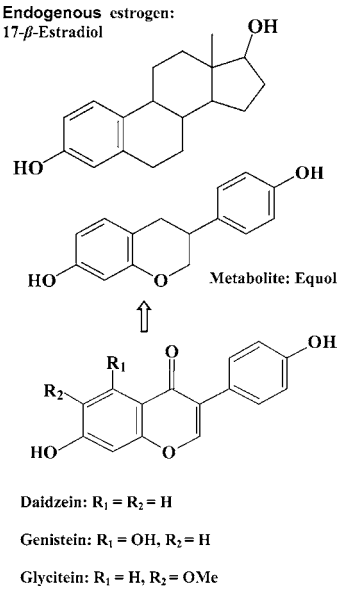


Figure 1. Soy isoflavones daidzein, genistein, and glycitein and the major metabolite equol showing the striking similarity to endogenous 17-β-estradiol.

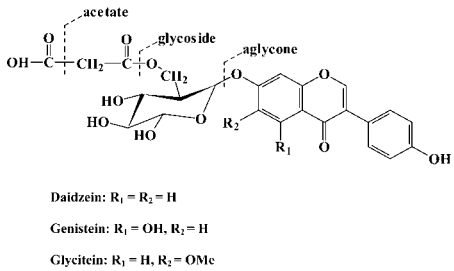


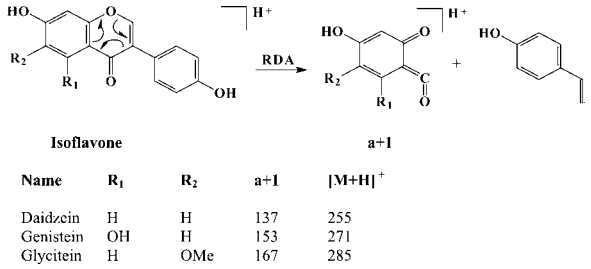
Figure 2. Chemical structures of soy isoflavones and their proposed MS fragment pathway.

fresh immature soybean used as a vegetable and the dried mature soybean (*8*, *9*).

In this study, using HPLC combined with UV and MS, the isoflavone profile and total isoflavones from 10 immature Edamame soybean varieties and 20 mature Tofu soybean varieties have been examined. Under optimized conditions, we were able to simultaneously identify 16 isoflavones including three aglycones, three glycosides, and eight glycoside malonates and two glycoside acetates in mature soybean seeds. In immature soybean seeds, 14 isoflavones were found, and acetyl derivatives were not detected. The quantitative part of this study allowed all three isoflavone aglycones in acidic hydrolyzed soybean seed extracts to be successfully quantified individually using RP-HPLC with UV and MS detector.

MATERIALS AND METHODS

Materials. Standard compounds, genistein, glycitein, and formononetin used as the internal standard were purchased from Indofine Chemical Company, Inc. (Somerville, NJ); and daidzein was purchased from Sigma Chemical Co. (St. Louis, MO) (structures shown in **Figure 3**). HPLC-grade methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), and concentrated hydrochloric acid (HCl) were procured from Fisher Scientific Co. (Fair Lawn, NJ); and formic acid was purchased from Acros Organics (NJ). HPLC-grade water (18MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA) and was used for preparing all solutions. All soybean varieties (*Glycine max*) were field grown at the Snyder Research Farm, Pittstown, New Jersey. The 10 soybean varieties (labeled as lines 1−10 in **Table 4**) were Edamame varieties introduced from Taiwan and harvested at the



Name	R_1	R_2	a+1	$\|M+H\|^+$
Daidzein	H	H	137	255
Genistein	OH	H	153	271
Glycitein	H	OMe	167	285

Figure 3. Chemical structures of three soy isoflavone aglycones and their proposed MS/MS fragment pathway (RDA) with the precursor of their molecular ions.

physiologically immature stage in the same manner as done commercially for human consumption and stored under −25 °C in a freezer. The mature soybean seeds labeled as lines 11−30 in **Table 4** are Tofu soybeans that came from Minnesota and Iowa and were harvested two months late after maturity.

Equipment. HPLC separation was performed on a Phenomenex ODS (*3*) prodigy column, 5-μm, 150- × 3.2-mm I. D. (Phenomenex Inc., Torrace, CA). For all LC/ESI-MS and LC/MS/MS experiments, an Agilent 1100 Series LC/MSD system (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump, diode 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.

