APPLICATION NOTE

Liquid Chromatography

Author:

Bryan McClanahan

PerkinElmer, Inc.
Shelton, CT

Analysis of Phytoestrogen Isoflavones in Dietary Supplements by HPLC/UV

Introduction

Isoflavones are a class of naturally occurring water-soluble compounds produced almost exclusively by members of the Fabaceae plant family. Found in a number of plants and food sources, isoflavones occur in several forms, including aglycones (daidzein, glycitein and genistein), glucosides (daidzin, genistin and glycitin), and their acetyl and malonyl conjugates. The aglycone species of isoflavones are biologically active, whereas the glucosides and conjugates undergo hydrolysis by bacterial beta-glucosidases in the intestines, releasing the corresponding aglycones. Figure 1 illustrates the structure of the three aglycones, as well as their corresponding glucosides.

Structurally similar to the female hormone estrogen, isoflavones are characterized as phytoestrogens, actively binding to estrogen receptors throughout the body. Although the estrogenic effects of phytoestrogens tend to be weaker than that of natural estrogen, the result can nevertheless result in both positive and negative effects on the human body. Phytoestrogens have been shown to reduce the potential of developing many cancers (such as breast, endometrial and prostate cancer), prevent osteoporosis, and improve the ratio of LDL to HDL cholesterol.[1-4] However, studies also suggest that the endocrine disrupting nature of phytoestrogens can exacerbate existing thyroid disorders.[5]

PerkinElmer®
For the Better

A variety of isoflavone supplements are available on the market today, many offered as nutraceuticals. Verifying label-claim accuracy is vitally important in the development and manufacturing of nutraceutical products to protect human health and ensure therapeutic levels of active ingredients are included in the supplements. Both the United States Pharmacopeia (USP) and the Association of Official Agricultural Chemists (AOAC) have established methods for the analysis of isoflavones in various supplements by high performance liquid chromatography (HPLC)[6-8]. However, these analytical methods can be inefficient for high throughput laboratories, and result in the consumption of significant amounts of solvents. Although a number of analytical methods for isoflavones exist, the goal of this work is to develop a simpler, faster and more reliable liquid chromatography method for the analysis of the most widely-used isoflavones in soy nutraceutical supplements. Method conditions and performance data, including linearity and repeatability, are presented.

Experimental

Hardware and Software

Chromatographic separation was achieved utilizing the PerkinElmer LC 300 UHPLC system, consisting of an LC 300 18k psi UHPLC pump, an LC 300 UHPLC autosampler with temperature-controlled sample compartment, and an internal column oven. Detection was achieved by an LC 300 photodiode array detector (PDA). Instrument control, analysis and data processing were performed utilizing the Simplicity™Chrom CDS software platform.

Method Parameters

The LC parameters are detailed in Table 1.

Table 1. LC Parameters.

Column	PerkinElmer Brownlee SPP C18, 2.7 µm, 75 x 2.1 mm (Part# N9308403)
Mobile Phase	**Solvent A:** HPLC or LC/MS Grade Water with 0.1% Formic Acid **Solvent B:** HPLC or LC/MS Grade Acetonitrile with 0.1% Formic Acid **Solvent Program:** Linear Gradient

Step	Time (min.)	Flow Rate (mL/min.)	%A	%B
1	0.00	0.600	88	12
2	0.10	0.600	88	12
3	6.00	0.600	70	30
4	6.99	0.600	70	30
5	7.00	0.600	88	12

Analysis Time	7.0 min.
Equilibration Time	2.0 min.
Pressure	~5500 psi/380 bar maximum
Oven Temp.	30 °C
Sample Temperature	Ambient
Injection Vol.	5 µL
Detection Wavelength	260 nm Bandwidth 15 nm Reference Wavelength: 400 Bandwidth 40 nm
Data Collection Rate	5 pts/sec (Hz)

