APPLICATION NOTE

Liquid Chromatography

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Analysis of Isoflavones in Soy Products by UHPLC with UV Detection

Introduction

Isoflavones are naturally occurring isoflavonoids, many of which act as phytoestrogens in mammals. These compounds are produced almost exclusively by the members of the Fabaceae (i.e., Leguminosae, or bean) plant family.[1] Isoflavones occur in foods in the form of water-soluble glucosides (daidzin, genistin and glycitin), being bound to sugar. To become biologically active, these glucosides must first undergo hydrolysis by bacterial beta-glucosidases in our intestine, releasing the corresponding bioactive aglycones (daidzein, genistein, and glycitein).[2,3] Figure 1 shows the structure of all six of these isoflavones.[3]

A great variety of isoflavone supplements and soy products, some additionally fortified, are available on the market today, many offered as nutraceuticals.[4,5,6] The elevated interest in these products stems from the known/potential health benefits, such as protection against breast cancer and prostate cancer, as well as the health concerns, such as heart disease and osteoporosis.[1,2]

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Considering the above, as well as the growing labeling requirements, there has been a considerable effort in the development of effective analytical techniques to determine both the type and amount of isoflavones in marketed products. Although a number of analytical methods for isoflavones exist[5,6], the goal of this work was to develop a simpler, faster and reliable LC method for the analysis of the six most widely-used isoflavones in soy products.

Method conditions and performance data, including linearity and repeatability, are presented.

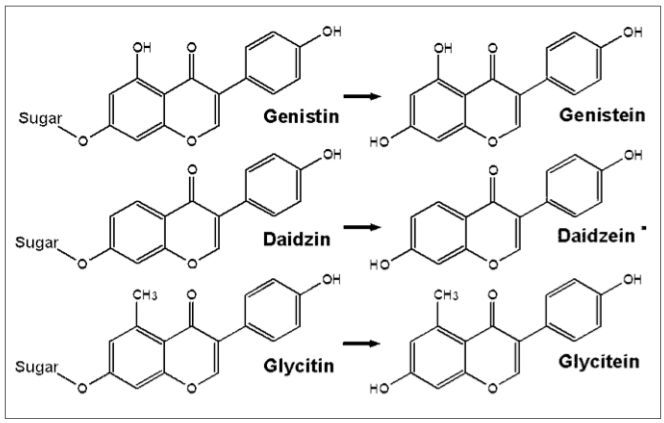

Figure 1. Chemical structure of the six isoflavone analytes.

Experimental

Hardware/Software

For the chromatographic separations, a PerkinElmer Altus™ UPLC® System was used, including the Altus A-30 Solvent/ Sample Module, integrated vacuum degasser, A-30 column heater, and Altus A-30 UV detector. All instrument control, analysis and data processing was performed using the Waters® Empower® 3 Chromatography Data Software (CDS) platform.

Method Parameters

The LC method parameters are shown in Table 1.

Solvents, Standards and Samples

All solvents, reagents, and diluents used were HPLC-grade or ACS grade and filtered via 0.45-µm filters. A mixture of 70/30 ACN/water was used for all diluents.

The individual isoflavone standards were obtained from ChromaDex®, Inc., Irvine, CA (www.chromadex.com). This included daidzin, daidzein, glycitin, glycitein, genistin and genistein.

Standard Preparation

A 400-µg/mL stock solution of each individual isoflavone was prepared by adding 10 mg of each isoflavone standard to a 25-mL volumetric flask. 15 mL of diluent was added to each flask and sonicated for 20 minutes. The flasks were then filled to volume with diluent. All stock solutions were filtered through 0.45-µm PVDF filters. Subsequently, a 16-µg/mL working standard (WS) was prepared by pipetting 1 mL of each stock standard into a 25-mL volumetric flask and filling to volume with diluent. To enhance stability, both the stock solutions and working standard were protected from light and refrigerated.

Table 1. LC Method Parameters.

Column:	PerkinElmer Brownlee™ SPP C18, 2.7 µm, 3.0 x 50 mm, Part # N9308408
Mobile Phase:	Mobile Phase A: 0.05% Trifluoroacetic Acid (TFA) in Water Mobile Phase B: 0.05% TFA in Acetonitrile (ACN) Solvent Program:

	Time (min.)	% MP A	% MP B	Curve
1	0.0	90	10	
2	4.0	65	35	6
3	5.0	65	35	6
4	10.0	90	10	6

Analysis Time:	10 min.
Flow Rate:	0.6 mL/min.
Pressure:	2300 psi/150 bar (maximum)
Oven Temp.:	40 °C
Detection:	UV at 260 nm
Injection Volume:	0.5 µL
Sampling (Data) Rate:	5 pts./sec

The working standard was used for the retention time reproducibility testing.

