Increased Throughput and Reduced Solvent Consumption for the Determination of Isoflavones by UHPLC

Liquid Chromatography

Authors:

Padmaja Prabhu, PerkinElmer
Wilhad Reuter, PerkinElmer

Studies suggest that the isoflavones found in soy can exert positive physiological effects.

Introduction

All plant foods are complex mixtures of chemicals including both nutrients and biologically active non-nutrients, referred to as phytochemicals. Soy is known for having high concentrations of several physiologically-active phytochemicals, including isoflavones, phytate (inositol hexaphosphate), saponins, phytosterols and protease inhibitors. The isoflavones are what makes soy unique. Soy isoflavones are non-steroidal molecules structurally and functionally related to 17β-estradiol. Soybeans and soy foods are the only natural dietary sources that provide nutritionally relevant amounts of isoflavones.

Clinical studies suggest that consumption of isoflavones can exert positive physiological effects[1]. Recent data has demonstrated that isoflavones have potent antioxidant properties, comparable to that of the well known antioxidant vitamin E[2]. Research in several areas of healthcare has linked isoflavones to lowering risks for disease, easing menopause symptoms, reducing heart disease and cancer risk, and improving prostate and bone health. As a result of the potential health benefits of isoflavones, many soy products and isoflavone supplements are available to consumers. These fall into a category of products known as nutraceuticals or functional foods, which provide a potential health benefit from a naturally occurring substance. This has created the need for an analytical technique which can qualify and quantify the type and amount of isoflavones in a nutraceutical product.

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For the Better

This application note will demonstrate a rapid method for the identification and quantification soy isoflavones using ultra high performance liquid chromatography (UHPLC). This UHPLC method is nearly 10x faster, and saves 92% of the mobile phase solvent, compared to conventional HPLC methods.

The focus will be on three major isoflavones found in soybeans, genistein, daidzein, and glycitein, and their glycosidic conjugates (Figure 1). In addition to qualitative and quantitative analysis, we will compare the analytical time and solvent use of this UHPLC application with a similar technique using conventional HPLC. The savings in both time and solvent consumption will be discussed. Lastly, three commercial formulations of supplements will be analyzed and isoflavone identification and content determined.
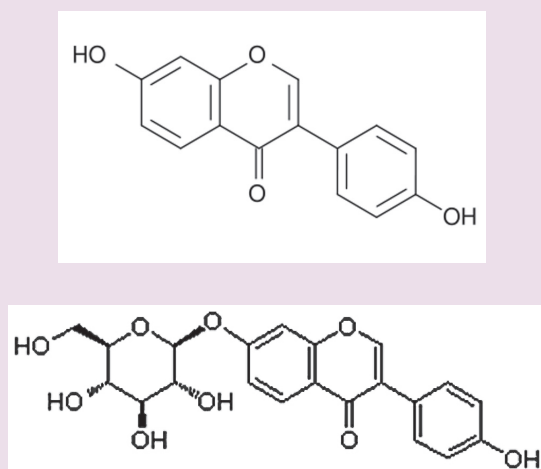
Figure 1. Chemical structure of a common soy isoflavone, daidzein, and its glycosidic conjugate, daidzin

Experimental

The PerkinElmer® Flexar™ FX-10 UHPLC system was used for this application. A 1.5 μm particle, 50 mm length, C18 column was used to separate the analytes of interest and matrix. This column required an operating pressure of approximately 8500 psi resulting in a mobile phase flow rate of approximately 0.7 mL/min. A Flexar FX-UV/Vis UHPLC detector was operated at 254 nm. Table 1 presents the detailed operating parameters of the UHPLC system. The instrument interaction, data analysis, and reporting was completed with the PerkinElmer, Chromera® data system.

Table 1: Detailed instrument conditions used in the determination of isoflavones.

