FAST ANALYSIS OF ISOFLAVONES IN DIETARY SUPLEMENTS

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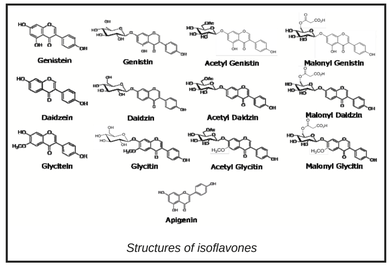
INTRODUCTION

- **Twelve major isoflavones were found in plants of soy (*Glycine max*), red clover (*Trifolium pretense*), and Kudzu (*Pueraria lobata*). These hormone-like compounds are often used in remedies to reduce menopausal and post-menopausal symptoms.**
- **Standard methods for isoflavones in dietary supplements use reversed-phase LC with C_{18} columns and ultraviolet and visible light (UV-Vis) spectroscopy for separation and quantitation. The chromatographic run time is over 70 minutes long.**
- **This work demonstrates the transfer of the USP isoflavone method onto an ACQUITY Arc™ UHPLC system and an ACQUITY UPLC H-Class system. The benefits of Mass Spectrometry in method transfer and sample analysis are highlighted.**

GOAL

- **Transfer the USP isoflavone HPLC method to faster UHPLC and UPLC methods to achieve higher sample analysis throughputs**



Structures of isoflavones

METHODS

Sample preparation:

Mix finely powder samples (NMT 5 mg of isoflavones), IS solution, 10 mL acetonitrile, and 6.0 mL water

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Shake for 60 min.

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Add 8.5 mL of water, mix, and centrifuge

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Filter the supernatant through a 0.45 µm PVDF membrane

UHPLC conditions:

System:	ACQUITY Arc system with 2998 PDA and ACQUITY QDa Detector (Performance)
Software:	Empower 3
Column:	CORTECS® C18 3.0 x 100 mm, 2.7µm (p/n 186007372)
Column Temp.:	30°C
Mobile phase A:	Water with 0.1% Formic acid
Mobile phase B:	Acetonitrile with 0.1% Formic acid
Inj Vol.:	2.0 µL
Run time:	18.0 min
UV detection:	260 nm

Elution Gradient:

	Time (Min)	Flow Rate (mL/Min)	%A	Curve
1	Initial	1.08	90	6
2	14.40	1.08	70	6
3	14.50	1.08	10	6
4	15.20	1.08	10	6
5	15.40	1.08	90	6
6	18.00	1.08	90	6

UPLC conditions:

System:	ACQUITY UPLC H-Class system with ACQUITY PDA and ACQUITY QDa Detector (Performance)
Software:	Empower 3
Column:	CORTECS® UPLC C18 2.1 x 75 mm, 1.6µm (p/n 186007094)
Column Temp.:	30°C
Mobile phase A:	Water with 0.1% Formic acid
Mobile phase B:	ACN with 0.1% Formic acid
Inj Vol.:	0.7 µL
Run time:	8.10 min

Elution Gradient:

	Time (Min)	Flow Rate (mL/Min)	%A	Curve
1	Initial	0.55	90	6
2	6.48	0.55	70	6
3	6.53	0.55	10	6
4	6.84	0.55	10	6
5	6.93	0.55	90	6
6	8.10	0.55	90	6

QDa Parameters:

Polarity:	ES+
Capillary (kV):	0.8
Cone (V):	15
Probe Temperature (°C):	600

Table 1. Masses of isoflavone molecular ions

Analyte	SIR Mass (Daltons)
Daidzin	417.1
Glycitin	447.0
Genistin	433.1
Daidzein	254.9
Glycitein	285.0
Genistein	270.9
Apigenin (IS)	270.9
Malonyl Daidzin	503.4
Malonyl Glycitin	533.1
Acetyl Daidzin	459.1
Acetyl Glycitin	489.0
Malonyl Genistin	519.0
Acetyl Genistin	475.1

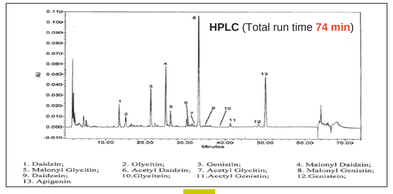
RESULTS

1) USP method transfer and optimization

The USP method (isoflavones powder extract) [1] was transferred to an ACQUITY Arc system with a CORTECS C18 column (2.7 µm 3 x 100 mm). The USP method's 74 minutes long mobile phase elution program was converted to a 18 minutes program using Waters UPLC Column Calculator. The mobile phase additive was changed from phosphoric acid (0.05%) to formic acid (0.1%), which is a mass spectrometry friendly additive. The column temperature was optimized to 30°C to meet the USP suitability criteria on peak resolution.