Calibration Standards and Quality Control (QC) Samples. Individual stock solutions of three standards, daidzein, genistein, and glycitein 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 then spiking the same amount of known internal standard. For UV detection, the calibration curve ranges were 14.16−29000 ng/mL for daidzein, 15.38−31500 ng/mL for genistein, and 11.72−24000 ng/mL for glycitein. Twelve different concentrations were used for each analyte in duplicate. For MS detection, the calibration curve ranges were 3.54−1812.5 ng/mL for daidzein, 3.85−1968.75 ng/mL for genistein, and 2.93−1500 ng/mL for glycitein, and 10 different concentrations were used for each analyte in duplicate. QC samples were then prepared by diluting separate analyte stock solutions with diluent and spiking with the same amount of known internal standard. Concentrations of lower limit of quantitation QC (LLQ), low QC (LQC), middle QC (MQC), and high QC (HQC) are indicated in **Tables 2** and **3** for UV and MS detection, respectively.

Sample Preparation. Frozen immature (fresh) and air-dried mature soy seeds were finely ground using a coffee grinder. Immediately after, for qualitative study, ∼1000 mg of the immature and ∼ 500 mg of the mature samples were then mixed with 10 mL of 80% methanol, extracted under 4 °C overnight, and stored at 4 °C until LC/MS analysis to prevent the possible decomposition of malonates (*20*). The extracts were filtered through a 0.45-μm filter, and a 20-μL extract was injected for each analysis. The extraction procedure for quantitative analysis was modified from prior published studies (*8*, *9*, *25*−*28*). Approximately 1000 mg of finely ground mature soybean sample and 2000 mg of immature soybean samples were 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 h, protected by N_2. The solution was then filtered to a 100-mL volumetric flask and carefully washed to final volume. Each hydrolyzed sample, 5 μL filtered over a 0.45-μm filter, was analyzed by duplicate injections.

LC/MS Conditions for Identification of Isoflavones. HPLC separation was performed with the mobile phase containing 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 35% B in 40 min. The wavelength of UV detection was 254 nm. Column compartment was set at 25 °C. The flow rate was 0.8 mL/min, and approximately 1/4 of its fluent was split into MSD. The electrospray ion mass spectrometer (ESI-MS) was operated

Table 1. Peak Assignments for the Analysis of Soybean Seed Extract

peak	t_R (min)	$[M + H]^+$ (m/z)	MS fragment ion (m/z)	identities
1	10.1	417	255	daidzein-G (daidzin)[a]
2	11.2	447	285	glycitein-G (glycitin)[a]
3	15.4	433	271	genistein-G (genistin)[a]
4a	15.8	503	255	daidzein-G-M
4b	16.1	519	433, 271	genistein-G-M
5a	16.4	503	255	daidzein-G-M
5b	16.6	533	285	glycitein-G-M
6	17.4	503	255	daidzein-G-M
7	18.0	533	285	glycitein-G-M
8	20.1	459	255	daidzein-G-A
9	21.6	519	433, 271	genistein-G-M
10	21.6	519	433, 271	genistein-G-M
11	24.1	255		daidzein[a]
12	25.6	285		glycitein[a]
13	26.3	475	271	genistein-G-A
14	32.7	271		genistein[a]

[a] Compared with the standard. [b] G = glucosyl/galactosyl moiety; M = malonate; A = acetate (in general, glucosyl group, occasionally galactosyl group was substituted on 7/4′ position of aglycone, acetyl or malonyl group was linked to 6″ position of sugar moiety).

Table 2. Accuracy and Precision of UV Detection[a]

