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Determination of Soy Isoflavones in Foods and Dietary Supplements by UPLC

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APPLICATION BENEFITS

- Accurate determination of soy isoflavone content in foods and dietary supplements
- Alternate selectivity to traditional C_{18} methods
- Significant time savings relative to currently accepted methods

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY® SQD System

XSelect™ HSS Cyano, XSelect HSS Cyano ***XP***, and ACQUITY UPLC HSS Cyano Columns

ACQUITY UPLC Columns Calculator

GHP Acrodisc® Minispike Filters

KEY WORDS

Soy, isoflavone, UPLC, XP, infant formula, method development, method transfer

INTRODUCTION

Isoflavones are a class of plant-derived compounds, produced almost exclusively by members of the *Fabaceae family*, that have been shown to have estrogenic activity in mammals. The major source of isoflavones in the human diet comes from soybeans, in which genistein and daidzein are the predominant components. Isoflavones remain the subject of many scientific studies with accepted methods established by organizations such as AOAC[1] and USP[2], utilizing traditional reversed-phase C_{18} columns. Recently, the National Institute of Standards and Technology (NIST) has developed a suite of soy-based candidate Standard Reference Materials (cSRMs) for the certification of soy isoflavones in foods and dietary supplements. For the analysis of those standards, they have developed a method utilizing a 60-minute gradient on a 5 µm cyano column which enables the separation of the soy isoflavones from other components not resolved using the standard C_{18} methods.[3] Using these conditions, they have demonstrated resolution of the three main soy isoflavones and their glycosides (structures shown in Figure 1), as well as the acetyl and malonyl conjugates of the glycosides. Since the conjugates are somewhat unstable in solution, they have added an additional sample preparation step to hydrolyze these conjugates to their associated glycoside, providing a more accurate, reproducible determination of total isoflavone content. Optimization of this method utilizing the XSelect HSS Cyano ***XP*** 2.5 µm Column provides a significant reduction in analysis time by traditional HPLC. Further optimization of this method to UPLC® with the ACQUITY UPLC HSS Cyano 1.8 µm Column provides additional savings in time, while maintaining the resolution for accurate determination of soy isoflavones.

EXPERIMENTAL

UPLC Conditions

System:	ACQUITY SQD with PDA detector
Column:	ACQUITY UPLC HSS Cyano, 2.1 x 50 mm, 1.8 µm, part number 186005986
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile
Column Temp.:	30 °C
Gradient:	10% (B) for 0.36 min, 10-30% (B) in 3.6 min, hold at 30% (B) for 0.36 min, re-equilibrate at 10% (B) for 1.8 min between injections
Flow Rate:	0.58 mL/min
Detection:	UV at 260 nm
Injection Volume:	3 µL
Strong Needle Wash:	50/50 acetonitrile/water
Weak Needle Wash:	10/90 acetonitrile/water

These UPLC conditions were scaled directly from the 5 µm HPLC method using the ACQUITY UPLC Columns Calculator. The calculator can be used to scale these conditions back to the HPLC conditions, for both the 5 µm and 2.5 µm materials.

MS Conditions

MS System:	Waters SQD
Ionization Mode:	ESI positive
Acquisition Range:	Single Ion Recording (SIR)
Capillary Voltage:	3.19 kV
Cone Voltage:	50 V
Desolvation Gas:	600 L/hr
Cone Gas:	0 L/hr
Source Temp.:	100 °C
Desolvation Temp.:	350 °C

