APPLICATION NOTE

UHPLC

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Analysis of Isoflavones with the PerkinElmer Flexar FX-15 UHPLC System Equipped with a PDA Detector

Introduction

Foods from plants are complex mixtures of chemicals including both essential nutrients and biologically active non-essential nutrients, referred to as phytochemicals. Soy is known for having high concentrations of several physiologically-active phytochemicals including isoflavones, phytates, saponins, phytosterols and protease inhibitors. Isoflavones are what make the soy unique. Soy isoflavones have estrogenic activities and are structurally and functionally related to 17β-estradiol. Soybeans and soy food 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. In fact, recent data have demonstrated that isoflavones have potent antioxidant properties comparable to that of vitamin E. Research in several areas of healthcare has linked isoflavones to reducing heart disease and cancer risk, easing menopause symptoms, and improving prostate and bone health. However, a study done in 2000 at the University of Missouri showed that injections of genistein in mice caused a decrease of thymocyte by 86% as well as a decrease of humoral immunity, thus raising the possibility that a high concentration of genistein in food could cause thymic and immune abnormalities in infants.

Because of the potential health benefit of isoflavones, many soy products and isoflavone supplements are available to consumers; these fall into the category of food products called nutraceuticals. The health benefit and concern as well as the labeling requirement have created the need for analytical techniques to determine the type and amount of isoflavone in nutraceutical products.

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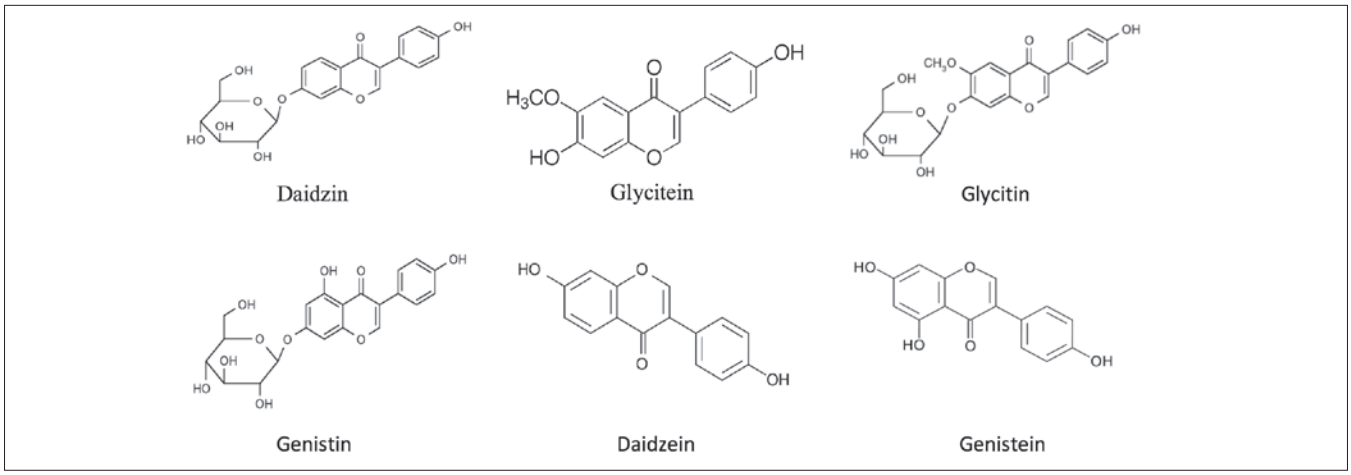

Figure 1. Molecular structure of isoflavones studied.

This application note presents a fast, robust, and sensitive reverse phase liquid chromatography method for the analysis of six widely used isoflavones (Figure 1). Method conditions and performance data including precision and linearity are presented. Two soy dietary supplement tablets are analyzed and the isoflavones type and amount are confirmed.

Experimental

A stock standard made of 0.2 mg/mL of each isoflavone was prepared by transferring about 5 mg of each into a 25 mL volumetric flask and diluting with a with 70:30 (v/v) methanol:water solution (diluent). The solution was vortexed for one minute and then sonicated for 15 minutes. For sample recovery calculation a calibration curve was determined from three points calibration of 8 µg/mL (working standard 1), 20 µg/mL (working standard 2) and 40 µg/mL (working standard 3) prepared by transferring and bringing to volume with diluent 1.0 mL of stock standard into 25, 10, and 5 mL, respectively. To further study the possible range of testing, from working standard 3, linearity samples ranging from 40 µg/mL to 0.25 µg/mL were prepared by successive dilutions.

Repeatability was studied with eight injections of the working standard 2. About 1.2 mg/mL of soy tablet A and 6 mg/mL of soy tablet B (theoretically equivalent to about 4 mg of isoflavones) were prepared in the same way as the stock standard. Samples and standard solutions were thoroughly mixed and filtered through a 0.2 µm filter prior to testing.

