UHPLC-UV Determination of Isoflavones in Soy Seed

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Abstract

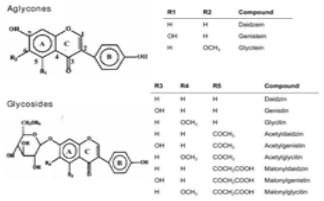
Isoflavones are extracted from ground soybean seed with a mixture of methanol and water. After being hydrolyzed with NaOH and neutralized with acetic acid, the acetyl and malonyl isoflavones are transformed to glycosides. A UHPLC/UV assay was developed to quantify the glycoside and aglycone forms including daidzin, daidzein, glycitein, glycitein, genistin and genistein using calibration curves of the six external standards. The linearity of this method is 0.1 µg/mL – 100 µg/mL, or 4.8 mg/Kg Dry Basis (DB) – 480 mg/Kg DB. Excellent accuracy and precision were obtained during method validation. At least forty samples can be analyzed per day using this method. The UHPLC run in this method takes 12.5 minutes which is much shorter than the HPLC test (44.5 min) of the AOAC method [1].

Aim

Develop and validate a high throughput method to determine isoflavones in soy matrices.

Background

Isoflavones are a group of phytoestrogens. In plant , they most often occur as glycosides or their respective malonates or acetyl conjugates. The most abundance isoflavones in soy seeds are the glycosides of daidzein, genistein, and glycitein.



R1	R2	Compound
H	H	Daidzein
OH	H	Genistein
H	OCH_3	Glycitein

R3	R4	R5	Compound
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	Acetyldaidzin
OH	H	$COCH_3$	Acetylgenistin
H	OCH_3	$COCH_3$	Acetylglycitin
H	H	$COCH_2COOH$	Malonyldaidzin
OH	H	$COCH_2COOH$	Malonylgenistin
H	OCH_3	$COCH_2COOH$	Malonylglycitin

Figure 1. Structure of the isoflavones in soy seeds.[2]

Methods

Sample Preparation [1, 3-5]

- Frozen soy seed samples were ground to pass 20 mesh.
- Ground samples were extracted twice with 6 mL of methanol: de-ionized water (80:20).
- After centrifuge, the supernatants were combined.
- Add 0.75 mL NaOH to the extracts for 15 min hydrolysis.
- Add 100 µL acetic acid to neutralize the samples.
- Dilute the samples.
- Filter and inject the samples.

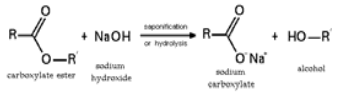


Figure 2. [6] Hydrolysis of the isoflavone esters.

UHPLC Analysis

- Waters ACQUITY UPLC®
- Column: ACQUITY HSS C18, 2.1 x 150 mm, 1.8 µm; Column Manager: 42ºC; Sample Manager: 4ºC.
- Calibration: 0.1-100 µg/mL for daidzin, glycitin and genistin; 0.1-10 µg/mL for daidzein, glycitein and genistein.
- Injection: the 50 µg/mL working standard is injected at both 2 and 4 µL. All other working standards and samples are injected at 2 µL. Loop option: Partial Loop with Needle Overfill.

Time (min)	Flow (mL/min)	%A	%B	Curve
0	0.4	95	5	
1.0	0.4	90	10	6
6.0	0.4	70	30	7
8.0	0.4	60	40	6
9.0	0.4	55	45	7
9.5	0.4	0	100	6
10.5	0.4	0	100	6
11.0	0.4	95	5	6
12.5	0.4	95	5	6

Table 1. UHPLC gradient. A = 0.5% formic acid in de-ionized water. B = 0.5% formic acid in acetonitrile.

Results

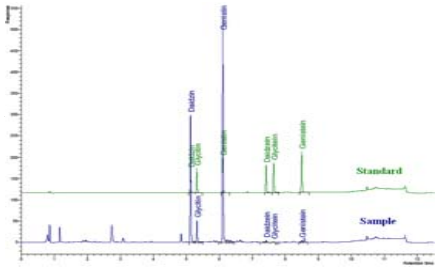


Figure 3. Chromatograms of a multi-isoflavone standard and a soy seed sample.

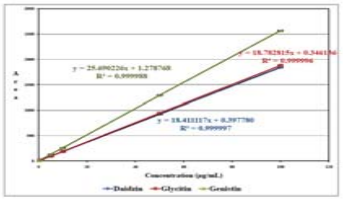


Figure 4. Linearity of daidzin, glycitin, and genistin.

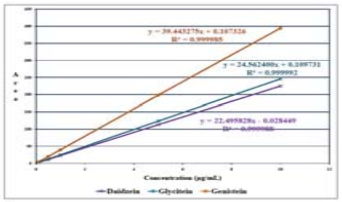


Figure 5. Linearity of daidzein, glycitein, and genistein.

	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
Content (%DB)	0.153	0.0238	0.200	0.00201	<0.0004	0.00111
% CV	2.86	8.70	1.54	3.97	1.76*	3.66
Recovery (%)			100		105	

*Table 2. Validation study of soy seed. %DB = % Dry Base. Moisture values were tested according to AOAC 925.09. The calculations were based on eight soy seed samples and four spikes. Spike: Genistin 1x; Glycitein 10✦(lowest standard). *Calculated using the spiked samples.*

Discussion & Conclusion

Sample Preparation

- The downscaled procedure saves both time and solvent.[1]
- The poor reproducibility of filter paper[7] was corrected by PTFE syringe filters or mini uniprep filter vials.
- Recoveries of glycitein (only dissolves in DMSO) and genistin (has the highest content in soy seed) are presented.
- Satisfactory results were obtained on accuracy and precision.

UHPLC Separation and Quantification

- Improved efficiency allows speed separation, compared with HPLC.
- Column conditioning or equilibrium is important for the best reproducibility.

Method Summary

Accurate; precise; reproducible; high-throughput.

	EPL method	AOAC 2001.10
Sample Preparation Time (hour)	5 ~ 6	14 ~ 16
Containers needed*	80	200
Containers to be washed*	0	160
Liquid Chromatography (LC) Analysis Time (min)	575	2000
Extraction Solvent (mL)	480	2000
Total LC Mobile Phase (mL)	230	800 or 3000**

*Table 3. Comparison of the EPL method with AOAC 2001.10. The calculations were based on the isoflavone analysis of 40 ground soy seed samples. *All the filtration needs of AOAC 2001.10 were not included in the calculation. **HPLC column of 2.1 mm or 4.6 mm ID.*

Literature Cited

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