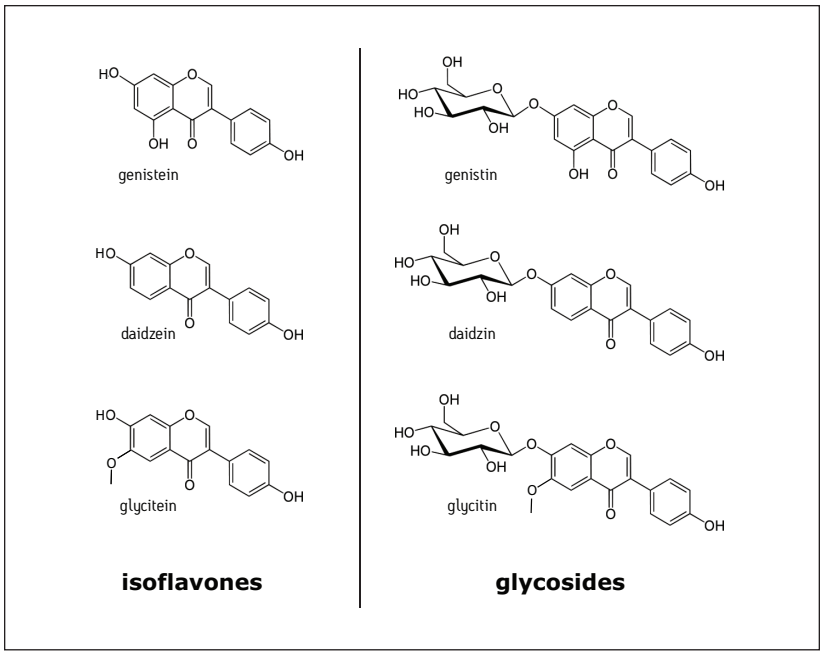


Figure 1. Structures of the three main soy isoflavones (genistein, daidzein, and glycitein) and their corresponding glycosides (genistin, daidzin, and glycitin).

SAMPLE PREPARATION

Standard Solution: Prepared from daidzin (25 ppm), glycitin (25 ppm), genistin (15 ppm), daidzein (25 ppm), glycitein (25 ppm), and genistein (15 ppm) using 10/90 acetonitrile/water diluent.

Samples:

Candidate Standard Reference Materials were obtained from the National Institute of Standards and Technology. Each sample was weighed into 12 ml centrifuge tubes (Table 1). For each tube, 4 mL of 80/20 methanol/water was added followed by sonication for 1 hour. Tubes were centrifuged for 2 minutes at 3000 rpm. A 2 mL aliquot of supernatant was collected from each tube and filtered using a 0.45 µm GHP syringe filter prior to analysis. The remainder of the sample in each tube was hydrolyzed using 150 µL of 2N sodium hydroxide. After mixing for 10 minutes, the solutions were neutralized with 50 µL of glacial acetic acid. The sample was again centrifuged for 5 minutes at 3000 rpm, with the collected supernatant filtered using a 0.45 µm GHP syringe filter prior to analysis.

Table 1. Samples used for analysis

	Weight
Soy flour (cSRM)	104.2 mg
Soy tablet (cSRM)	111.9 mg
Soy protein isolate (cSRM)	365.6 mg
Soy protein concentrate (cSRM)	973.7 mg
Soy-based infant formula (commercially-available)	1119.2 mg

RESULTS AND DISCUSSION

Based on the method presented by NIST, we developed a method using the soy standard solution with an XSelect HSS Cyano, 4.6 x 150 mm, 5 µm Column, resulting in chromatography with excellent resolution of the soy isoflavones and glycosides. To maximize productivity of the HPLC system, this method was transferred, using the ACQUITY UPLC Columns Calculator, to an XSelect HSS Cyano ***XP***, 4.6 x 75 mm, 2.5 µm Column. This resulted in an HPLC method with a 76% reduction in run time, relative to the 5 µm column. To achieve the greatest benefit, the method was then transferred to UPLC using an ACQUITY UPLC HSS Cyano, 2.1 x 50 mm, 1.8 µm Column. Figure 2 shows the chromatography of the isoflavone standards under HPLC and UPLC conditions. These chromatograms demonstrate the ability to scale between different column configurations by maintaining the same ratio of column length to particle size (L/d_p), resulting in similar chromatography but with a significant decrease in analysis time (~88% decrease in run time for the UPLC column relative to the 5 µm HPLC column).

