Quantitative Determination of Coumestrol in Soybean Seed by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

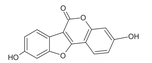
Coumestrol is a common phytoestrogen found in soybean seed. Soybeans contain several poylphenolic compounds known to possess weak estrogenic, anti-estrogenic and hypocholesterolemic effects in mammals. The physiological effects include adverse reproduction in livestock fed large amounts of soy meal (1), but also include potential beneficial anti-carcinogenic action in humans (2). Among the most common of the soybean phytoestrogens are the isoflavones daidzein, glycitein and genestein, and their conjugated forms: glucoside, acetylglucoside or malonylglucoside. Also included in this class of polyphenols is coumestrol. It is known that coumestrol concentrations in whole soybeans are several orders of magnitude less than the other isoflavones. However, it has been reported that coumestrol possesses bioactivity that is 30-100 times greater than the more prevalent soybean phytoestrogens (3). Reported concentrations of coumestrol in whole soybeans range from 0.05 (4) to 1.2 µg/g (ppm) (5) on a fresh weight basis. A new procedure for analysis of soybean seed utilizing ultra performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS) was developed and validated. The UPLC column selected for the assay, a 1.7µm, 2.1 x 50 mm Bridged Ethylene Hybrid (BEH) C18, improved resolution, sensitivity and speed. The current procedure provided a UPLC run time of only 3.5 minutes per injection. The HPLC-MS/MS assay precision was 13% (relative standard deviation). Spiking recovery values ranged from 72-120%. The method limit of quantitation (LOQ) was 0.1 ppm and the limit of detection (LOD) was 0.03 ppm.

Objective

To validate an analytical method for the determination of coumestrol in soybean seed.

Chemicals and Reagents

The chemical structure of coumestrol:



- Coumestrol analytical standard, >99%, was purchased from Sigma Chemical Company (St. Louis, MO).
- Acetonitrile (ACN) was purchased from Columbus Chemical Industries (Columbus, WI) and was HPLC grade.
- Reagent alcohol, >90% ethanol, was purchased from Honeywell Burdick & Jackson® (Morristown, NJ) and was HPLC grade.
- Concentrated hydrochloric acid (HCl) was purchased from VWR (Radnor, PA), a distributor of BDH ARISTAR® high purity acids, and was ACS grade.
- Formic acid, 88%, was purchased from Fisher Scientific (Fair Lawn, NJ) and was ACS grade. Dimethyl sulfoxide
- (DMSO) was purchased from Sigma Chemical Company and was capillary GC grade.
- ACS grade 2,6-di-tert-butyl-4-methylphenol (BHT) was purchased from Sigma Chemical Company.
- Deionized (DI) water was obtained from a Barnstead (Dubuque, IA) NANOpure® water system.

Equipment

- Balances, 0.1 and 1 mg accuracy-Mettler-Toledo, Models XP6035 and XP204, Columbus, OH
- Customized Glass Tubes of at least 20 mL volume, about 155 mm x 10 mm, with threaded Teflon plugs and rubber O-rings
- Graduated Cylinders
- UPLC autosampler vials-Waters Corporation Milford, MA
- UPLC Column, Waters BEH C18, 1.7 µm, 2.1 x 50 mm or equivalent -Waters Corporation Milford, MA
- UPLC, Waters Acquity-Waters Corporation Milford, MA
- Mass Spectrometer Detector, Waters TQ

Equipment

- Oven capable of maintaining a temperature of 108-llic
- Scintillation vials
- Syringe filters, Teflon (PTFE), 0.45 µm x 25 mm VWR, PALL, Batavia, IL
- Volumetric flasks, Class A
- Vortex mixer
- Retsch Centrifugal Grinder with 0.75mm screen, Hahn, Germany

Test Matrices

The method was validated using a commercial variety of soybean seed (*Glycine max*). The sample matrix was ground using a Retsch Centrifugal grinder. Ground seed was stored in vacuum packed plastic bags at approximately -50°C when not needed for laboratory experiments.