A PerkinElmer® Flexar® FX-15 UHPLC system fitted with a Flexar FX PDA photodiode array detector was the platform for this experiment. The separation was achieved using a PerkinElmer Brownlee™ SPP C-18, 2.7 µm, 50 x 2.1 mm column. The run time was about six minutes with a back pressure of approximately 5100 PSI (352 bar).

Results And Discussion

The U.S. Pharmacopeia (USP) compendial method is a very long and complicated gradient of 74 minutes, and the method calls for the use of a 3 mm x 250 mm column with a 5 µm particle size and a flow rate of 0.7 mL/min. By using a shorter column with smaller particle size (PerkinElmer Brownlee SPP C-18, 50 x 2.1 mm, 2.7 µm particle size) suitable for UHPLC, a six minutes run time was achieved. Prior to running samples, from one injection of the standard, the maximum wavelength for each peak was determined and the wavelength recording setting was optimized accordingly (see Figures 2 and 3).

***Table 1.* Detailed UHPLC system and chromatographic conditions.**

Autosampler:	Flexar FX UHPLC
	Setting: 50 µL Loop and 15 µL needle volume, partial loop mode, 350 µL mixer
	Injection: 2 µL; injector wash: water
PDA Detector:	Scanned from 190 – 400 nm, recording setting 255 nm
UHPLC Column:	PerkinElmer Brownlee SPP C-18, 2.7 µm, 50 x 2.1 mm Part No. N9308402 at Ambient temperature
Mobile Phase:	B: 0.05% TFA in Acetonitrile A: 0.05% TFA in Water

Time (min.)	Flow rate (mL/min.)	B %	Curve
4	0.4	10-35	1
2.5	0.4	35	1

	3 minutes equilibration after each injection (HPLC grade solvent and ACS grade reagent)
Software:	Chromera Version 3.0
Sampling Rate:	5 pts/s

PerkinElmer's Chromera® software helps in assessing the purity of each peak in the standard solution by comparing the spectra on the upslope and the down slope of the peak. Because a pure peak has matching spectra throughout the peak, a ratio of upslope/down slope absorbance greater than 1.5 could be an indication of a co-elution or lack of purity. The purities of the isoflavones in the standard solution based on their spectra ratio are presented in Figure 4 and a representative chromatogram of the soy tablets tested are presented in Figures 5 and 6.

In liquid chromatography with UV/Vis detectors peak identification is usually based on the retention time. Chromera's ability to collect and store spectra offers another way of identification by matching any peak spectrum to spectra stored in its library. This feature of Chromera adds another level of confidence in the analysis as the same relative retention time does not necessarily mean the components are the same. Confirmation of the presence of Daidzin, Glycitin, Genistin, and Glycitein, in the soy tablet sample is shown in Figure 7. In that figure, the spectra at the peak apex of each peak is compared with the spectra in the standard solution previously stored in the library. When a match is made, the name of the matching spectrum appears on each peak in question, confirming its identity.

When compared to the current USP compendia method, the analysis using a UHPLC resulted in thirteenfold reduction in chromatographic run time. Furthermore the flow rate was reduced to 0.4 mL/min. from 0.7 mL/min. called by the USP method resulting in 95% reduction in solvent usage. Thus by moving to the UHPLC method testing time and solvent usage were dramatically reduced. This is important not only because of the relatively high cost of HPLC-grade solvents, but also because far less solvent is disposed of as waste. This results in a much lower cost of ownership and a much greener laboratory operation.

Excellent method performance was achieved. From 0.25 µg/mL to 400 µg/mL method was perfectly linear with an R-squared of 1 for all six components. Precision values ranged from 0.6 – 1.1% RSD, and resolution between closest consecutive peaks were not less than 1.5. Column temperature set a 35 ˚C or 40 ˚C did not change the chromatography and the results; thus, showing an impressive robustness. Details of the method performance and results of the samples tested are presented in Table 2.

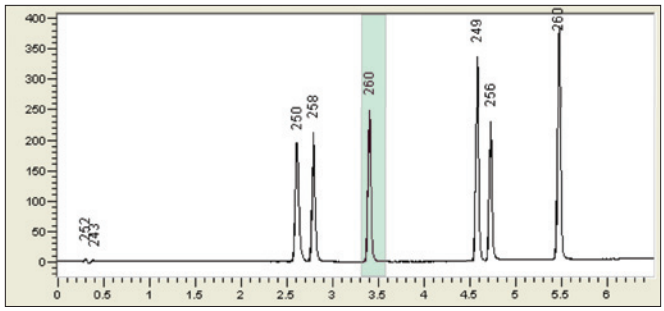

Figure 2. Chromatogram from the analysis of the standards with maximum absorbance for each peak.