Eight calibration levels were prepared via serial dilution of the working standard with diluent. The concentration at each level is provided in Table 2.

Samples Preparation

Two types of product were tested, a solid and a liquid. Soy isoflavones tablets were purchased at the local chain store. Organic soy milk was purchased at the local supermarket.

To prepare a tablet extract, two random tablets, labeled Tablet 1 and Tablet 2, were weighed individually and then crushed into a fine powder. The entire contents of each tablet was quantitatively transferred into a 50-mL centrifuge tube and filled to volume with diluent. Each tube was vigorously shaken for one minute and sonicated for 30 minutes.

At each 10-minute interval, the tubes were vortexed for 30 seconds. Following, the tubes were centrifuged at 5000 rpm for 10 minutes. Each supernatant was then collected and filtered through a 0.45-µm PVDF filter. 0.5-mL of each filtered supernatant was transferred into an individual 10-mL volumetric flask and filled to volume with diluent. Following, each solution was transferred into a HPLC vial and injected in triplicate.

Table 2. Concentration of individual isoflavone at each calibration level.

Compound	Level 1 (µg/mL)	Level 2 (µg/mL)	Level 3 (µg/mL)	Level 4 (µg/mL)	Level 5 (µg/mL)	Level 6 (µg/mL)	Level 7 (µg/mL)	Level 8 (WS) (µg/mL)
Daidzin	0.0775	0.1550	0.3101	0.7752	1.550	4.651	7.752	15.50
Glycitin	0.0780	0.1560	0.3120	0.7800	1.560	4.680	7.800	15.60
Genistin	0.0783	0.1566	0.3133	0.7832	1.566	4.699	7.832	15.66
Daidzein	0.0796	0.1590	0.3184	0.7960	1.592	4.776	7.960	15.92
Glycitein	0.0738	0.1475	0.2950	0.7376	1.475	4.426	7.376	14.75
Genistein	0.0765	0.1530	0.3059	0.7648	1.530	4.589	7.648	15.30

For the soy milk, two 1-mL aliquots of soy milk, labeled Soy Milk 1 and Soy Milk 2, were transferred into 10-mL conical centrifuge tubes. These were then filled to volume with diluent. The tubes were vigorously shaken for one minute, vortexed for two minutes, sonicated for five minutes, and then centrifuged for five minutes at 5000 rpm. The supernatant was collected and subsequently filtered through a 0.45-µm PVDF filter into an HPLC vial and injected in triplicate.

Results and Discussion

Using the method parameters described in Table 1, Figure 2 shows the chromatographic separation of the level-8 calibrant, containing the six isoflavones, all well resolved in less than five minutes. The additional five-minute downward ramp back to initial mobile phase conditions was necessary to eliminate any potential late-eluting matrix components while also reducing the re-equilibration time between injections.

Figure 3 shows the overlay of 12 replicate injections of WS (Level-8), demonstrating excellent reproducibility. All retention time % RSDs were ≤ 0.085.

For calibration, the calibration set was based on a series of WS dilutions. The individual analyte concentrations at each level are shown in Table 2.