HPLC System	PerkinElmer Flexar FX-10 UHPLC
Autosampler	Flexar UHPLC Autosampler
Detector	Flexar FX UV/Vis UHPLC Detector
Column	Grace Vision HT C18 (50 mm x 1.5 μ, 2.1 mm i.d.)
Column Temperature	30 C
Detector Wavelength	254 nm
Injection Volume	2 μL (partial loop)
Flow Rate	1 mL/min
Mobil Phase A	Water pH adjusted to 3.0 with orthophosphoric acid
Mobil Phase B	Acetonitrile Gradient Program

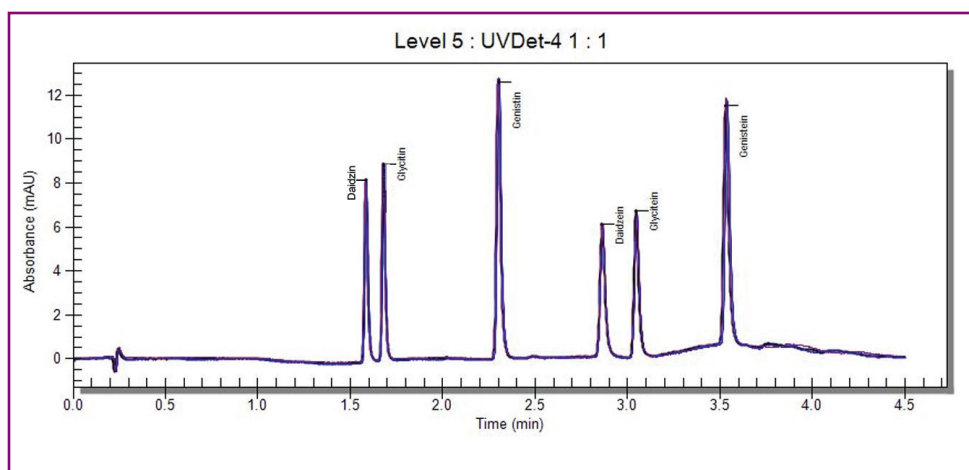| Type	Time (min)	Flow (mL/min)	% A	% B	Curve
Equil	0.5	0.7	90	10	0
Run	0.3	0.7	90	10	0
Run	0.3	0.7	85	15	0
Run	0.8	0.7	80	20	0
Run	0.9	0.7	75	25	0
Run	0.5	0.7	65	35	0
Run	0.5	0.7	90	10	0
Run	1.2	0.7	90	10	0

Figure 2. Resultant chromatograms of the analysis of reference material under the instrument conditions presented here (overlay of three replicates).

Standard preparation: The reference standards were procured from Chromadex (Irvine, CA).

Stock Solution: 1 mg of each of daidzin, glycitin, genistin, daidzein, glycitein and genistein were dissolved in 10 mL of water:acetonitrile (1:1), making a stock solution at a concentration of 100 μg/mL.

Calibration curve: The stock solution (100 μg/mL) was diluted in 9:1 (water:acetonitrile) to create an 8 level calibration (Table 2). The three low calibration points were serially diluted from the 10 μg/mL level, to reduce inaccuracies in the measurement to small volumes. The diluent in the calibration curve was used so that the solvent composition was as close as possible to the mobile phase composition at the time of injection. This will minimize baseline disturbance associated with injection. This is especially important in UHPLC where peak shapes can be distorted as a result of disturbance of the mobile phase composition.

Table 2: Scheme used for the creation of an eight level calibration.

Calibration Level	Concentration (μg/mL)	Volume of Standard Solution Added (mL)	Final Volume (mL)
1.	0.5	0.5*	10
2.	1	1*	10
3.	2	2*	10
4.	4	0.4	10
5.	6	0.6	10
6.	8	0.8	10
7.	10	1.0	10
8.	12	1.2	10

Calibration: The UV detector was calibrated across the range of 0.5 to 12 μg/mL, each calibration point was run in triplicate to demonstrate the precision of the system. The average coefficient of determination for a line of linear regression was 0.9965 for all 6 compounds. The calibration curves for daidzein and daidzin are pictured in Figure 3. Also in Figure 3 is the percent relative standard deviation (%RSD) for each calibration point (n=3). The precision of the system across the calibration range is excellent, the %RSD for diadzein and diadzin with an average of approximately 0.5%.