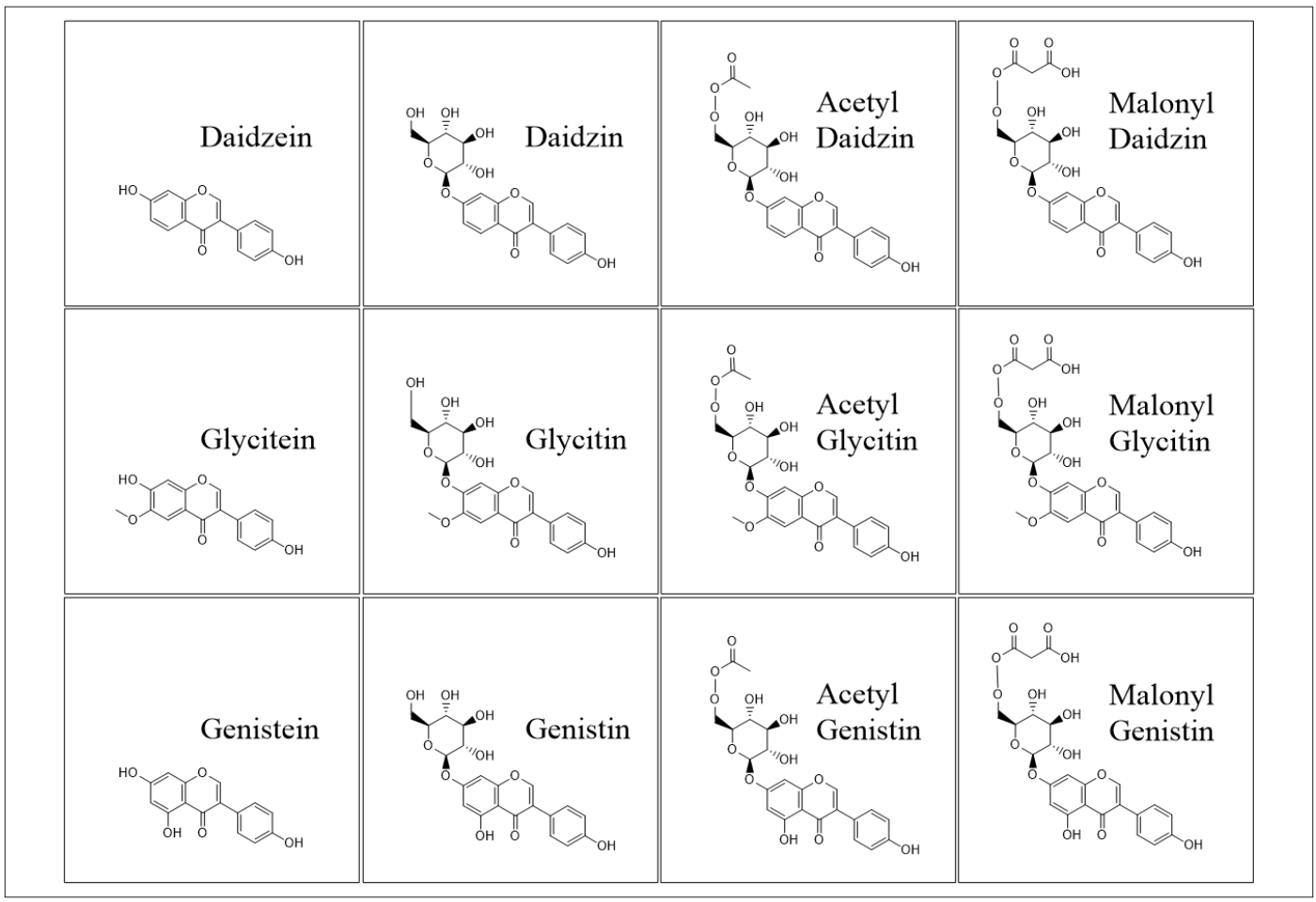

Figure 1. Chemical structures of the isoflavones analyzed in this study.

Solvents and Standard Preparation

All solvents and diluents used were HPLC or LC/MS grade.

The three aglycone and three glucoside standards were obtained from Sigma-Aldrich®, Inc (St. Louis, MO) in a powder form. These standards included daidzin, daidzein, glycitin, glycitein, genistin and genistein. USP defatted powdered soy reference material was also obtained from Sigma-Aldrich®, Inc (St. Louis, MO).

Individual stock solutions of approximately 400 µg/mL were prepared by dissolving 5-10 mg of each standard into acetonitrile.

A mixed standard solution was prepared by combining appropriate aliquots of the individual stock solutions, and diluting to the final volume with water and acetonitrile such that the solvent was 50:50 water:acetonitrile. Calibrants were prepared by serially diluting the mixed standard with 75:25 water:acetonitrile. Final concentrations of the standards are shown in the Table 2 below.

Table 2. Group A compounds are daidzin, daidzein, genistin and genistein. Group B compounds are glycitin and glycitein.

Solution Name	Group A Conc. (µg/mL)	Group B Conc. (µg/mL)
STD-A	10.00	5.000
STD-B	5.000	2.500
STD-C	2.500	1.250
STD-D	1.250	0.6250
STD-E	0.6250	0.3125
STD-F	0.3125	0.1563
STD-G	0.1563	0.07813
STD-H	0.07813	0.03906

Acetyl and malonyl retention time check solutions were prepared using USP defatted powdered soy reference material, as described in the USP monograph for soy isoflavone capsules. One 200 mg portion was heated in an open container at 120 °C for two hours. A second portion of the reference material was simply weighed into an appropriate vial. Both were prepared by adding 2 mL acetonitrile and 1.2 mL water, and mixed for one hour. An additional 1.8 mL of water was added to each vial, thoroughly mixed, centrifuged, and filtered using a 0.45-µm PVDF filter. Aliquots were further diluted 1:1 with water prior to analysis.