		analyte		
		daidzein	glycitein	genistein
LLQ (ng/mL)	nominal concn	14.16	11.72	15.38
	mean concn	14.06	12.20	14.88
	SD	0.89	0.68	0.92
	accuracy (%)	–0.72	3.92	–3.38
	precision (%)	6.32	5.56	6.20
LQC (ng/mL)	nominal concn	58	48	63
	mean concn	54.38	45.78	58.83
	SD	1.43	1.57	1.51
	accuracy (%)	–6.65	–4.84	–7.08
	precision (%)	2.63	3.43	2.57
MQC2 (ng/mL)	nominal concn	580	480	630
	mean concn	535.62	445.40	584.88
	SD	2.18	2.89	2.37
	accuracy (%)	–8.29	–7.77	–7.71
	precision (%)	0.41	0.65	0.40
MQC1 (ng/mL)	nominal concn	1160	960	1260
	mean concn	1110.98	951.42	1213.18
	SD	3.59	3.75	3.03
	accuracy (%)	–4.41	–0.90	–3.86
	precision (%)	0.32	0.39	0.25
HQC (ng/mL)	nominal concn	11600	9600	12600
	mean concn	11506.95	9871.86	12353.97
	SD	38.08	57.29	41.33
	accuracy (%)	–0.81	2.75	–1.99
	precision (%)	0.33	0.58	0.33

[a] $n = 10$.

under positive ion and auto MS/MS mode (Threshold, 30 000) and optimized collision energy level of 80%, scanned from m/z 100 to 600. ESI was conducted using a needle voltage of 3.5 kV. High-purity nitrogen (99.999%) was used as dry gas and at a flow rate of 8 L/min, capillary temperature at 325 °C. Helium was used as Nebulizer at 40 psi. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity.

LC/MS Conditions for Quantification of Isoflavones. Elution was carried out at a flow rate of 1.0 mL/min with the solvent system containing solvents 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 20 to 35% B in 30 min. MS detection was conducted under collision energy level of 80% and scanned from m/z 100 to 320. Other MS parameter and LC conditions were the same as described above. Under MRM mode (multiple reaction monitoring), protonated $[M + H]^+$ ions were isolated for each isoflavone aglycone. The mass spectrometer was set into two time segments: (1) from 0 to 14 min for daidzein and glycitein with isolation of m/z 255 and 285; (2) from 14 to 30 min for genistein and formononetin of m/z 271and 269. 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 ratio of analyte/internal standard acquired from UV and MS detector versus analyte concentration. The concentrations of the QC and hydrolyzed soy samples were calculated from these linear equations.

Table 3. Accuracy and Precision of MS Detection[a]

		analyte		
		daidzein	glycitein	genistein
LLQ (ng/mL)	nominal concn	3.54	2.93	3.85
	mean concn	3.95	2.56	3.41
	SD	0.16	0.16	0.37
	accuracy (%)	10.30	–14.40	–12.90
	precision (%)	4.07	6.34	10.97
LQC (ng/mL)	nominal concn	14.5	12	15.75
	mean concn	12.86	11.39	15.39
	SD	0.42	0.60	0.80
	accuracy (%)	–12.74	–5.31	–2.32
	precision (%)	3.26	5.24	5.20
MQC2 (ng/mL)	nominal concn	58	48	63
	mean concn	54.51	49.74	64.52
	SD	4.18	3.84	4.23
	accuracy (%)	–6.41	3.51	2.36
	precision (%)	7.67	7.73	6.56
MQC1 (ng/mL)	nominal concn	116	96	126
	mean concn	107.90	91.39	121.17
	SD	7.04	4.78	9.79
	accuracy (%)	–7.50	–5.05	–3.98
	precision (%)	6.52	5.23	8.08
HQC (ng/mL)	nominal concn	1160	960	1260
	mean concn	1148.04	873.43	1301.01
	SD	43.75	45.41	47.66
	accuracy (%)	–1.04	–9.91	3.15
	precision (%)	3.81	5.20	3.66

[a] $n = 10$.

RESULTS AND DISCUSSION

Identification of Isoflavones in Soybean Seeds by LC/MS/MS. Representative auto MS/MS total ion chromatogram and processed chromatograms of soy seed 80% methanol extracts are shown in **Figure 4**. The identities, retention time, protonated $[M + H]^+$ and characteristic fragment ions for individual peaks are presented in **Table 1**. The isoflavone aglycones, daidzein, genistein, and glycitein, and the three isoflavone glucosides, daidzin, genistin, and glycitin, were identified by comparison of the retention time and mass spectral data with those of standards.