Sample preparation: Three commercially available supplements were analyzed with the method developed here. The samples are referred to as: sample 1, sample 2 and sample 3. The sample preparation used was relatively straightforward. A 0.5 gram sample of each supplement was ground with a mortar and pestle. The ground sample was extracted in 100 mL of (1:1) water: acetonitrile, in an ultrasonic bath. The sample extracts were filtered through a 0.2μm nylon filter. Following filtration, 2 mL of sample extract was diluted to 10 mL final volume in 9:1 (water:acetonitrile), this reduced the concentration of the isoflavones in the extract within the range of the calibration curve and made the diluents and mobile phase more alike.
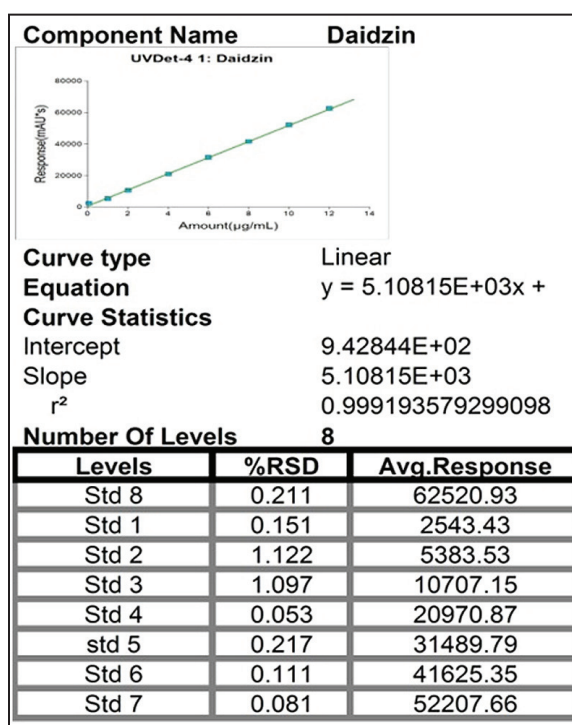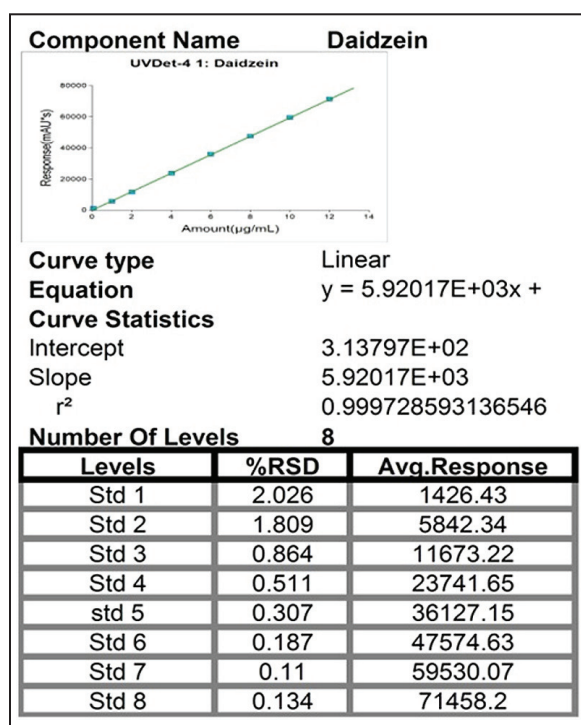

Component Name — Daidzein

UVDet-4 1: Daidzein

Curve type	Linear
Equation	y = 5.92017E+03x +
Curve Statistics	
Intercept	3.13797E+02
Slope	5.92017E+03
r²	0.999728593136546
Number Of Levels	8

Levels	%RSD	Avg.Response
Std 1	2.026	1426.43
Std 2	1.809	5842.34
Std 3	0.864	11673.22
Std 4	0.511	23741.65
std 5	0.307	36127.15
Std 6	0.187	47574.63
Std 7	0.11	59530.07
Std 8	0.134	71458.2

Component Name — Daidzin

UVDet-4 1: Daidzin

Curve type	Linear
Equation	y = 5.10815E+03x +
Curve Statistics	
Intercept	9.42844E+02
Slope	5.10815E+03
r²	0.999193579299098
Number Of Levels	8