Sample Preparation

Three different dietary supplements containing soy isoflavones were purchased from a local health food store. One contained only the soy isoflavones of interest, while the other two also contained a variety of supplemental herbal ingredients. The contents of five capsules from each supplement were pooled and weighed. An amount of the pooled material was weighed to give approximately 5 mg of isoflavone based on the label-claim amount. The samples were prepared as described in the USP monograph by adding 10 mL acetonitrile and 6 mL water, and subsequently mixing for one hour.[6] An additional 9 mL of water was added to each vial, thoroughly mixed, centrifuged, and filtered using a 0.45-µm PVDF filter. Aliquots were further diluted 1:1 with water prior to analysis.

Results and Discussion

The chromatogram of the high concentration standard (Std-A, 10.0/5.0 µg/mL) is shown in Figure 2.

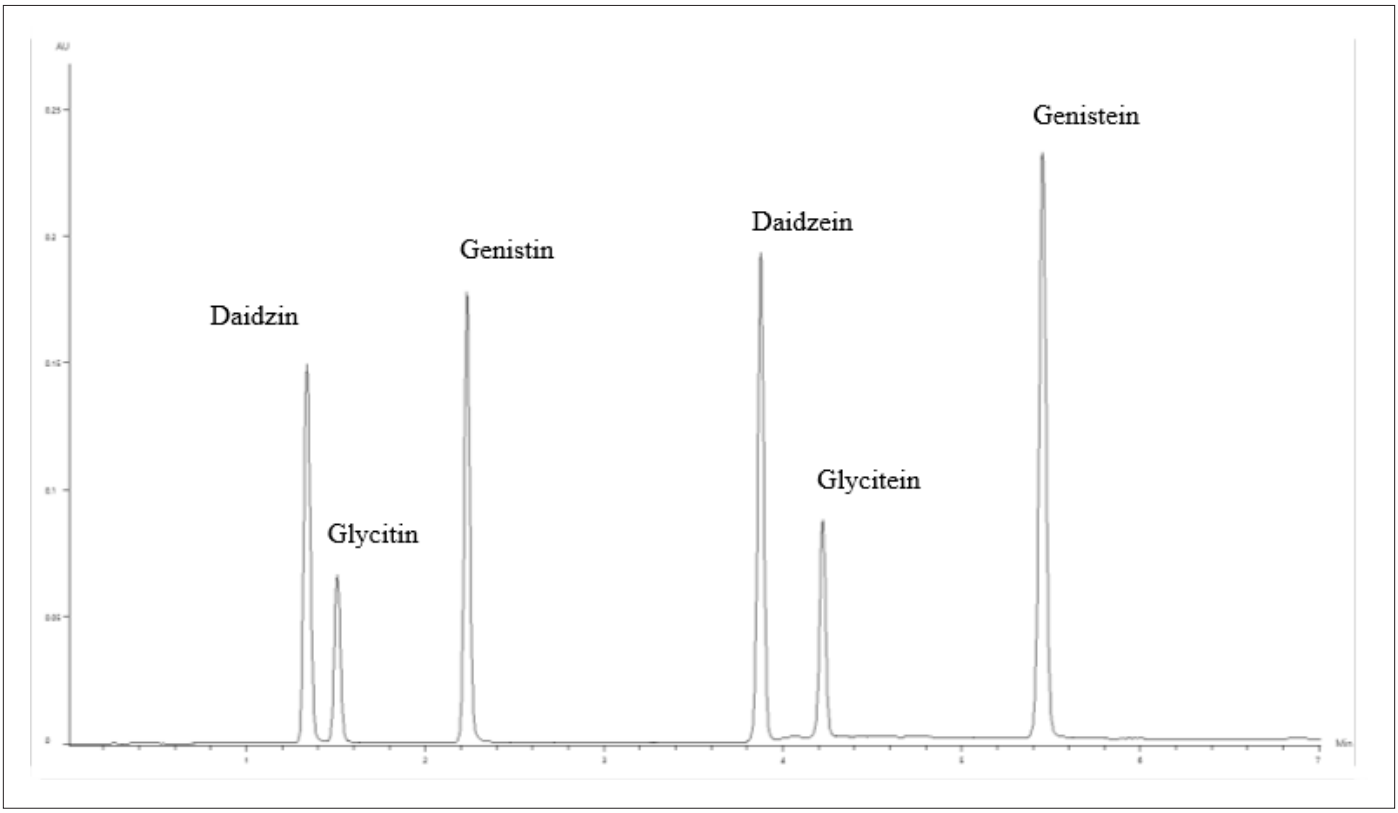

Figure 2. Chromatogram of the the 10.0/5.0-µg/mL isoflavone standard.