Procedures

- The extraction and hydrolysis procedure was based on work by Franke et al (6).
- Subsamples (0.500 gram) of ground soybean seed were weighed and transferred to 155 x 10 mm glass tubes.
- Subsamples designated as laboratory fortified samples were spiked with 0.5 mL of a 1 µg/mL coumestrol standard solution, producing a fortified concentration of 1 ppm.
- 15 mL of Ethanol/HCl/BHT were added. Each tube was capped with a threaded Teflon® cap and rubber o-ring. The samples were mixed using a vortexing apparatus and then heated in an oven at 110°C for two hours.
- After allowing the samples to cool to room temperature in the oven, the tubes were vortex-mixed again, and the contents were quantitatively transferred to a 50 mL volumetric flask with reagent alcohol. It is critical that the 155 x 10 mm glass tubes be allowed to cool to room temperature following the two-hour extraction/hydrolysis period. Handling of hot tubes is extremely hazardous as they are under very high pressure and could explode if disturbed.
- The flasks were brought to volume with reagent alcohol and mixed.

Procedures

- An aliquot, approximately 20 mL, was filtered through a 0.45 µm Teflon® syringe filter into a glass vial.
- A five-fold dilution was performed by mixing 200 µL of the filtered sample extract with 800 µL of reagent alcohol in a 1.5 mL autosampler vial.
- The additional dilution was necessary to eliminate MS ion source suppression caused by co-eluting matrix compounds. The diluted extracts were then analyzed by UPLC-MS/MS.
- Instrument calibration solutions were prepared by pipetting 0.1 mL of the 1,000 µg/mL stock solution into a 155 x 10 mm glass tube and processing as described above for extraction and hydrolysis of the ground soybean subsamples.
- After bringing to a final volume of 50 mL, an intermediate standard solution containing 2,000 ng/mL (2 µg/mL) resulted. Serial dilution of this standard with reagent alcohol produced instrument calibration solutions ranging in concentration from 0.5-20 ng/mL.
- Calibration standards were produced in this manner to compensate for minor losses during sample hydrolysis.

UPLC-MS/MS Parameters

- UPLC analyses were carried out on a BEH™ (Bridged Ethylene Hybrid) C18 column (2.1 x 50 mm i.d.; 1.7 µm) reversed phase column (Waters Corporation, Milford, MA). The column was maintained at a temperature of 40°C.
- Mobile Phase:

  A: 0.1% Formic Acid

  B: 0.1% Formic Acid in ACN

Time, minutes	mL/minute	%A	%B	Curve
0.00	0.5	95	5	1
0.20	0.5	95	5	1
2.50	0.5	50	50	6
2.51	0.5	5	95	1
3.00	0.5	5	95	1
3.01	0.5	95	5	1
3.50	0.5	95	5	1

UPLC-MS/MS Parameters

- Detector: Waters TQ
- Injection Volume: 5 µL
- MS Polarity: Negative Ion
- Multiple Reaction Monitoring (MRM) Program

Compound	Parent (m/z)	Daughter (m/z)	Dwell Time (s)	Cone Voltage	Collision Energy
Coumestrol	267	211	0.1	45	25

UPLC-MS/MS Parameters

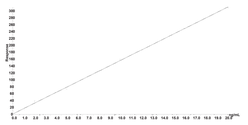
- The expected retention time for coumestrol was 2.2 min. Retention times were typically consistent to ± 0.01 min within the course of a run sequence. Mobile phase flow was diverted to waste for the first two minutes following injection, then redirected to the MS detector from 2-3 min to allow detection of the eluted coumestrol band. The flow was again diverted to waste from 3-3.5 min. The MS detector only scanned during the 2-3 min retention time range of peak elution. Diversion of the mobile phase prevented the rapid build-up of non-volatile matrix solutes in the MS detector ionization source, thus reducing the need for frequent cleaning of the source components. An electrospray ionization (ESI) source was employed.
- Calibration standard solutions were injected periodically over the course of an analysis sequence containing soybean extracts. A linear regression calibration curve of the standard peak areas (y-axis) and the calibration standard solution concentrations (ng/mL, x-axis) were plotted to generate a calibration curve. A 1/x weighting function was used. A calibration standard concentration range of 0.5-20 ng/mL was injected as part of each sample set. The linear regression calibration curve equation ($y = mx + b$), where y is the coumestrol peak area, m is the slope of the linear regression curve, x is the concentration (ng/mL) and b is the y-intercept of the linear regression curve, was used to calculate sample extract concentrations in units of ng/mL.