Previous chemical studies indicated that the principal soy isoflavones include daidzein, genistein, and glycitein, and their derivatives of glycosides, malonates, and acetates (*17*, *22*). In general, the glucosyl group is substituted at the 7/4′ position of aglycones, acetyl/malonyl group is linked to the 6″ position of sugar moiety. As a consequence, we can confirm their structures from their molecular ions and specific fragment ions of $[M + H - \text{malonyl/acetyl}]^+$ and $[M + H - \text{malonyl/acetyl-glucosyl}]^+$. **Figure 2** indicates the MS fragmentation pathway of soy isoflavone glycoside malonate conjugates. **Figure 5** illustrates the representative MS spectra of soy isoflavones of aglycones (**A**), glycosides (**B**), glycoside acetates (**C**), and glycoside malonates (**D**). Ion peaks at m/z 255, 271 and 285 in **Figure 5A** are the positive molecular ions of three isoflavone aglycones of daidzein, genistein, and glycitein corresponding to peaks 11, 14, and 12 in **Figure 4**, respectively. **Figure 5B** shows the

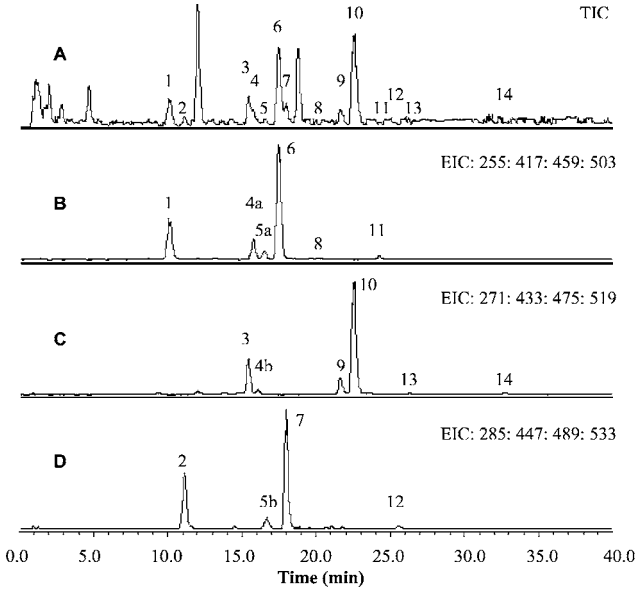


Figure 4. Representative auto MS/MS chromatograms of soybean seed extract. (**A**) Total ion chromatogram; (**B**) reconstructed ion chromatogram for daidzein and its derivatives; (**C**) reconstructed ion chromatogram for genistein and its derivatives; (**D**) reconstructed ion chromatogram for glycitein and its derivatives. The identities, t_R value and MS of each peak are listed in **Table 1**.

molecular ions of *m*/*z* 417, 433, and 447 and specific fragments of $[M + H - glucosyl]^+$ at *m*/*z* 255, 271 and 285 for daidzin, genistin and glycitin, which are related to peaks 1, 3, and 2 in **Figure 4**, respectively. The molecular ions at *m*/*z* 459 and 475 and fragment ions of $[M + H - acetyl - glucosyl]^+$ at *m*/*z* 255 and 271 in **Figure 5C** indicate the presence of daidzein and genistein glycoside acetates shown in **Figure 4** as peaks 8 and 13, respectively. The molecular ions of *m*/*z* 503, 519, and 533 and characteristic fragments of $[M + H - malonyl - glucosyl]^+$ at *m*/*z* 255, 271, and 285 for daidzein, genistein, and glycitein malonate conjugates as shown in **Figure 5D** correspond to peaks 6, 10, and 7 in **Figure 4**, respectively. The identification of each compound was also further confirmed by their MS/MS spectral data. **Figure 6** shows the representative MS/MS spectra of soy isoflavones with the precursors of *m*/*z* 255 (**A**), 271 (**B**), and 285 (**C**). Ion peaks at *m*/*z* 137, 153, and 167 are RDA (retro-Diels−Alder) fragment ions of daidzein, genistein, and glycitein, respectively. The RDA fragment pathway is illustrated in **Figure 3**.