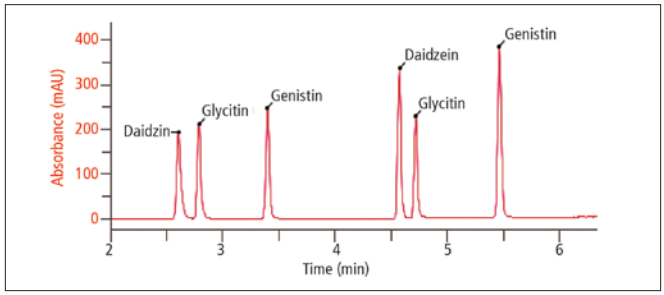

Figure 3. Chromatogram from the analysis of the standards.

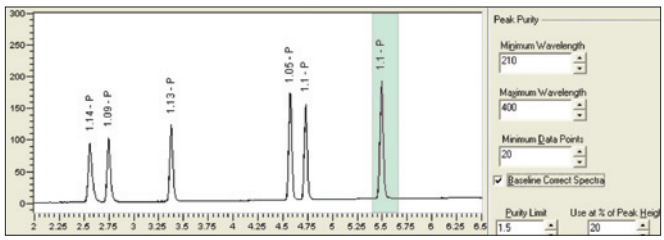

Figure 4. Ratio upslope, down slope spectra or purity of peak in the standards.

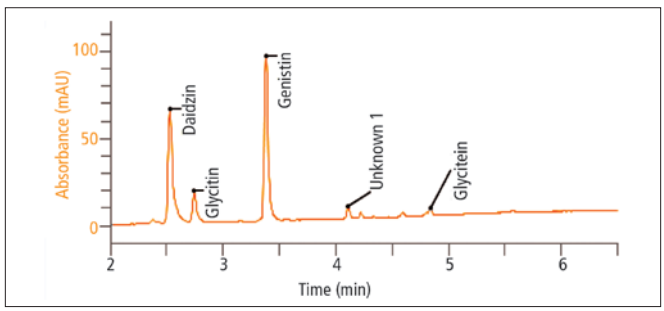

Figure 5. Chromatogram from the analysis of a soy tablet A.

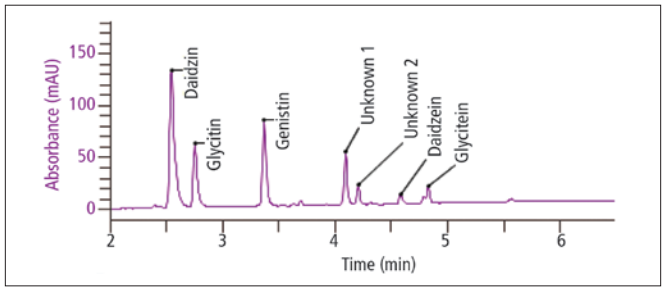

Figure 6. Chromatogram from the analysis of a soy tablet B.

Table 2. **Precision, linearity and amount in sample.**

	%RSD (n=8)	Resolution	Linearity r^2	Range (µg/mL)	Tablet A (mg/g)	Tablet B (mg/g)
Daidzin	0.6	NA	1	0.25 - 40	10.7	5.1
Glycitin	0.6	2.8	1	0.25 - 40	3.3	2.5
Genistin	0.7	NA	1	0.25 - 40	12.6	2.3
Daidzein	1.2	NA	1	0.25 - 40	ND	0.2
Glycitein	1.1	2.9	1	0.25 - 40	0.6	0.5
Genistein	1.0	NA	1	0.25 - 40	ND	ND
Total					27.2	10.6