Figure 3 displays the chromatogram of the heated sample of the defatted powdered soy reference material. This, along with the non-heated sample, were used to identify the retention times of the acetyl and malonyl conjugated forms of the isoflavones. These compounds are relatively unstable, and reference standards are not readily available. As these compounds were not included in the calibration standards, the amounts were calculated using the method described in the USP monograph. The corresponding glucoside calibration curve was used to calculate a concentration which was adjusted using a correction factor.

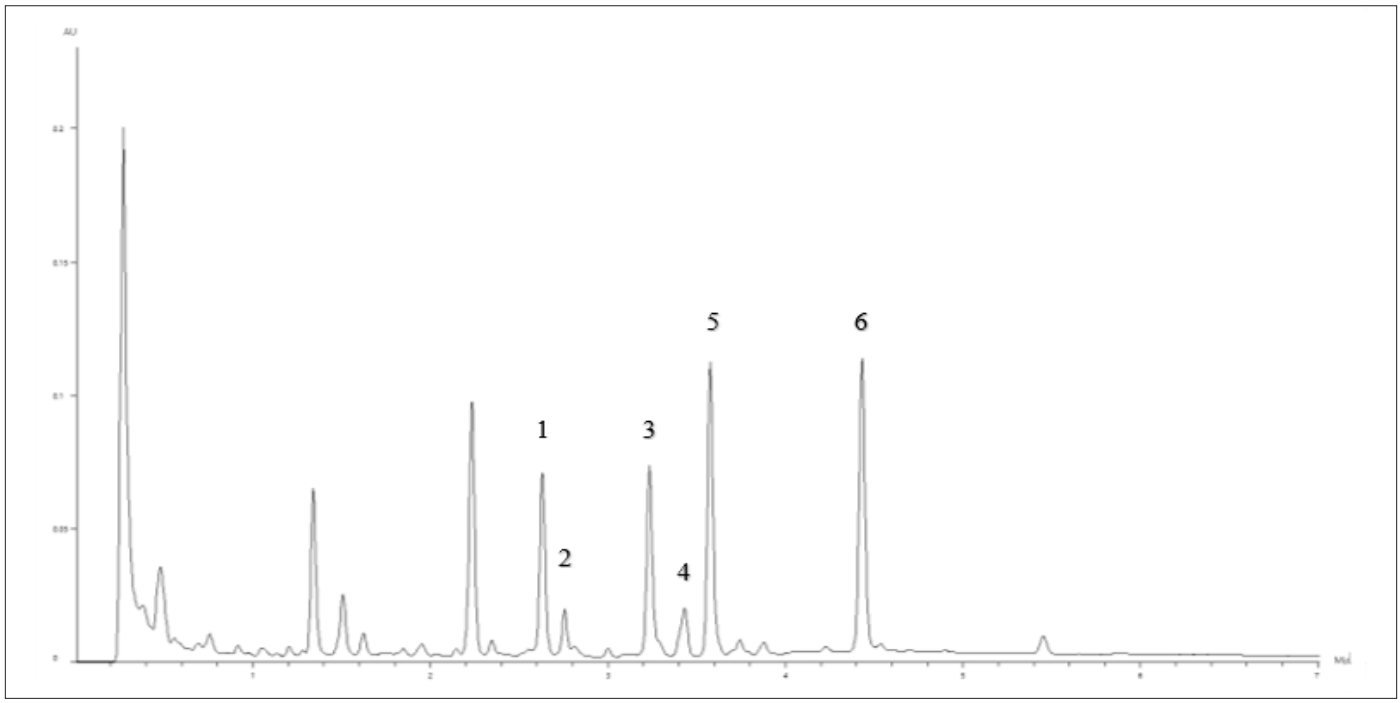

Figure 3. Heated USP Defatted Powdered Soy showing peak identification: 1) Malonyl Daidzin 2) Malonyl Glycitin 3) Acetyl Daidzin 4) Acetyl Glycitin 5) Malonyl Genistin 6) Acetyl Genistin.

Figure 4 shows the overlay of six replicate 2.5/1.25-µg/mL isoflavone standard injections, demonstrating exceptional reproducibility. Retention time repeatability was below 0.01 min and peak area %RSDs were below 0.5% for all analytes.