On the basis of these MS and MS/MS data analyses, a total of 16 isoflavones, including three aglycones, three glycosides, two glycoside acetates, and eight glycoside malonates were identified from soybean seeds (**Table 1**). The major soy isoflavones are daidzein and genistein glycosides and their malonate conjugates. Trace amounts of daidzein and genistein acetyl glycosides were found only in mature soybean seeds. No acetyl conjugate was detected in immature fresh soybean seeds. Soy isoflavone acetates were reported commonly available in variety of soy processed products (*10*−*19*). As we did not detect any acetate conjugate in immature fresh soybean seeds and only found trace amounts of acetates in mature soybean seeds, most of the isoflavone acetates in soy supplements might be metabolized from isoflavone malonates during processing. Isoflavonoid isomers are commonly occurring in leguminous plants (*29*, *30*). From the soybean seeds of 30 cultivars, some

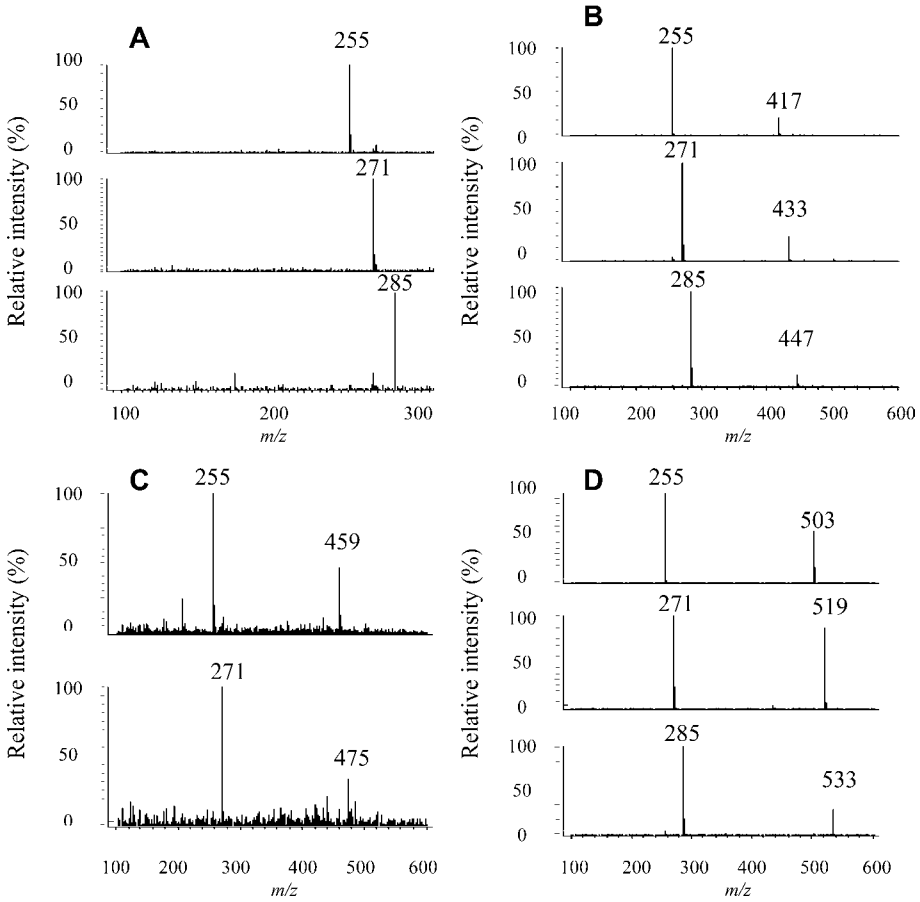


Figure 5. Representative MS spectra of soy isoflavone aglycones, daidzein, genistein, and glycitein (**A**), and their derivatives, glycosides (**B**), glycoside acetylates (**C**), and glycoside malonates (**D**).