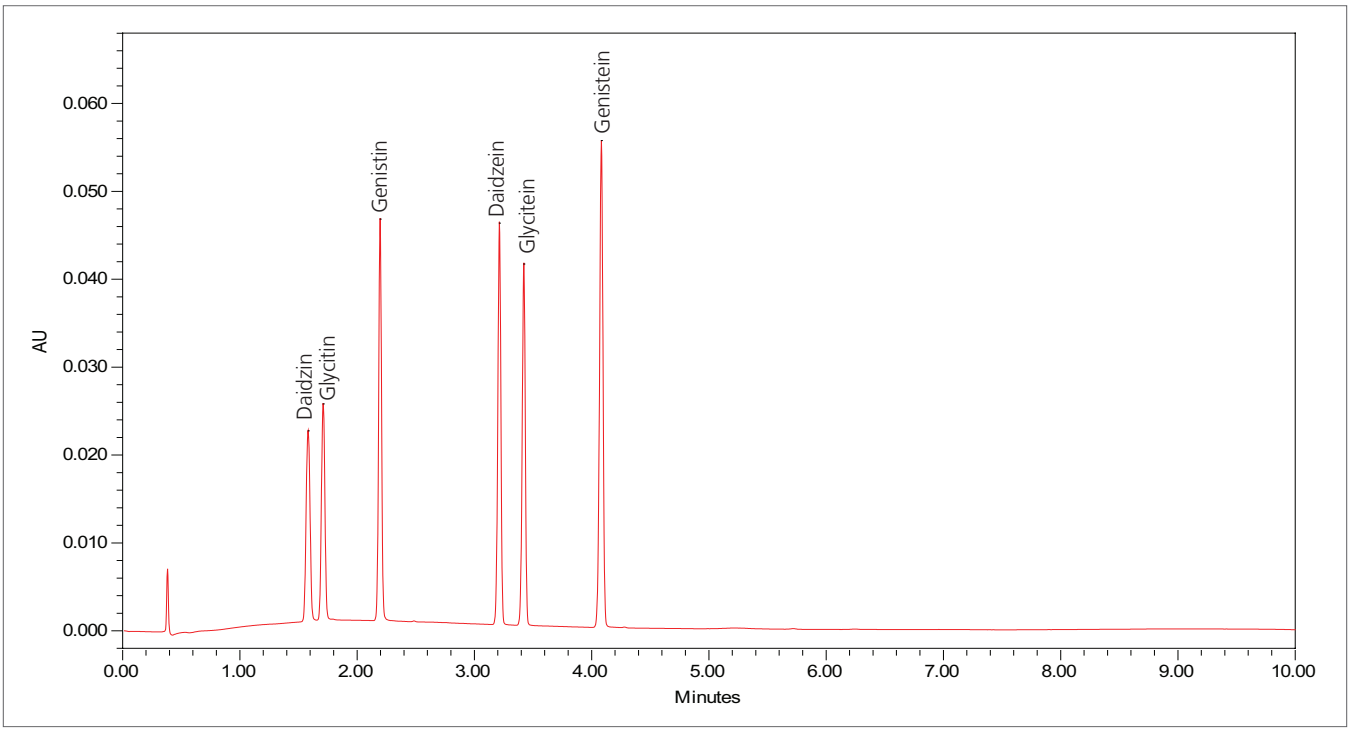

Figure 2. Chromatogram of the level-8 calibrant; UV at 260 nm.