This USP method was also transferred to an ACQUITY UPLC H-Class system with a CORTECS UPLC C18 (1.6µm, 2.1 x 75 mm). The total run time in UPLC was shortened to 8.1 min, which is about one tenth of the HPLC run time.

Figure 1. The reference HPLC chromatogram for USP defatted powder soy RS. The run time is 74 min. This chromatogram was provided by USP.



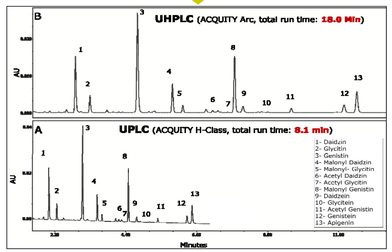


Figure 2. Chromatograms of USP defatted powder soy RS in A) UPLC and B) UHPLC conditions. The peak assignment were confirmed by mass spectrometry data that obtained from a Waters QDa mass detector.

2) Method performance

Table 2 shows the UV calibration results for the UPLC method. The square of the correlation coefficients (R^2) between the responses (peak area ratio) and the standard concentrations (ppm) for all compounds were better than 0.998. The relative standard deviation (RSD) in retention time for all compounds were less than 0.27%.

Table 3 shows the isoflavone results for the NIST 3238 SRM and a comparison to its certified and reference values. Relative difference of less than 14% was obtained for the genistin, glycitin, daidzin, genistein, and glycitein. The accuracy for the daidzein, genistein, and glycitein was also evaluated by a spiking experiment (Table 4). Recovery of 95% to 102% were obtained for these analytes.

Table 2. *Isoflavones retention times and their relative standard deviation, calibration equations, R^2, and linear ranges for the UPLC method.*

Analyte	RT Min	RT RSD (%)	Equation	R^2	Range (ppm)
Daidzin	2.03	0.21	$y = 2.12 \times 10^{-3} X + 3.93 \times 10^{-3}$	0.999	0.075–10
Glycitin	2.27	0.27	$y = 2.45 \times 10^{-3} X + 4.54 \times 10^{-3}$	0.998	0.075–10
Genistin	3.07	0.21	$y = 4.56 \times 10^{-3} X + 5.30 \times 10^{-3}$	0.998	0.075–10
Daidzein	4.76	0.11	$y = 2.61 \times 10^{-3} X + 4.50 \times 10^{-3}$	0.998	0.075–10
Glycitein	5.15	0.14	$y = 4.97 \times 10^{-3} X + 5.69 \times 10^{-3}$	0.998	0.05–10
Genistein	6.32	0.09	$y = 4.88 \times 10^{-3} X + 4.87 \times 10^{-3}$	0.998	0.05–10

Table 3. Comparison of determined isoflavone values to the certified and reference values of NIST 3238 SRM (UPLC method).

	NIST value (mg/kg)	Determined value (mg/kg)	Rel. difference
Genistin	12700±530	11432	-10%
Glycitin	3760±180	3669	-2%
Daidzin	13400±2400	15102	13%
Daidzein	241±5	262	9%
Genistein	108±10	93	14%
Glycitein	211±5	190	10%

Table 4. Recovery results from a spiking experiment (UPLC method).

	Original value (mg/kg)	Spiked level (mg/kg)	Determined value (mg/kg)	Recovery (%)
Daidzein	317	499	827	102%
Genistein	159	499	634	95%
Glycitein	165	499	665	100%

ANALYSIS OF ISOFLAVONES IN DIETARY SUPPLEMENTS

The isoflavone contents in three isoflavone dietary supplement samples were measured by this 8-minute UPLC-UV method. The sample forms included tablets, capsules, and powder. The USP calibration and quantitation protocols were followed in the data processing. The conversion factors for the acetyl and malonyl derivatives that were specified in the USP HPLC-UV method were used in this analysis. Table 6 shows the determined individual and total isoflavone contents, as well as the total isoflavone contents on labels. For an easy comparison, the label values were converted to the same concentration unit (mg/kg). Two of the three samples (C and D) showed good agreement between the determined values and their label values, while one sample (B) had much less measured total isoflavone content than its label value. The reason for this low total isoflavone content in sample B compared to its label claim is unknown.