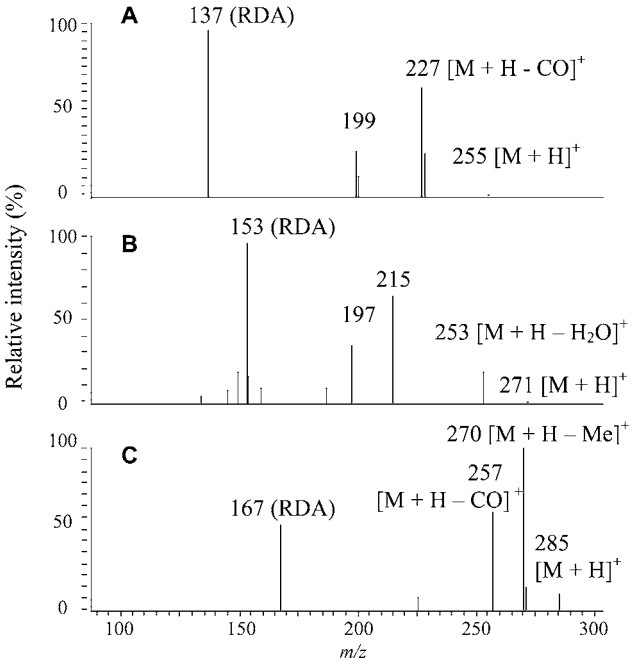


Figure 6. Representative MS/MS spectra of soy isoflavones with the precursors of *m/z* 255 (**A**), 271 (**B**), and 285 (**C**). Ion peaks at *m/z* 137, 153, and 167 are RDA fragment ions of daidzein, genistein, and glycitein, respectively.

isomers of glycoside malonates of daidzein, genistein, and glycitein were also detected (**Table 1**).

Quantification of Isoflavones in Acidic Hydrolyzed Soybean Seed Extracts by LC/UV/MS. The qualitative studies described above compared with prior reports revealed that soybean seeds contained large amount of isoflavones mainly available as malonate conjugates (e.g., glycoside malonates of daidzein and genistein) (*22*, *23*). The malonates are of biological interest in plants because this conjugated form can be utilized to store the less soluble isoflavone aglycones, and upon microbial infection, the aglycones are generated from the malonate conjugates (*31*, *32*). Isoflavones are the common form of phytoestrogens. The glycosides and glycoside malonates in their original plants are inactive (*32*). Clinical and preclinical studies found that the isoflavone aglycones, daidzein, and genistin may be absorbed or further metabolized to many specific metabolites including equol (**Figure 1**) (*1*, *2*, *33*−*36*). Thus, the presence of isoflavone aglycones may be directly related to their bioavailability. Moreover, the malonate conjugates are not stable during processing (*20*−*23*), and it is very difficult to procure and maintain pure standards. Therefore, in this work, to facilitate the quantification and accurately evaluate the total isoflavones in soybean seeds, plant samples were hydrolyzed during extraction. Under optimized HPLC conditions, all three isoflavone aglycones, considered to be the major form of phytoestrogens, were successfully quantified in hydrolyzed extract by using the internal standard of formononetin by UV and MS detection. For the soybean seed extracts and spiked QC samples, all analytes were carried out in duplicate. The isoflavone concentrations in QC and soybean seed samples were calculated from the regression equations.

Using the HPLC conditions optimized under multiple preliminary assays, this system enables total separation of the three target isoflavone aglycones, daidzein, genistein, and glycitein, along with the internal standard of formononetin within 25 min. **Figure 7** shows a chromatogram of a typical hydrolyzed soybean seed extract under UV detection at wavelength of 254 nm. Peak assignments were made with single compound injections and MS spectral data. Baseline separation was successfully achieved for analytes.

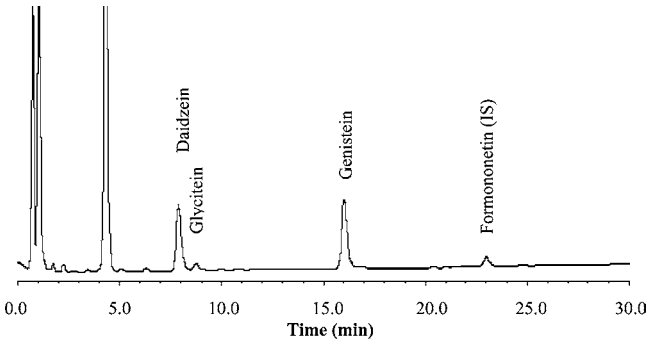


Figure 7. Representative HPLC-UV_{254} chromatogram of hydrolyzed soybean seed extract spiked with formononetin as the internal standard (IS).