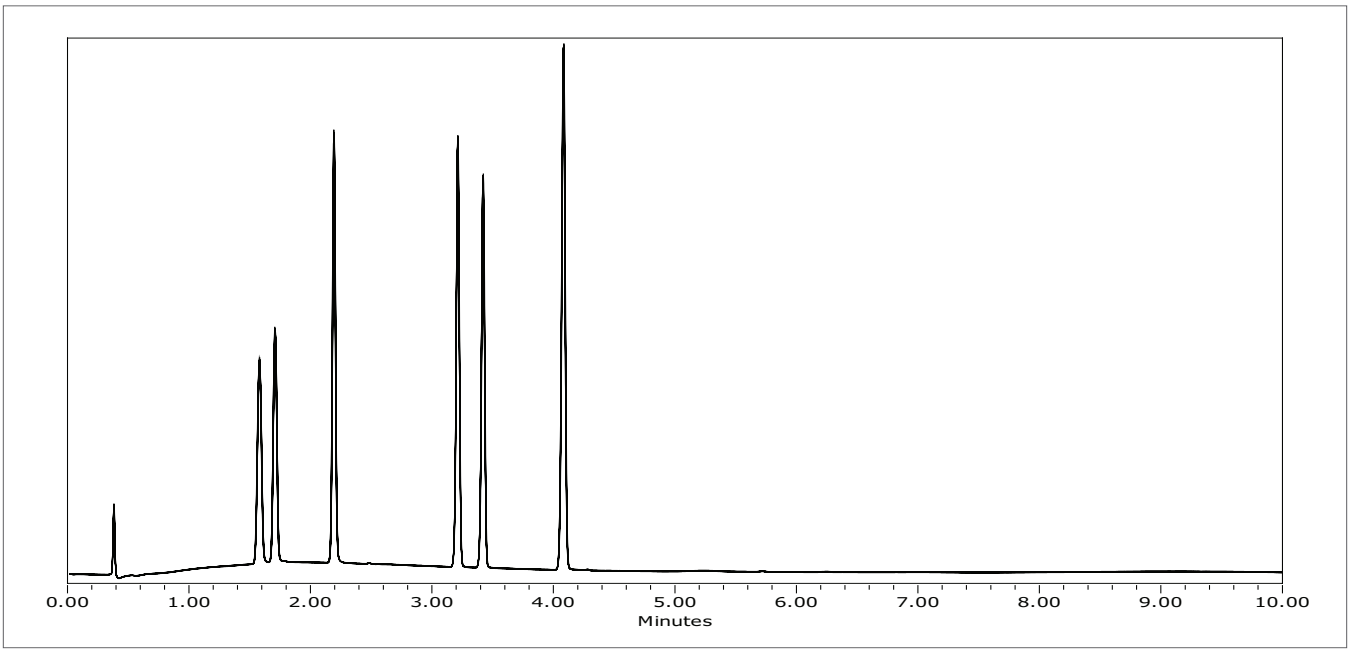

Figure 3. Overlay of 12 replicates of WS (Level-8); UV at 260 nm.

Figure 4 shows the calibration results for the six isoflavones. All isoflavones exhibited an exceptional linear fit (coefficient of determination, $R^2 > 0.9999$; n=3).

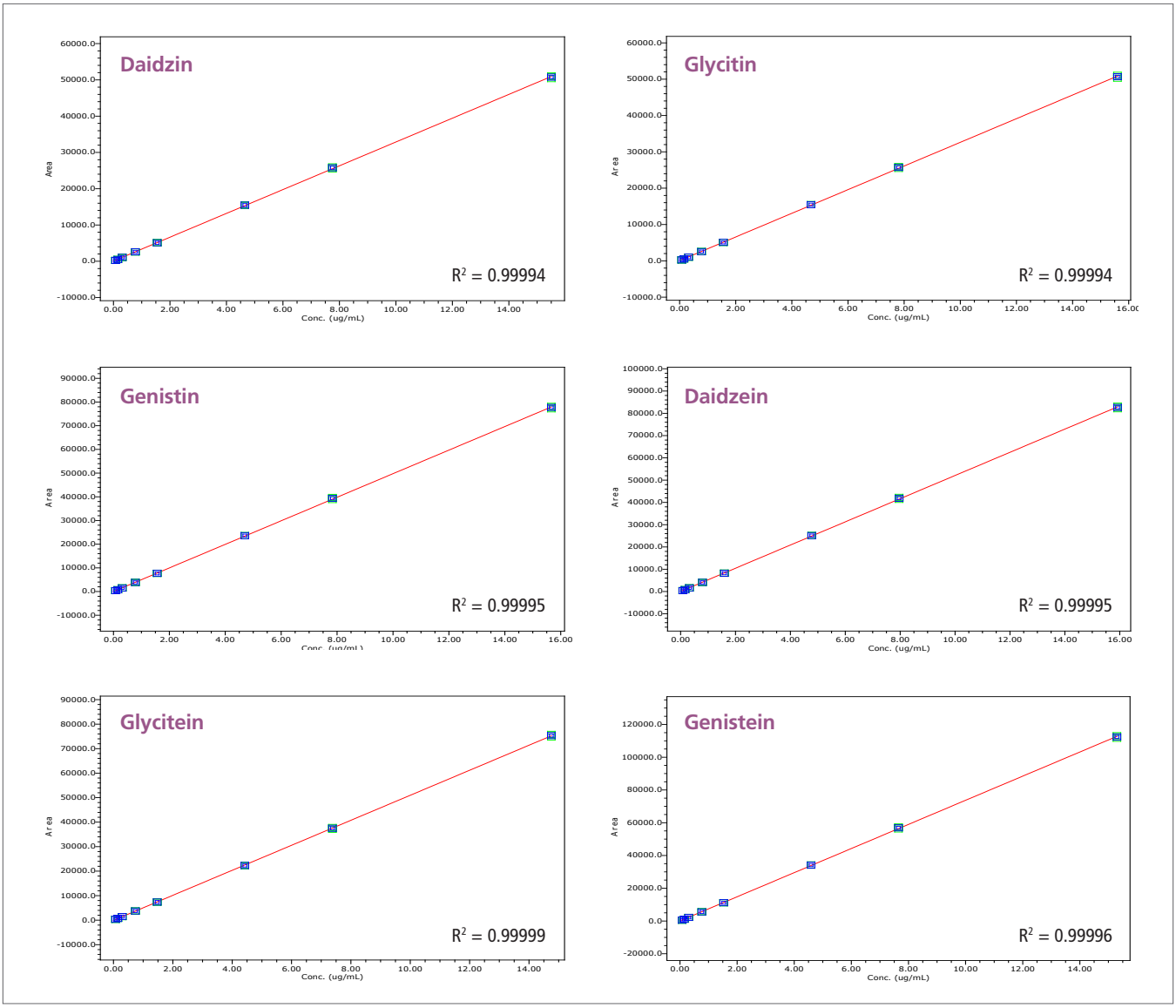

Figure 4. Results of 8-level calibration sets for the individual isoflavones.