Results/Discussion

Assay Linearity. A typical calibration curve appears in Figure 1. A coefficient of determination (r^2) value of at least 0.99 was achieved for each calibration curve. Detector response provided acceptable linearity over a 40-fold concentration range (0.5-20 ng/mL). This concentration range represents a mass range of 2.5-100 pg injected.

Figure 1: Calibration Curve

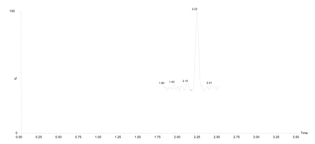
Correlation Coefficient (r^2): 0.996
Linear Regression Equation: 15.4233 * x - 0.515
Response Type: External Standard, Peak Area
Curve Type: Linear, Exclude Origin, 1/x Weighting



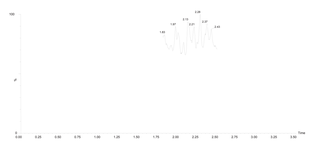
Results/Discussion (continued)

- **Quantitation and Detection Limits.** The method LOQ was established at 0.1 ppm based on a signal to noise ratio (S/N) of 10. The method LOD was established at 0.03 ppm (S/N=3).
- **Accuracy and Precision.** Fortification of soybean subsamples and calculation of recovery was used to assess the method's accuracy. See Chromatogram C. A target fortification level of 1 ppm was chosen based on reported levels of coumestrol in whole soybeans. The actual range of fortification levels was 0.83-1.33 ppm on a dry sample basis. Assays were carried out over a period of approximately one year.
- Mean fortification recovery: 92% (n = 31)
- Recovery Standard Deviation 12% (n=31)
- Relative Standard Deviation (RSD): 13% (n=31)
- These data met the accuracy (recovery range of 70-120%) and precision (RSD ≤ 15%) requirements established by our facility for analytical methods used to support nutrient composition studies with biotech seeds. Unfortified control subsamples were analyzed with the fortified samples to assess background levels of coumestrol. All control subsamples (n = 21) yielded non-detectable levels of coumestrol (< 0.03 ppm), (Chromatogram B).

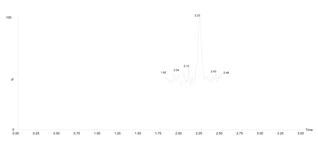
Chromatogram A: UPLC-MS/MS chromatogram of a 2 ng/mL coumestrol calibration standard with a retention time of 2.22 min



Chromatogram B: Unfortified soybean control with no detectable coumestrol.



Chromatogram C: Fortified (1 ppm) soybean sample with a retention time of 2.23 min.



Results/Discussion (continued)

- **Advantages of UPLC.** The developed UPLC procedure provides many advantages over conventional HPLC. The analysis time from injection to completion of the gradient, including column re-equilibration, was 3.5 min. The analysis time for our facility's previous method was 40 min. This represents more than a 10-fold reduction in run time. The superior selectivity of the TQ detector provides for simpler processing of the chromatographic data. The absence of matrix peaks in the chromatogram simplifies the peak integration process and makes peak identification definitive (Figure 4). Finally, the UPLC-MS/MS procedure provides excellent sensitivity without the need for time-consuming analyte concentration or cleanup steps. A simple five-fold dilution prior to instrumental analysis is all that was required to eliminate matrix ion source suppression.
- The developed UPLC-MS/MS procedure provides a new avenue for the analysis of phytoestrogens in plant tissues, particularly in high throughput environments such as those encountered in regulatory studies required for approval of new biotech seeds

Conclusions

An analytical method for the determination of coumestrol in soybeans was successfully validated. The method demonstrated acceptable accuracy, precision, sensitivity, selectivity and linear range. A method LOQ of 0.1 ppm was determined and the LOD was 0.03 ppm.

References

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