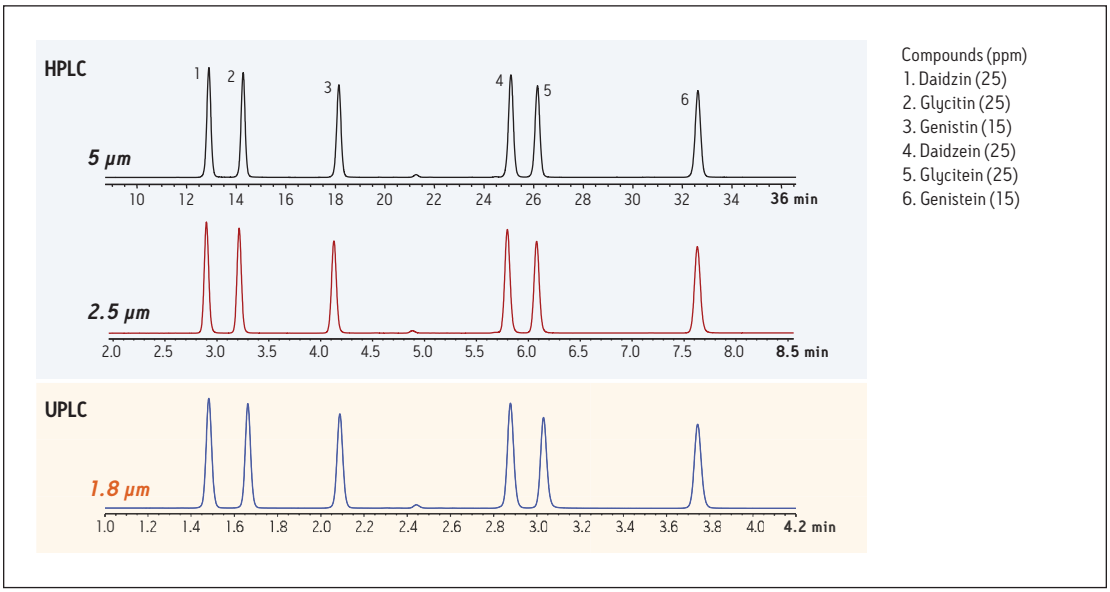


*Figure 2. HPLC and UPLC separations (UV) of soy isoflavone standards on cyano columns: XSelect HSS Cyano, 4.6 x 150 mm, 5 µm (top); XSelect HSS Cyano **XP**, 4.6 x 75 mm, 2.5 µm (middle); and ACQUITY UPLC HSS Cyano, 2.1 x 50 mm, 1.8 µm (bottom). The gradient profiles, flow rates, and injection volumes were scaled for each method using the ACQUITY UPLC Columns Calculator. The flow rates were 1.0 mL/min, 2.0 mL/min, and 0.58 mL/min, respectively.*

The UPLC method was used to analyze each of the candidate SRM extracts, both before and after hydrolysis of the glycoside conjugates (Figure 3). The identity of the peaks in the UV chromatograms were assigned based on retention times and m/z values from the LC/MS analyses (Soy Flour cSRM example shown in Figure 4). The disappearance of the glycoside conjugate peaks after sample treatment with sodium hydroxide confirms the hydrolysis of these conjugates was successful and complete, which allows for simplified determination of the total isoflavone content.