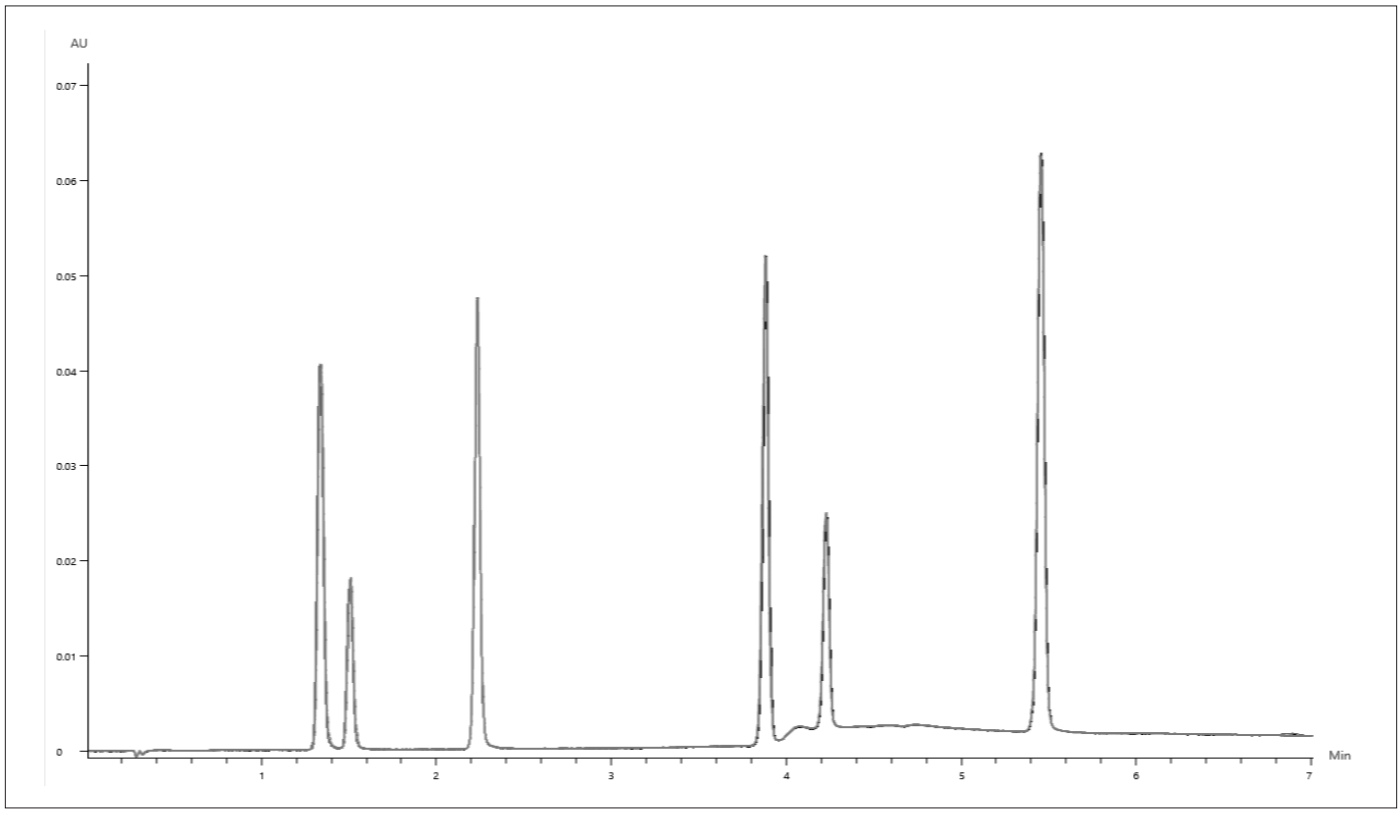

Figure 4. Chromatographic overlay of 6 replicates of the 2.5/1.25-µg/mL isoflavone standard.

Figure 5 details the calibration results for all six isoflavone compounds over the tested concentration range. All six compounds showed a good linear (1st order) fit and had R^2 coefficients above 0.999.