Table 3 presents the estimated limits of quantitation and detection (LOQ and LOD) for all tested isoflavones, prepared in diluent. These limits were derived using the signal-to-noise (s/n) results obtained during calibration, using an average of three replicates per level.

As can be seen from the above table, the estimated LOD and LOQ for daidzin and glycitin are similar and almost twice as high as those for the other four isoflavones.

Using the same chromatographic conditions, the soy milk and soy tablet samples were then analyzed.

Table 3. Calculated LOQs and LODs for each isoflavone prepared in diluent.

Compound	LOD (µg/mL) (s/n ≥ 3/1)	LOQ (µg/mL) (s/n ≥ 10/1)
Daidzin	0.0107	0.0355
Glycitin	0.0093	0.0311
Genistin	0.0051	0.0171
Daidzein	0.0052	0.0173
Glycitein	0.0054	0.0179
Genistein	0.0041	0.0137

The chromatographic results for Soy Milk 1 are shown in Figures 5 and 6. The chromatogram for Soy Milk 2 is not shown, as it was virtually identical to that of Soy Milk 1.

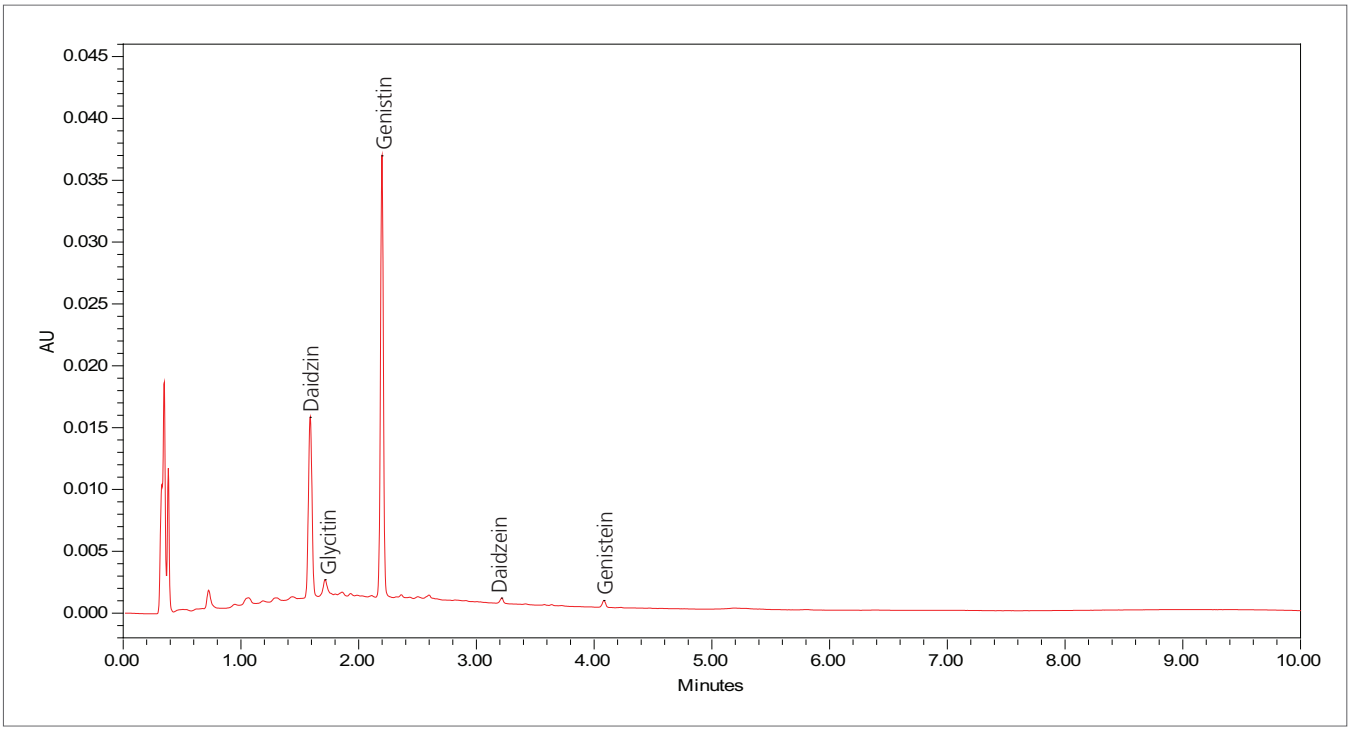

Figure 5. Chromatogram of Soy Milk 1; UV at 260 nm.

