Quantitative Determination of the Mycotoxin Oosporein in Maize Grain and Soybean Seed by Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry

Fred Claussen (claussen@eplbas.com)

EPL BIO ANALYTICAL SERVICES

Abstract

An analytical method for the determination of oosporein in maize grain (*Zea mays*) and soybean seed (*Glycine max*) was developed and validated. Oosporein is a nephrotoxic secondary fungal metabolite produced by *Penicillium*, *Acremonium*, *Oospora*, *Beauveria* and *Chaetomium* species. Environmental conditions which may allow for the formation of oosporein in grains are not known. In fact, little is known about the natural occurrence of this compound. Analytical methods for accurate determination of oosporein in agricultural substrates are also lacking. Oosporein is a highly polar molecule containing four acidic functional groups. It is a relatively strong acid with a pKa, of 2.42 (1). These chemical characteristics complicate the development of sample extraction and chromatographic methods for its quantitative determination. The validated method included extraction of ground grain and seed samples using a Britton-Robinson buffer (pH 5.5)/methanol solution, dilution of the raw extracts and filtration. Filtered sample solutions were analyzed by Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS). The chromatographic separation was carried out on a C18 column utilizing an aqueous formic acid/acetic acid mobile phase gradient with methanol. Oosporein was detected using electrospray ionization (ESI) in negative ion mode and quantified by multiple reaction monitoring (MRM). Assay precision was 8.5% (relative standard deviation), and the mean spiking recovery was 100% for maize grain. For soybean seed, the RSD was 11%, and the mean spiking recovery was 100%. The limit of quantitation (LOQ) was 400 µg/kg (ppb) and limit of detection (LOD) was 120 ppb for both matrices.

Objective

The objective of the research was to develop and validate an analytical method for the determination of oosporein in maize grain and soybean seed with a target limit of quantitation of 400 ppb. Analysis of this mycotoxin is required as part of a multi-toxin screen to support animal feeding trials conducted with laboratory diets formulated with genetically modified (GM) seed. The developed analytical procedure was based on the extraction procedure described by Seger *et. al.* (2).

Chemicals and Reagents

The chemical structure of oosporein:

- Oosporein Analytical Standard, Apollo Scientific Ltd., Bredbury, UK. 98%
- A stock standard solution of oosporein was prepared in methanol at a target concentration of 100 µg/mL.
- Subsequent dilutions of the stock solution were prepared in 30:70 Britton-Robinson buffer (pH 5.5):methanol to achieve concentrations needed for laboratory fortification and instrument calibration.
- All standard solutions were stored in amber glass vials in a refrigerator (ca. 4°C) when not needed in the laboratory.
- Britton-Robinson Buffer, pH 5.5 (BR 5.5) was prepared by weighing 2.30 g of sodium hydroxide, 1.70 g of acetic acid, 2.80 g of phosphoric acid and 1.80 g of boric acid into a 1 L container containing 500 mL of deionized (DI) water. The pH was adjusted to 5.5 using concentrated phosphoric acid. The solution was brought to a final volume of 1 L in a volumetric flask.

Chemicals and Reagents

- 30:70 BR 5.5:Methanol was prepared by mixing 300 mL of BR 5.5 with 700 mL of methanol for each liter of solution needed.
- Acetic acid, glacial, BDH/VWR, Radnor, PA
- Boric acid, ACS Reagent Grade, Sigma-Aldrich, St. Louis, MO
- Buffer solutions for pH meter calibration, pH 2, 7 and 10, BDH/VWR, Radnor, PA
- Formic acid, 88%, ACS Reagent Grade, Fisher Scientific, St. Louis, MO
- Phosphoric acid, concentrated, VWR, Radnor, PA
- Methanol, HPLC grade, EMD, Billerica, MA
- Sodium hydroxide, ACS Reagent Grade, Macron/Avantor, Center Valley, PA
- DI water was obtained from a Barnstead NANOPure water system, Barnstead/Thermolyne, Dubuque, IA

Equipment

- Analytical balance, capable of weighing to the nearest 0.01 mg (stock standard preparation)
- Top-loading balance, capable of weighing to the nearest mg (sample weighing)
- Class A volumetric glassware
- Pipetters, fixed and adjustable volume
- Centrifuge
- Centrifuge tubes, plastic, 1.5 mL
- Platform shaker with orbital rotation
- Syringe filters, 0.2 µm, Nylon
- Syringes, plastic, 3 cc capacity