For UV detection, the calibration was based on the duplicate analysis of each working solution across 12 concentration levels. The concentrations for the calibration curves of three standards ranged from 14.16 to 29000 ng/mL for daidzein, from 15.38 to 31500 ng/mL for genistein, and from 11.72 to 24000 ng/mL for glycitein, and regressions for each were greater than 0.999. The accuracy (deviation from nominal concentration) and precision (RSD) of this method was assessed by analyzing the QC samples of LLQ, LQC, MQC, and HQC ($n = 10$). Results show that the assay accuracy and precision for the three analytes was within 10% (**Table 2**). The LLQs for the three analytes were established at 14.16 ng/mL for daidzein, 15.38 ng/mL for genistein, and 11.72 ng/mL for glycitein, the lowest concentrations of analytes in the calibration curves (**Table 2**). Using the UV method developed here, we further evaluated 30 soybean varieties from our research Rutgers University farm in New Jersey. The contents of individual and total isoflavones in the 30 samples were quantified, respectively (**Table 4**). The total isoflavone concentration expressed as aglycone contents in the 10 Edamame immature fresh soybean seeds ranged from 0.02 to 0.12% and from 0.16 to 0.25% among the 20 different Tofu soybeans harvested at a mature stage of development.

In this research with soybeans, a more sensitive MS method compared to UV detection was also developed and validated. Under MRM mode, protonated $[M + H]^+$ ion was isolated for individual target compounds of daidzein at *m/z* 255, genistein at *m/z* 271, and glycitein at *m/z* 285, and internal standard of formononetin at *m/z* 269. **Figure 8** illustrates the processed MS chromatograms of hydrolyzed soybean seed extract spiked with an internal standard of formononetin with selected ion monitoring (SIM) that demonstrates baseline separation of the four components in complex plant matrix within 25 min. The calibration curves were constructed by injecting working solutions between 3.54 and 1812.5 ng/mL for daidzein, between 3.85 and 1968.75 ng/mL for genistein, and between 2.93 and 1500 ng/mL for glycitein at 10 concentration levels in duplicate, and regressions values for all were greater than 0.99. The accuracy and precision of MS method was also confirmed by analyzing the QC samples of LLQ, LQC, MQC, and HQC ($n = 10$). The accuracy and precision for all three analytes were found to be less than 15% (**Table 3**). The LLQs for the three analytes were established at 3.54 ng/mL for daidzein, 3.85 ng/mL for genistein and 2.93 ng/mL for glycitein, the lowest concentrations of analytes in the calibration curves (**Table 3**). The sensitivity of this MS method is significantly higher than that of UV detection.

Table 4. Comparative Content of Isoflavones in 30 Soybean Seed Varieties

sample codes[b]	content (μg/g)[a]			
	daidzein	glycytein	genistein	total
1	549.0 ± 3.4	40.5 ± 0.4	619.9 ± 2.5	1209.4
2	409.6 ± 3.1	43.9 ± 0.2	458.6 ± 2.1	912.1
3	310.0 ± 1.3	36.7 ± 0.2	333.4 ± 2.3	680.1
4	441.9 ± 2.6	51.8 ± 0.6	561.7 ± 3.5	1055.4
5	133.2 ± 0.8	31.1 ± 0.4	237.9 ± 1.9	402.1
6	454.1 ± 2.9	42.1 ± 0.1	498.2 ± 2.2	994.4
7	498.1 ± 3.3	48.9 ± 0.3	620.7 ± 3.7	1167.7
8	114.3 ± 1.1	27.4 ± 0.3	157.2 ± 0.7	298.9
9	356.7 ± 2.2	44.1 ± 0.2	390.6 ± 2.3	791.5
10	251.1 ± 2.1	28.3 ± 0.4	309.0 ± 1.6	588.3
11	1093.2 ± 5.4	101.1 ± 0.7	999.0 ± 5.1	2193.3
12	1093.4 ± 4.7	95.2 ± 0.5	1045.8 ± 4.7	2234.4
13	1228.8 ± 5.3	118.3 ± 0.8	1175.0 ± 3.2	2522.1
14	996.1 ± 5.1	115.3 ± 0.3	954.4 ± 3.6	2065.8
15	1062.9 ± 6.6	73.3 ± 0.2	1059.1 ± 4.8	2195.3
16	1063.0 ± 3.7	113.4 ± 0.4	1090.7 ± 7.6	2267.0
17	1087.8 ± 4.6	131.9 ± 0.6	1105.9 ± 3.4	2325.6
18	1111.5 ± 3.2	104.4 ± 0.7	1143.6 ± 4.9	2359.5
19	1155.8 ± 3.9	118.7 ± 0.4	1129.0 ± 3.1	2403.6
20	974.1 ± 4.5	134.6 ± 0.5	958.8 ± 2.6	2067.5
21	769.5 ± 2.3	77.6 ± 0.5	962.7 ± 3.5	1809.8
22	678.4 ± 1.3	93.5 ± 0.6	870.9 ± 1.6	1642.8
23	942.1 ± 3.5	100.6 ± 0.7	1156.1 ± 2.5	2198.8
24	686.4 ± 2.2	66.3 ± 0.1	919.4 ± 6.4	1672.0
25	844.9 ± 2.3	96.2 ± 0.2	1041.0 ± 5.3	1982.1
26	824.0 ± 3.1	97.0 ± 0.5	1051.4 ± 3.8	1972.4
27	779.3 ± 4.2	96.5 ± 0.3	1100.5 ± 4.6	1976.3
28	695.0 ± 1.5	99.8 ± 0.6	931.9 ± 4.3	1726.8
29	964.3 ± 6.1	105.7 ± 0.6	1177.9 ± 5.6	2247.9
30	769.0 ± 3.8	74.5 ± 0.4	931.6 ± 3.3	1775.2