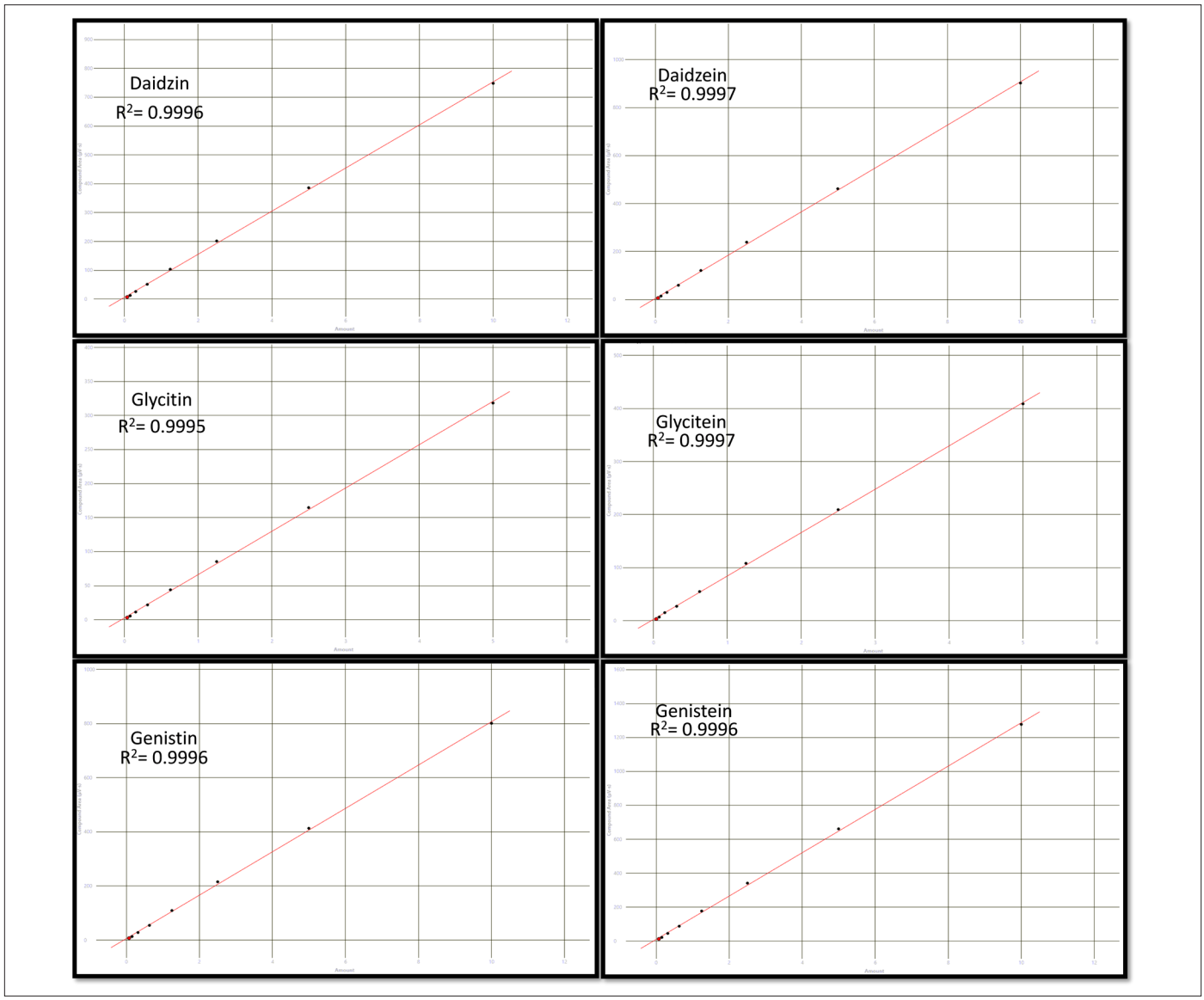

Figure 5. Results of the 8-level calibration sets for the three glucoside and three aglycone isoflavones.

As listed in Table 3, the limit of detection (LOD) and limit of quantitation (LOQ) were obtained for each analyte, and were calculated from the standard deviation of the response measured at the low standard (STD-H, n=6) and the slope of the calibration curve for each analyte.

Sample Results

Using the same chromatographic conditions, the three prepared supplement samples were analyzed. The chromatographic results for supplements A and B were visually similar with most of the isoflavone content in the form of the glucosides daidzin and genistin, as shown in Figure 6. Sample C showed different relative concentrations of the measured analytes, specifically a higher proportion of acetyl and malonyl conjugated glucosides, as shown in Figure 7.

Table 3. LODs and LOQs for the six analytes, in order of elution.

Analyte	Calculated LOD (µg/mL)	Calculated LOQ (µg/mL)
Daidzin	0.0099	0.0330
Glycitin	0.0063	0.0209
Genistin	0.0092	0.0306
Daidzein	0.0028	0.0095
Glycitein	0.0062	0.0208
Genistein	0.0026	0.0085