Equipment

- Vortex mixer
- Vials, glass, 20 mL capacity (for extract storage)
- Nitrogen evaporation unit, N-Evap, Organomation, Berlin, MA
- HPLC autosampler vials, 1.5 mL, glass, screw caps, Waters Corporation, Milford, MA
- HPLC column, BEH Shield RP18, 50 mm x 2.1 mm x 1.7 µm, Waters Corporation, Milford, MA
- UPLC, Acquity, Waters Corporation, Milford, MA
- Instrument data system, MassLynx Version 4.1, Waters Corporation, Milford, MA
- Mass spectrometer detector, TQ, Waters Corporation, Milford, MA
- Centrifugal mill grinder, Retsch, Haan, Germany

Test Portion Extraction

- Weighed 0.5 g (±0.025 g) test portion of ground sample into a 1.5 mL plastic centrifuge tube.
- Laboratory fortified test portions were spiked with 0.2 mL of a 1 µg/mL oosporein working standard solution with a resulting fortification level of 400 ppb or with 0.01 mL of a 98 µg/mL oosporein stock solution with a resulting fortification level of 2,000 ppb.
- 5 mL of 30:70 BR 5.5:methanol were added and the tube was vortex mixed for 5-10 seconds.
- Oosporein was extracted from the test portion by shaking horizontally on a platform shaker at a setting of 350 rpm for 30 minutes.
- After centrifuging at 3,000 rpm for 10 minutes, the supernatant extract was decanted into a clean 20 mL glass vial.
- The sample extract was diluted by combining 1 mL of the supernatant extract with 4 mL of 30:70 BR 5.5:methanol.
- An aliquot of the diluted extract was filtered through a 0.2 µm Nylon syringe filter into a glass autosampler vial for UHPLC-MS/MS analysis.

Test Matrices

The method was validated using non GM commercial varieties of dent corn grain and soybean seed. A bulk sample of each matrix was ground and homogenized using a centrifugal mill grinder to pass a 0.75 mm sieve. Ground grain was stored in a freezer (ca. -20°C) when not needed in the laboratory.

UHPLC-MS/MS Parameters

- Flow Rate: 0.5 mL/min.
- Injection Volume: 5 -10 µL
- Column Temp.: 50°C
- MS Source: Electrospray
- MS Polarity: Negative
- MS Mode: Multiple Reaction Monitoring (MRM)
- Expected Retention Time (Figures 1a-1g): Approximately 1.7 min.
- HPLC Gradient: Linear

Mobile Phase Gradient Program

Time (min.)	%A	%B
0.00	95	5
1.00	95	5
1.50	5	95
2.50	5	95
2.51	95	5
4.00	95	5

A: 0.9% Formic Acid, 0.1% Acetic Acid in DI Water. B: Methanol

MRM Program for the Detection of Oosporein (Figure 2)

Ion Type	Ion (m/z)		Dwell Time (s)	Cone Voltage	Collision Energy
	Precursor	Product			
Quantitative	305	277	0.3	35	15
Qualifier	305	261	0.3	35	15

- Calibration: Linear, external standards
- Calibration Range: 5-100 ng/mL
- Weighting: 1/x
- Minimum Correlation Coefficient (r). 0.99
- MS data for the qualifier ion is not collected unless oosporein is found at levels above the LOQ in submitted grain or seed samples.

Results/Discussion

Method Validation: Accuracy and intra-laboratory precision (repeatability) were determined by analysis of two sample sets by two analysts on two separate days. For each sample set, a single reagent blank, a single unfortified test portion of each matrix and triplicate fortifications of each matrix at two concentrations were extracted and analyzed. The fortification concentrations included the target LOQ (400 ppb) and 5x LOQ (2,000 ppb). Analysis of reagent blanks and unfortified control test portions yielded no detectable oosporein (<120 ppb). Certified reference materials for oosporein could not be located. Therefore, only spike and recovery could be used to ascertain the method's accuracy and repeatability. The method demonstrated acceptable accuracy and repeatability (Tables 1 and 2).