[a] Mean value ± SD in duplicate. [b] Samples 1–10 are varieties of immature soybean seeds and 11–30 are varieties of mature soybean seeds.

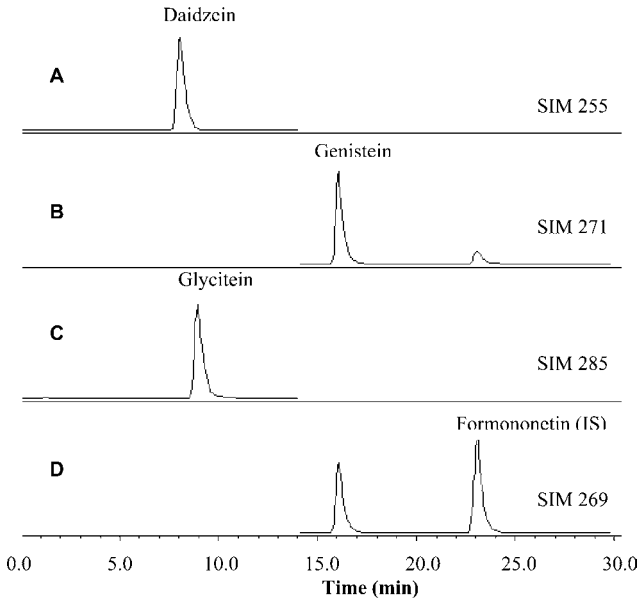


Figure 8. Processed MS chromatograms of hydrolyzed soybean seed extract spiked with formononetin as the internal standard (IS). Reconstructed ion chromatogram of *m/z* 255 for daidzein (**A**), 271 for genistein (**B**), 285 for glycitein (**C**), and 269 for internal standard, formononetin (**D**).

Specificity. Soybean seed samples were analyzed with and without the internal standard of formononetin to determine whether any endogenous constituents interfered with the internal standard. The degree of interference was assessed by inspection of the MS chromatograms. No significant interfering peaks from the soybean seed extracts were observed at the retention times and in the ion channels of the internal standard.

In conclusion, a reliable and robust LC/UV/MSD method has been developed for the determination of isoflavones in both Edamame and Tofu, immature and mature soybeans, respectively. Under the optimized HPLC and MSD conditions, 16 isoflavones in mature dry soybean seeds including three isoflavone aglycones, three glycosides, eight glycoside malonates, and two minor glycoside acetates have been tentatively identified by analysis of their molecular ions and characteristic fragment ion peaks under MS and MS/MS mode, and also in comparison with standard isoflavones. There were no acetyl glycoside isoflavones detected in immature fresh soybean seeds. A simple, reliable, and sensitive method was also established for quantitation of total isoflavones in hydrolyzed soybean seed extracts by HPLC with UV and MS detectors. Using this method, all three targeted isoflavone aglycones, daidzein, genistein, and glycitein were fully separated and eluted individually and distinct from the internal standard, formononetin within 25 min. Validation of this method led to the LLQ at ~12 ng/mL for UV detection and ~3 ng/mL for MS detection, respectively. The accuracy and precision (RSD) for this method were well below 10% for UV detection and 15% for MS detection at the concentration of LLQ. This technique will facilitate large-scale screening of soybeans irrespective of maturity.

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