3) Analysis of isoflavones in dietary supplements

The isoflavone content in three isoflavone dietary supplement samples were measured by this 8-minute UPLC-UV method. The sample forms included tablets, capsules, and powder. The USP calibration and quantitation protocols [1] were followed in the data processing. The

Sample (mg/kg)	B Mean	B %RSD	C Mean	C %RSD	D Mean	D %RSD
Daidzin	3095	1.0	12837	0.2	310	4.3
Glycitin	617	2.0	4792	0.2	0	
Genistin	271	3.2	1766	0.6	664	1.1
Malonyl daidzin	97	7.8	655	2.0	0	
Acetyl daidzin	1634	1.7	7391	0.3	0	
Acetyl glycitin	390	1.5	2202	0.04	0	
Malonyl genistin	0		0		221	1.2
Daidzein	6418	0.8	407	0.7	0	
Glycitein	0		179	1.4	0	
Acetyl genistin	162	2.1	1042	0.4	0	
Genistein	0		0		0	
Total isoflavones	12,684		31,270		1,195	
Label claim value	>31,250		>25,000		867–2,600	

conversion factors for the acetyl and malonyl derivatives that were specified in the USP HPLC-UV method were used in this analysis. Table 5 shows the determined individual and total isoflavone contents, as well as the total isoflavone contents on labels. For an easy comparison, the label values were converted to the same concentration unit (mg/kg). Two of the three samples (C and D) showed good agreement between the determined values and their label values, while one sample (B) had much less measured total isoflavone content than its label value. The reason for this low total isoflavone content in sample B compared to its label claim is unknown.

Table 5. UPLC-UV method determined Isoflavone content in dietary supplements and their label claim values.

4) Mass *detection helps to reduce co-eluting errors in UV detection*

It is important to note that due to the complex sample matrix, co-elution often occurs in isoflavones analysis. Figure 3 shows the UV peak fronting for the daidzein in NIST 3238 SRM. The quantitation error that is caused by

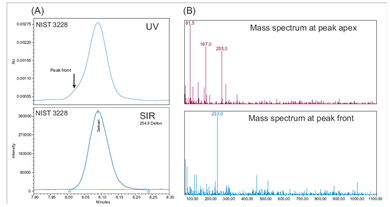


Figure 3. (A) Comparison of the UV (top) and the SIR (bottom) peaks for the daidzein in NIST 3238 SRM. A small peak fronting is observed in the UV, but not in the SIR peaks. (B) Comparison of the mass spectra (background subtracted) from the MS scan channel at daidzein peak's apex (top) and the peak front (bottom) for NIST 3238 SRM. The mass spectrum at peak front is different from the spectrum at peak apex. This confirms that the peak front is caused by a compound other than the daidzein.

CONCLUSION

The USP isoflavone method was successfully transferred to UHPLC (on ACQUITY Arc system) and to UPLC (on ACQUITY H-Class system) systems

The total run time is shortened from 74 min (HPLC) to 18 min (UHPLC) and to 8.1 min (UPLC)

Analysis results for the NIST reference material showed good agreement between the reference values and the experiment results

Mass detection is extremely useful in the analysis of complex samples. Its highly selective detection can reduce interferences, and the mass spectrum data is invaluable in troubleshooting

Dietary supplement samples have been analyzed by the UHPLC and the UPLC methods. One sample was found that its total isoflavone content was much lower than its label claim

References

1. USP Monograph. Powdered Soy Isoflavones Extract, USP39–NF34 S1 [6841], The United States Pharmacopeial Convention.
2. L. X. Zhang, C. Q. Burdette, M. M. Phillips, C. A. Rimmer, and R. K Marcus, Determination of isofalvone content in SRM 3238 using liquid chromatography-particle beam/electron ionization mass spectrometry, J AOA Int. 2015; 98(6): 1483 -1490

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