Levels	%RSD	Avg.Response
Std 8	0.211	62520.93
Std 1	0.151	2543.43
Std 2	1.122	5383.53
Std 3	1.097	10707.15
Std 4	0.053	20970.87
std 5	0.217	31489.79
Std 6	0.111	41625.35
Std 7	0.081	52207.66

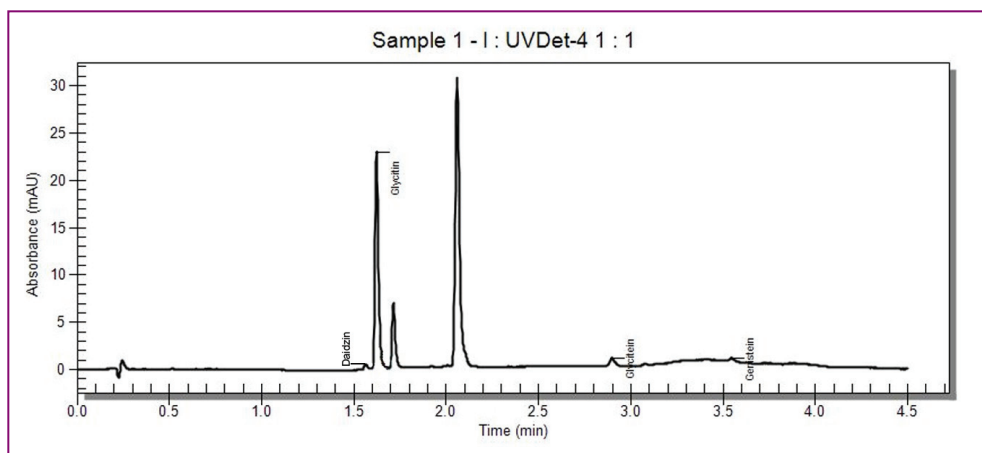*Figure 3.* Example calibration results, via Chromera CDS.

Figure 4: Example chromatogram of sample 1.

Results

Under the conditions presented here, the analytical run was 4.5 minutes long with an elution order of daidzin, glycitin, genistin, daidzein, glycitein and genistein. In similar applications performed with conventional HPLC, the analytical run time was 43 minutes. Therefore, this method has reduced the run time by 38.5 minutes, while maintaining complete resolution of all analyte peaks. The minimum resolution (critical pair) of analytes in this separation was 2.8, occurring between daidzin and glycitin.

The analysis of samples 1 and 2 resulted in detection of significant levels of isoflavones, with 49 and 52 mg of isoflavones in each sample, respectively. The label on the bottle for both samples 1 and 2 stated that each contained 55 mg of isoflavones per tablet, the determined values for each sample equate to 89% and 95% recovery. The sample analysis is summarized in Table 3. The analysis of sample 3 resulted in no detection of isoflavones; this was expected, as sample 3 was a multi-vitamin that did not list any isoflavones on its label.

Table 3. Summary of the results determined in the analysis of supplement samples for isoflavone content.

	Measured Isoflavone Content (mg)	Labeled Isoflavone Content (mg)	Percent Recovery
Sample 1	49	55	89%
Sample 2	52	55	95%
Sample 3	ND	0	n/a

Conclusions

The technique presented in this application note applies UHPLC instrumentation to the determination of isoflavones in nutraceutical supplements. A commercial reference standard was used to identify 3 isoflavones and their glycosidic conjugates by retention time. The separation used a short small particle (1.5 μm) LC column and achieved adequate resolution of all isoflavone peaks commonly found in soy materials. A multilevel calibration curve using the UV/Vis detector at 254 nm was used to quantitatively determine the amount of isoflavone in three dietary supplements. In addition to providing a precise and accurate result for the determination of isoflavones in supplements, this UHPLC application has reduced the analytical runtime by nearly 10x and eliminated nearly 40 mL of solvent use per sample. When compared to conventional HPLC, this directly translates into solvent savings of 92%.

References

1.   www.soyconnection.com, IsofavonesFactSheet.pdf

2.   http://www.isoflavones.info

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