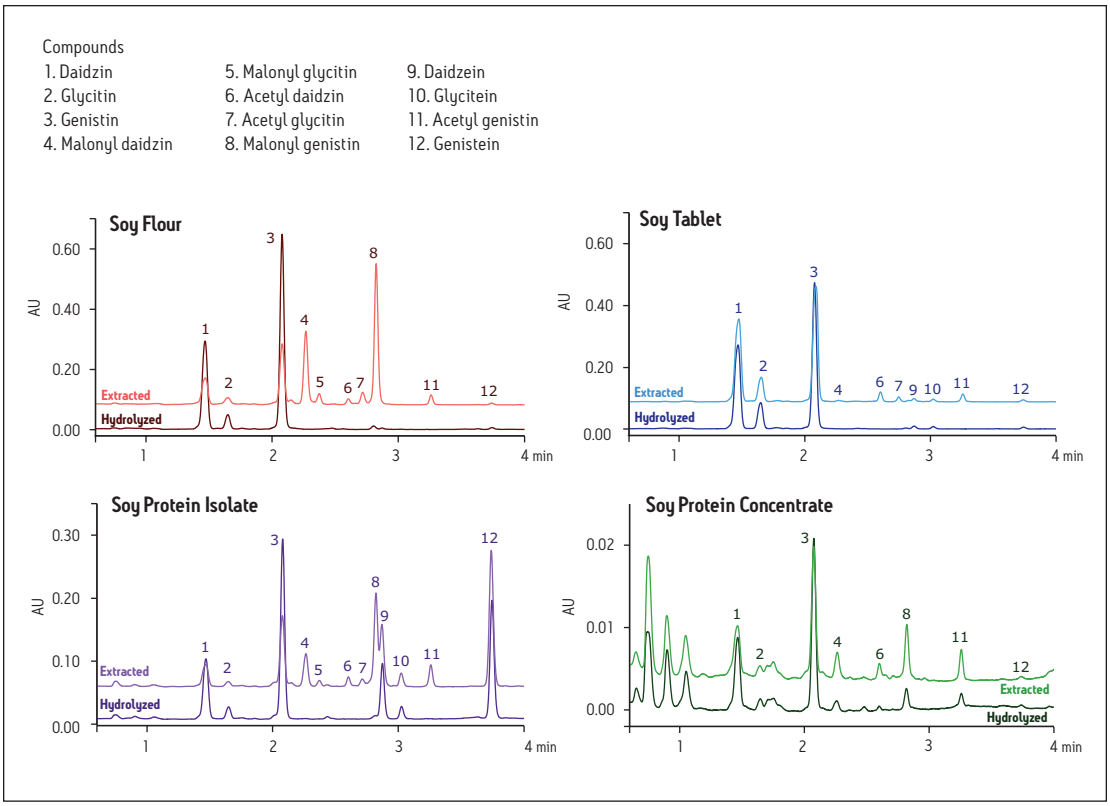
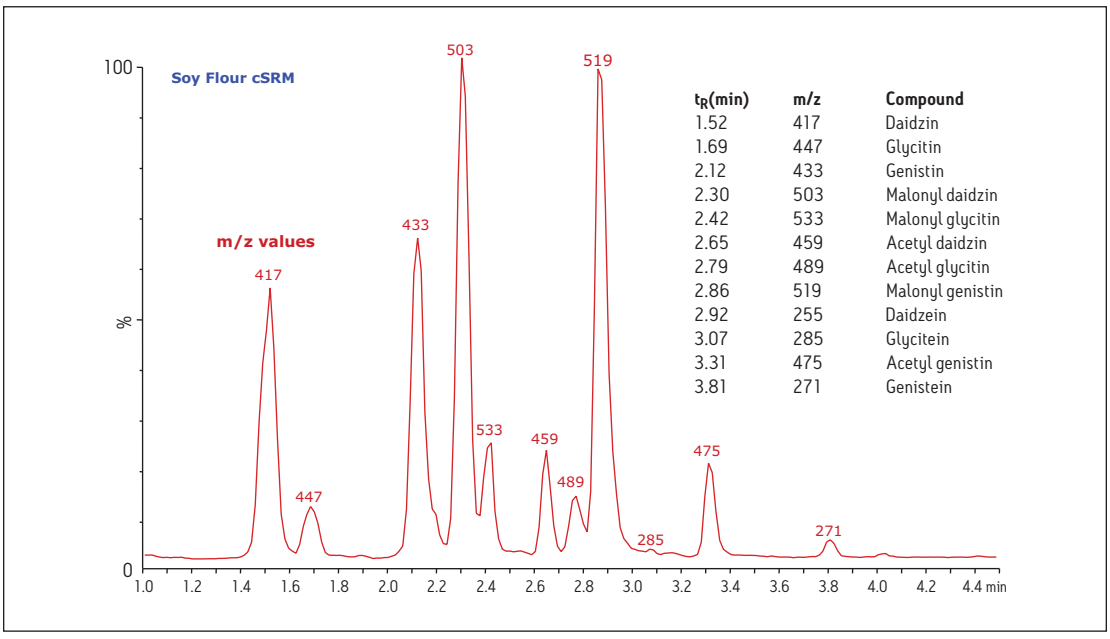


Figure 3. UPLC analysis of extracted (top chromatograms) and hydrolyzed (bottom chromatograms) candidate Standard Reference Materials (cSRMs).



t_R(min)	m/z	Compound
1.52	417	Daidzin
1.69	447	Glycitin
2.12	433	Genistin
2.30	503	Malonyl daidzin
2.42	533	Malonyl glycitin
2.65	459	Acetyl daidzin
2.79	489	Acetyl glycitin
2.86	519	Malonyl genistin
2.92	255	Daidzein
3.07	285	Glycitein
3.31	475	Acetyl genistin
3.81	271	Genistein

Figure 4. ESI+ LC/MS confirmation of peak identity for the Soy Flour cSRM, using single ion recording (SIR).