Per Figure 5, it appears that Soy Milk 1 contains a number of isoflavones, though no quantitatable glycitein (3.4 min.). Elution times were confirmed by overlaying the chromatogram of Soy Milk 1 with that of the 16 µg/mL working standard, as shown in Figure 6.

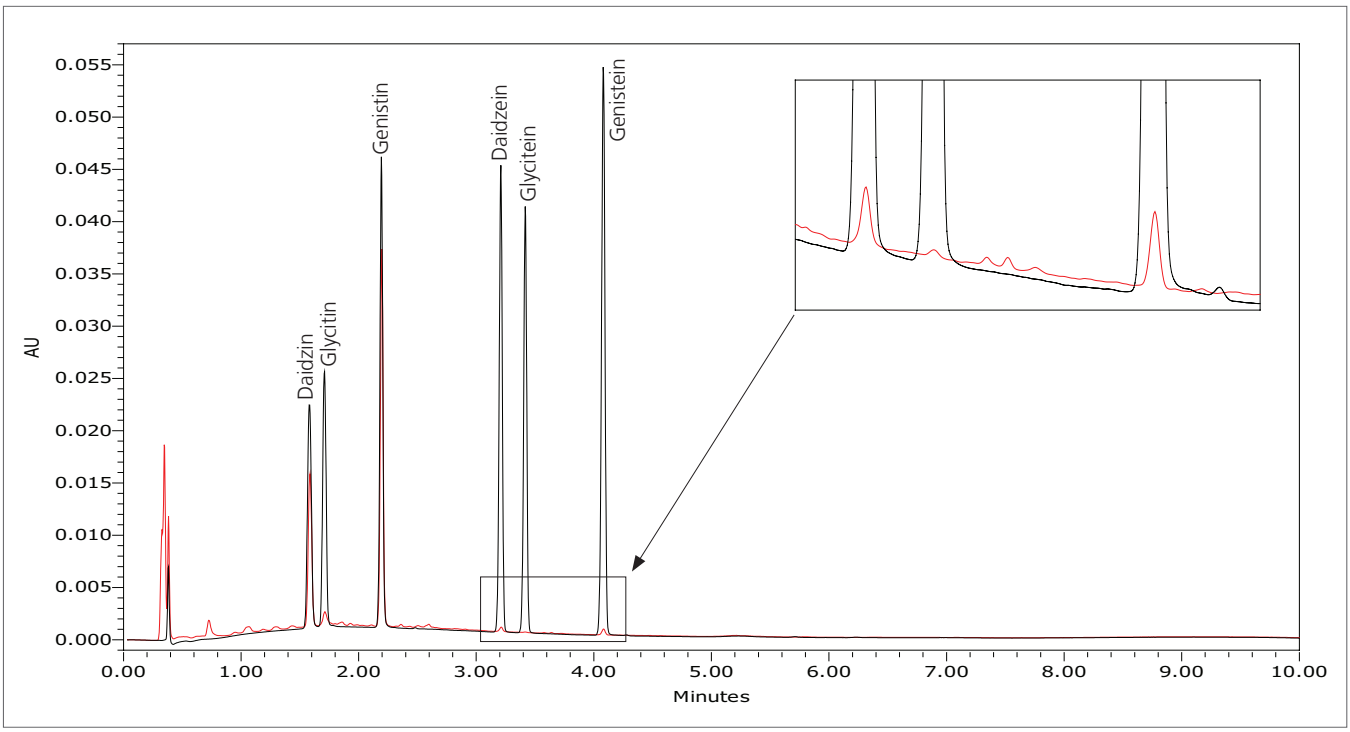

Figure 6. Overlaid chromatograms of Soy Milk 1 (red) and working standard (Level-8) (black); UV at 260 nm.

Table 4 presents the quantitative results, including LOD and LOQ values, for each of the isoflavones in soy milk. As expected, there are significant amounts of water-soluble glucosides (daidzin, glycitin, and genistin) and very little aglycones, as the latter are only produced upon metabolism of the glucosides.[3]

Per the chromatogram in Figure 7, besides the two unidentified excipient peaks between 2.5 and 3.0 minutes, Tablet I contained all six isoflavones, all quantitatable (see Table 5). Figure 8 shows the overlaid chromatograms of Tablet 1 and the 16 µg/mL working standard, confirming the elution times for these analytes. The chromatogram for Tablet 2 is not shown, as it was virtually identical to that of Tablet 1.