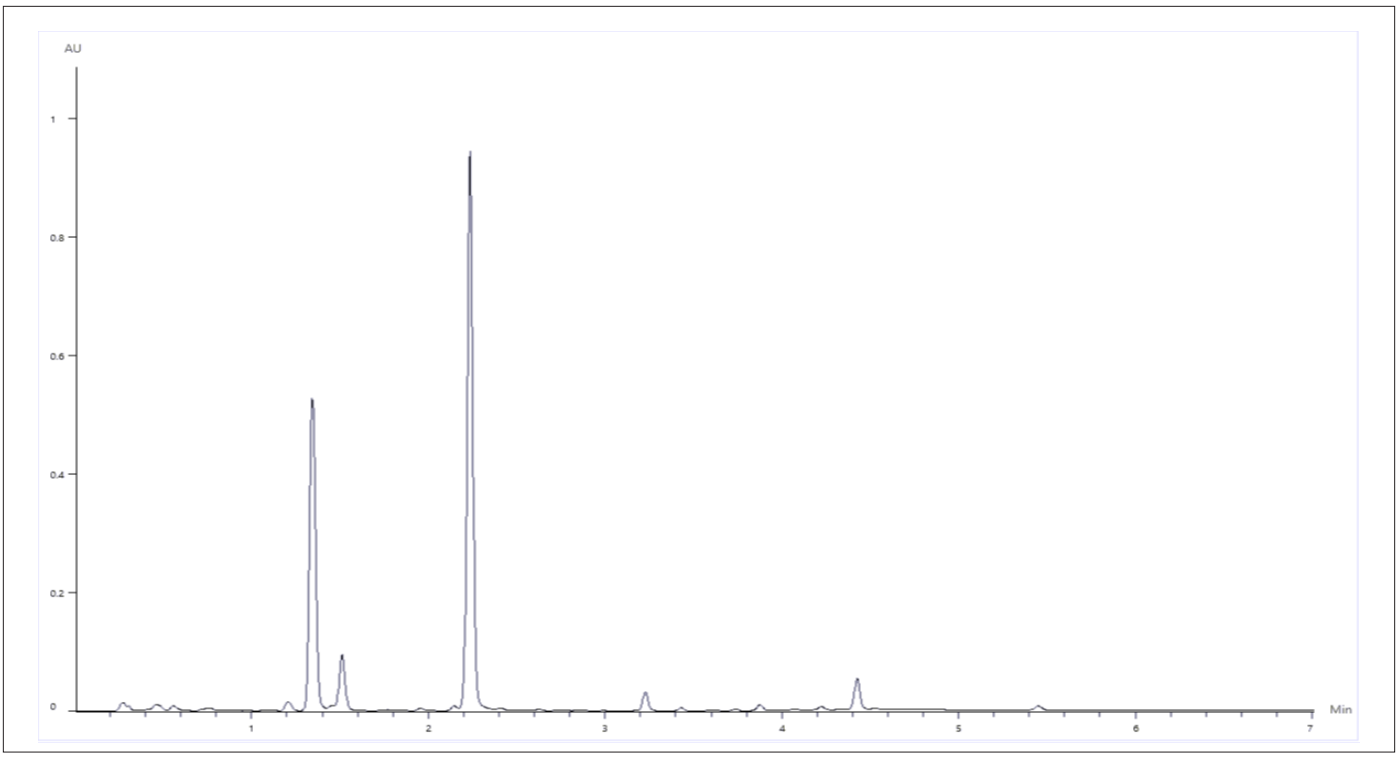

Figure 6. Representative Chromatogram of Supplements A and B.