Because of their biological activity and the pervasiveness of soy-based food products, isoflavones have been the focus of many nutritional studies. Some studies advocate the nutritional benefit of isoflavones, asserting that consumption of soy products leads to decreased menopausal symptoms and lowers the risk of certain cancers.[4] Other researchers have reached alternate conclusions, with one such study of the isoflavone genistin on mice resulting in significant thymic and immune abnormalities, raising concerns for the use of soy-based infant formulas.[5] Because of these concerns, understanding the isoflavone profiles of food products is of critical interest. Using the current method, a sample of commercially-available soy-based infant formula was prepared and analyzed by both LC/UV and LC/MS. Analysis of the extracted sample revealed, in addition to the isoflavones and their glycosides, only a minor concentration of the malonyl genistin conjugate. After hydrolysis, all evidence of the conjugate is gone, revealing only the isoflavones and their glycosides (Figure 5). Using this method, the concentrations of soy isoflavones can be evaluated and monitored, facilitating fundamental research, as well as the quality control of food and nutraceutical consumer products.

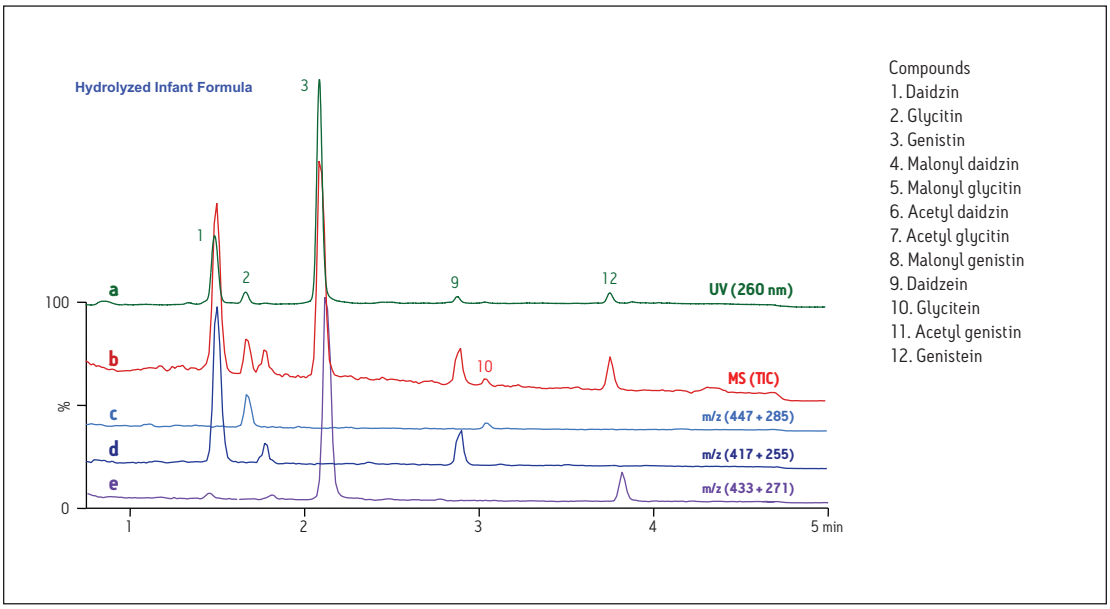


Figure 5. UPLC analysis of isoflavones in a commercially-available, soy-based infant formula (after hydrolysis); a) UV [260 nm], b) MS - Total Ion Chromatogram [TIC], c) extracted ion chromatogram for m/z 447 + 285 [glycitin and glycitein], d) extracted ion chromatogram for m/z 417 + 255 [daidzin and daidzein], and e) extracted ion chromatogram for m/z 433 + 271 [genistin and genistein].

CONCLUSIONS

Analysis of soy isoflavones using the HSS Cyano stationary phase offers complimentary selectivity to traditional C_{18} based methods. Hydrolysis of the glycoside conjugates simplifies the resulting chromatography and the determination of total isoflavone content of a sample. The availability of the HSS Cyano phase in 5-, 3.5-, 2.5-, and 1.7-µm particle sizes facilitates scaling between different instrument platforms (HPLC and UPLC). Method conditions can be easily scaled for different column configurations using the ACQUITY UPLC Columns Calculator. Scaling methods to utilize the XSelect HSS Cyano ***XP*** 2.5 µm Columns maximizes productivity with existing HPLC instrumentation. Additional method transfer to UPLC conditions offers the greatest savings in time and resources.

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