Table 4. Concentrations and LODs/LOQs of isoflavones in soy milk samples.

Compound	RT (min.)	Concentration (µg/mL)		LOD (µg/mL)	LOQ (µg/mL)
		Soy Milk 1	Soy Milk 2		
Daidzin	1.587	93.77	92.67	0.0824	0.2745
Glycitin	1.714	10.80	10.04	0.1052	0.3506
Genistin	2.197	124.3	123.3	0.0457	0.1520
Daidzein	3.213	1.620	1.650	0.0504	0.1681
Glycitein	NQ*	---	---	---	---
Genistein	4.083	1.510	1.520	0.0361	0.1200
Total Isoflavones		232.0	229.1		

* For glycitein, although the peak was visually observed, it was below the LOQ.

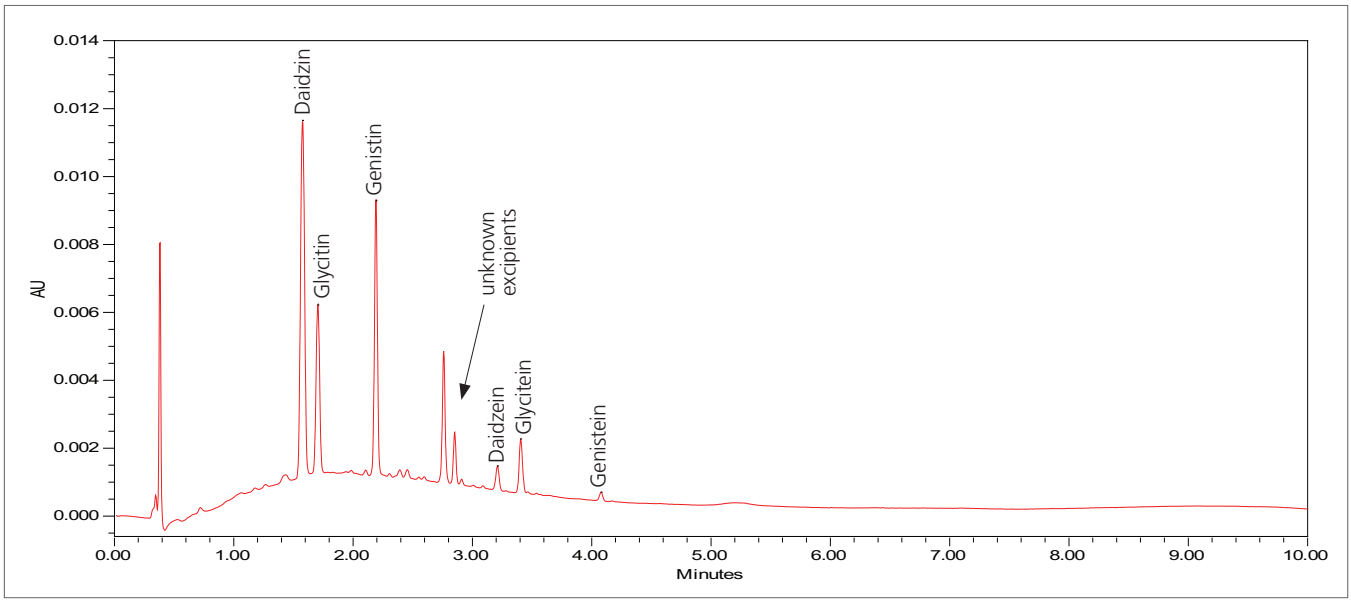

Figure 7. Chromatogram of Tablet 1; UV at 260 nm.