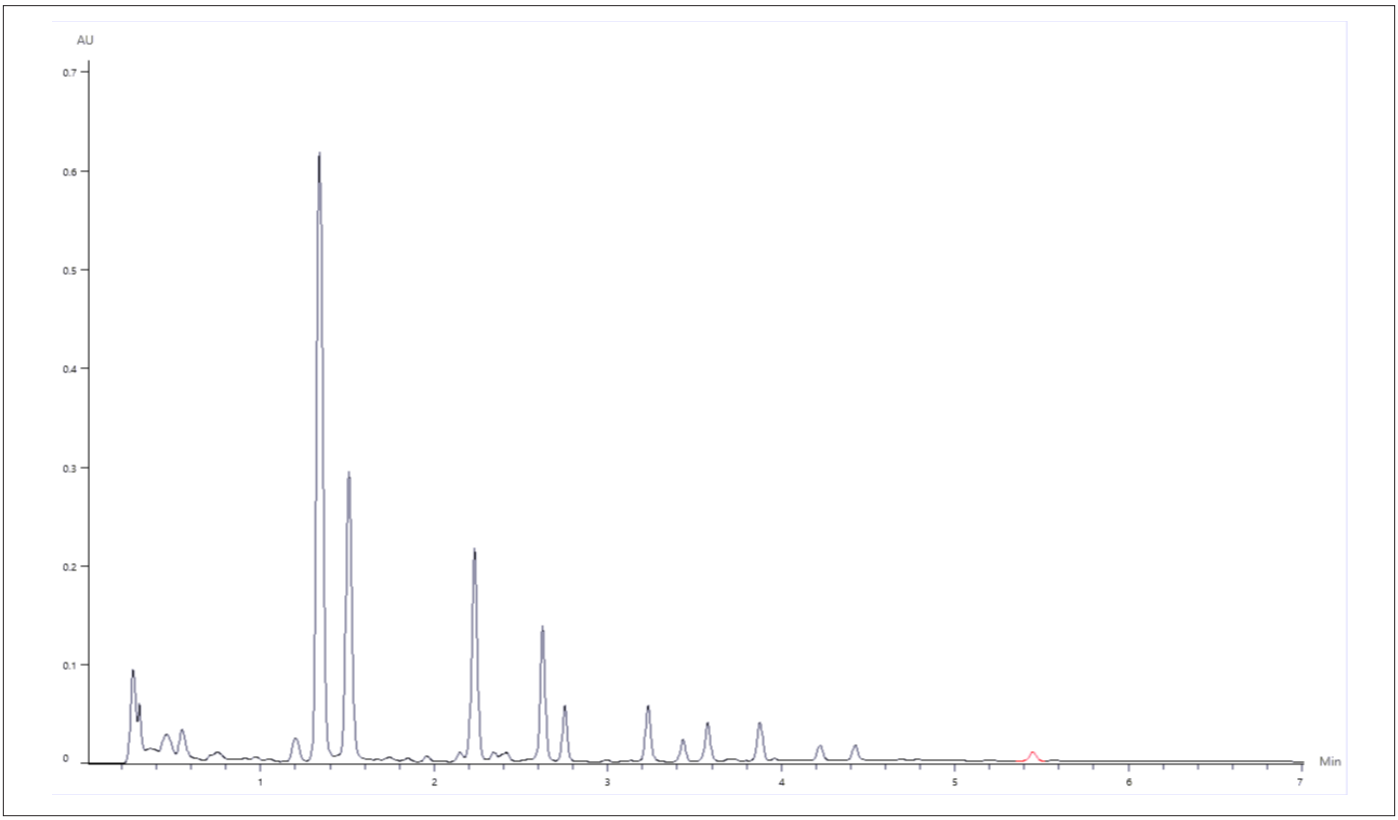

Figure 7. Representative Chromatogram of Supplement C.

Quantitative results for the three capsule samples are shown in Table 4 below. Samples A and B showed very similar profiles chromatographically and quantitatively. Both products are produced by the same manufacturer, and list the same ingredient source. Sample C is produced by a different manufacturer and lists a different ingredient source. The three glucoside compounds in each sample were measured above the high calibration standard, and as such, extrapolated values are reported based on the linearity of the tested curve range and the known wide linear range of the LC 300 PDA detector.

Table 4. Detected isoflavone concentrations in the three samples.

Analyte	Sample A (µg/mL)	Sample B (µg/mL)	Sample C (µg/mL)
Daidzin	76.6[a]	67.9[a]	84.8[a]
Glycitin	10.3[a]	14.4[a]	47.3[a]
Genistin	106[a]	105[a]	25.0[a]
Malonyl Daidzin	0.364	0.378	18.1
Malonyl Glycitin	0.156	0.134	8.04
Acetyl Daidzin	4.74	4.10	7.51
Acetyl Glycitin	0.520	0.723	3.23
Malonyl Genistin	ND	ND	4.98
Daidzein	0.941	0.887	3.794
Glycitein	0.351	0.512	1.74
Acetyl Genistin	7.45	6.39	1.64
Genistein	0.540	0.481	0.637
Total Isoflavone	**208**	**201**	**207**
Label Claim Isoflavone (per weight)	**202 (26.1 mg)**	**203 (43.3 mg)**	**202 (189.5 mg)**
Percent of Label Claim	**103%**	**99.1%**	**102%**

[a] *value extrapolated above calibration curve*

Conclusion

This work has demonstrated the fast and robust chromatographic separation and quantitation of soy isoflavones including glucoside, acetyl-glucoside and malonyl-glucoside conjugates using a PerkinElmer LC 300 18K UHPLC system with PDA detection. The results exhibited very good retention time repeatability, as well as excellent linearity and reproducibility over the tested concentration ranges. Samples were prepared in accordance with the USP monograph for soy isoflavones in dietary supplement capsules, and the analytical results were consistent with the label-claim for each sample. The method affords LOQs ≤ 0.03µg/mL for most analytes, which is four times lower than the level detected for any analyte in the three samples.

References

1. Linus Pauling Institute, Micronutrient Information Cetner at Oregon State University. https://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals/soy-isoflavones. Accessed March 2020.
2. Anderson JW, Johnstone BM, Cooke-Newell ME. Meta-analysis of the effects of soy protein intake on serum lipids. N Eng J Med. 1995;333:276-281.
3. Baum JA, Teng H, Erdman JW Jr, et al. Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. Am J Clin Nutr. 1998;68:545-551.
4. Marini H, Minutoli L, Polito F, et al. Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women. Ann Intern Med. 2007;146:839-847.
5. Sathyapalan, Thozhukat, et al. "The Effect of Soy Phytoestrogen Supplementation on Thyroid Status and Cardiovascular Risk Markers in Patients with Subclinical Hypothyroidism: A Randomized, Double-Blind, Crossover Study." The Journal of Clinical Endocrinology &; Metabolism, vol. 96, no. 5, 2011, pp. 1442–1449., doi:10.1210/jc.2010-2255.
6. USP Monograph Soy Isoflavones Capsules; page reference USP43-NF38 – 5276; document ID GUID-683A08A4-E4C7-4DB4-A080-B97DFF5500C3_1_en-US.
7. Collison, Mark W., Determination of Total Soy Isoflavones in Dietary Supplements, Supplement ingredients and Soy Foods by High-Performance Liquid Chromatogrphy with Ultraviolet Detection: Collaborative study. J AOAC Int. 2008; 91(3) 489-500. Accessed Online March 2020.
8. Daems, Frederic, Analytical methods used to quantify isoflavones in cow's milk: a review. Dairy Sci. & Technol. 2016 96:261-283.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

71686 PKI