NA: not applicable, ND: none detected

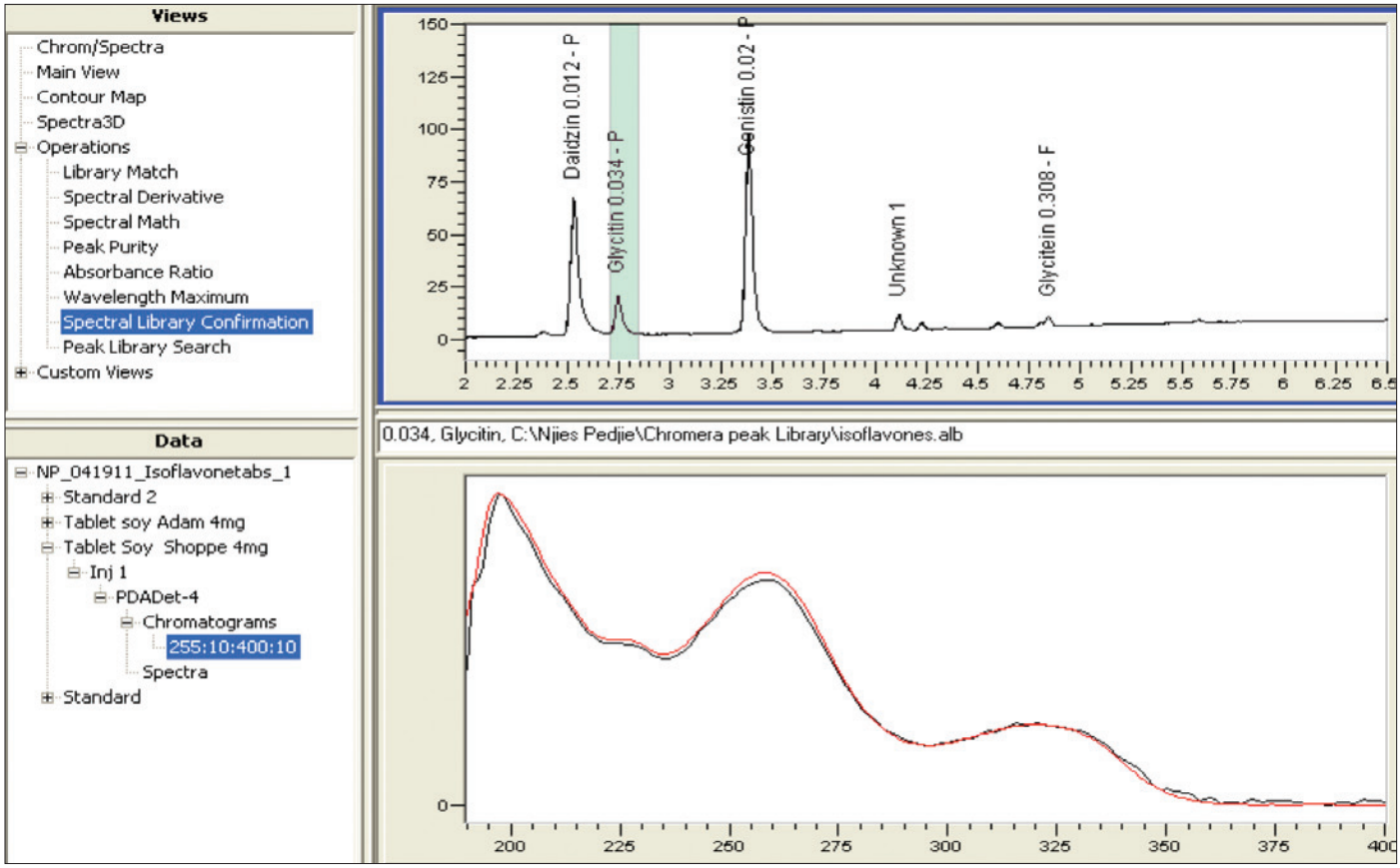

Figure 7. Peak identification in sample using the Chromera spectral library.

Conclusion

When compared to the current USP compendial method, the UHPLC analysis of isoflavones resulted in 90% or more than thirtyfold reduction in run time as well as 95% reduction in solvent usage. The PerkinElmer Flexar FX-15 UHPLC system and PerkinElmer Brownlee SPP C-18, 2.7 µm, 50 x 2.1 mm resolved all the six isoflavones with a resolution between closest consecutive peaks more than the cut off limit of 1.5. The method was shown to be linear. Total isoflavones were 27 mg/g on soy tablet A and 11 mg/g on soy tablet B, which is less than the label claim of 75 mg/g and for A and 13 mg/g for B.

PerkinElmer FX PDA detector provides rugged and accurate detection over a range of 190 nm to 700 nm, encompassing UV and visible wavelengths. PerkinElmer's Chromera software offers many data acquisition and processing features: spectral library creation, and peak purity, spectra 3D and contour maps, which are powerful tools for interrogating the information content of a 3D photodiode array chromatogram. The spectra library search function allowed the storage of standard peaks spectra that were later used for peak identification confirmation in the samples.

References

1. www.soyconnection.com, IsoflavonesFactSheet.pdf
2. www.isoflavones.info
3. 2011 USPC Official Dietary Supplement: Powdered Soy Isoflavones.
4. Yellayi S., Naaz A., Szewczykowski M.A., *et al.* (May 2002). "The phytoestrogen genistein induces thymic and immune changes: a human health concern?". *Proc. Natl. Acad. Sci. U.S.A.* 99 (11): 7616–21. http://www.pnas.org/cgi/content/full/99/11/7616.
5. Prabhu, Padmaja, PerkinElmer, Inc. Throughput and Reduced Solvent Consumption for the Determination of Isoflavones by UHPLC.

Note: This application is subject to change without prior notice.

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