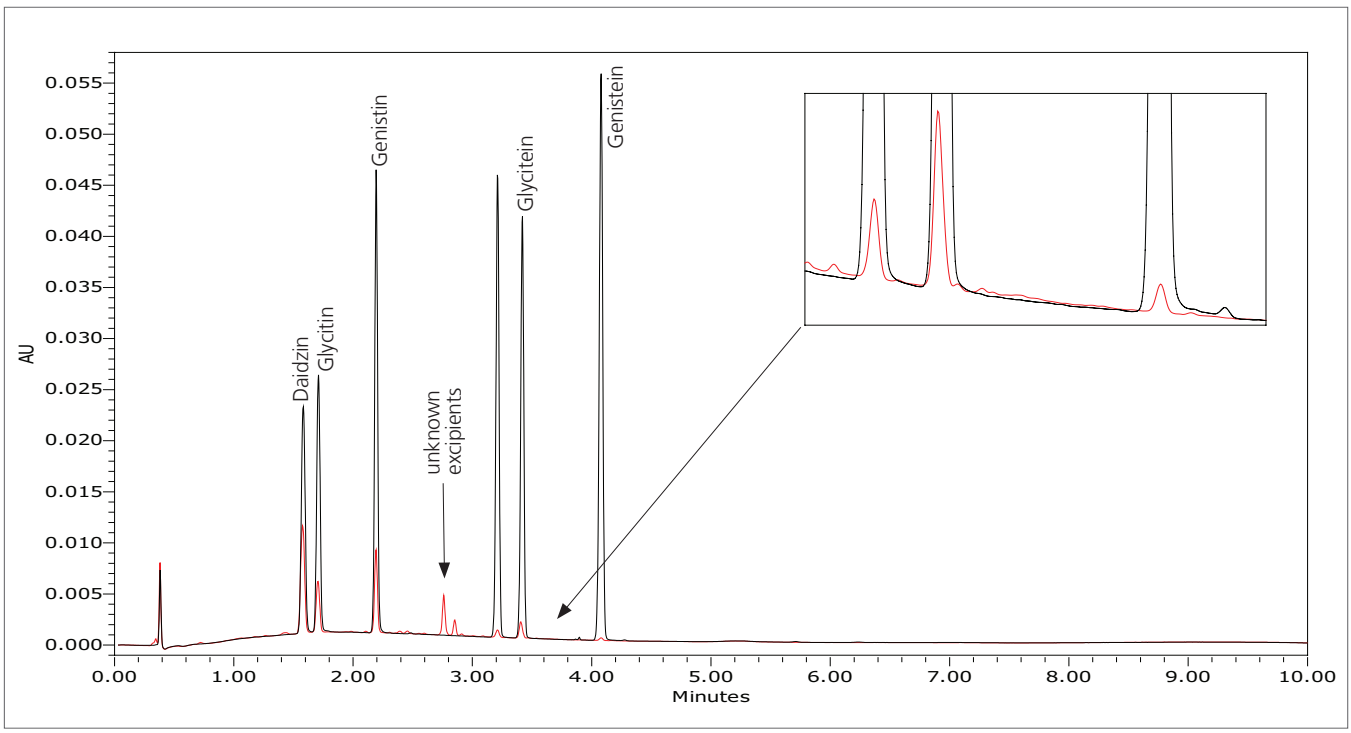

Figure 8. Overlaid chromatograms of Tablet 1 (red) and working standard (Level-8) (black); UV at 260 nm.

Table 5 presents the quantitative results, including LOD and LOQ values, for each of the isoflavones in the two soy tablet samples. Compared to the soy milk samples, though there were again higher levels of water-soluble glucosides than aglycones in soy tablets, both soy tablets contained quantitatable amounts of all three aglycones.

Table 5. Concentrations and LODs/LOQs in soy tablet samples.

Compound	RT (min.)	Concentration (mg/gm)		LOD (mg/gm)	LOQ (mg/gm)
		Tablet 1	Tablet 2		
Daidzin	1.580	5.7	5.7	0.006	0.022
Glycitin	1.707	2.3	2.3	0.006	0.019
Genistin	2.195	2.1	2.1	0.003	0.010
Daidzein	3.211	0.21	0.20	0.003	0.011
Glycitein	3.405	0.44	0.45	0.003	0.011
Genistein	4.080	0.05	0.05	0.002	0.008
Total Isoflavones		10.8	10.8		

Conclusion

The results obtained confirm the applicability of this method for the efficient, routine, and robust chromatographic analysis of the most commonly found isoflavones in soy products. All six isoflavones were completely separated from other matrix components in under five minutes by UHPLC using UV detection. The results showed excellent retention time repeatability, as well as very good linearity, over the tested concentration range.

Thereupon, and considering the LOD and LOQ levels for all isoflavones and the test results from actual samples, this application was found to be effective for the monitoring of isoflavones in both soy milk and tablet supplements. It also reduced the analytical runtime which, with a flow rate of 0.6 mL/min, translates directly into low solvent consumption and greater savings.

As the chromatographic separation was performed under 2500 psi, this application could be successfully performed using conventional HPLC instrumentation.

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