Results/Discussion

Table 1. Oosporein Accuracy and Repeatability for Maize Grain

Analyst	Fortification Level (ppb)	Mean Recovery (%)	Std. Dev.	RSD (%)
1	400 (n=3)	99.8	12.7	12.7
2	400 (n=3)	102	8.07	7.90
Pooled 400 ppb (n=6)		101	9.55	9.46
1	2000 (n=3)	99.5	6.82	6.85
2	2000 (n=3)	98.4	10.4	10.5
Pooled 2000 ppb (n=6)		99.1	8.01	12.4
Pooled Overall (n=12)		100	8.46	8.46

Results/Discussion

Table 2. Oosporein Accuracy and Repeatability for Soybean Seed

Analyst	Fortification Level (ppb)	Mean Recovery (%)	Std. Dev.	RSD (%)
1	400 (n=3)	101	13.5	13.3
2	400 (n=3)	94.8	3.37	3.56
Pooled 400 ppb (n=6)		98.2	9.43	9.60
1	2000 (n=3)	105	7.17	6.86
2	2000 (n=3)	99.5	19.4	19.5
Pooled 2000 ppb (n=6)		102	13.5	13.2
Pooled Overall (n=12)		100	11.3	11.3

Results/Discussion

- ***Limit of Quantitation (LOQ)***: The method LOQ was established at 400 ppb. The LOQ was based on a peak signal to noise ratio (S/N) for grain and seed extracts. S/N at the LOQ was approximately 10. The LOD was estimated to be 120 ppb, or about 30% of the LOQ providing a S/N of approximately 3.
- ***Linearity***: The linear concentration range for oosporein was approximately 5-100 ng/mL (Figure 3).

Conclusions

A method for the extraction and UHPLC-MS/MS analysis of oosporein in maize grain and soybean seed was developed and validated. The method demonstrated acceptable accuracy, precision, sensitivity and linear range. The validated method is now routinely employed to support toxin screens of laboratory animal diets formulated with GM seeds.

Figure 1a. UHPLC-MS/MS Chromatogram: Oosporein Calibration Standard (20 ng/mL)

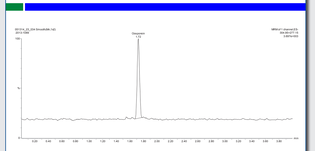


Figure 1b. UHPLC-MS/MS Chromatogram: Unfortified Maize Grain Sample

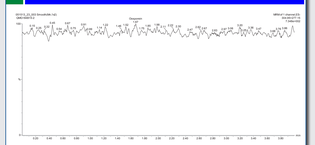


Figure 1c. UHPLC-MS/MS Chromatogram: Laboratory Fortified Maize Grain Sample (400 ppb)

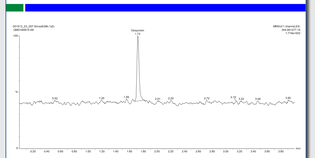


Figure 1d. UHPLC-MS/MS Chromatogram: Laboratory Fortified Maize Grain Sample (2000 ppb)

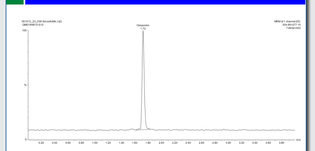


Figure 1e. UHPLC-MS/MS Chromatogram: Unfortified Soybean Seed Sample

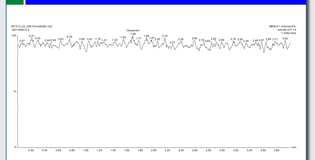


Figure 1f. UHPLC-MS/MS Chromatogram: Laboratory Fortified Soybean Seed Sample (400 ppb)

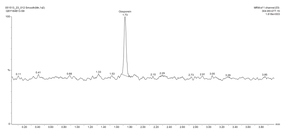


Figure 1g. UHPLC-MS/MS Chromatogram: Laboratory Fortified Soybean Seed Sample (2000 ppb)

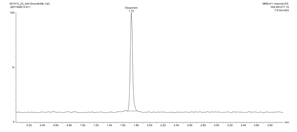
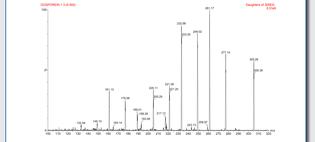
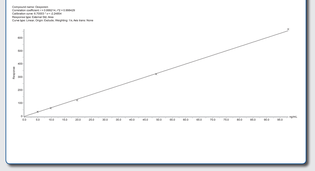


Figure 2. Product Ion Spectrum of Oosporein



Precursor Ion: m/z 305, Quantitation Ion: m/z 277, Qualifier Ion: m/z 261

Figure 3. Oosporein Linear Range



References

(1) Seger, C.; Erlebach, D.; Stuppner, H.; Griesser, U.; Strasser, H. Physiochemical Properties of Oosporein, the Major Secreted Metabolite of the Entomopathogenic Fungus Beauveria brongniartii. *Helvetica Chimica Acta*. 2005. 88(4): 802-810.

(2) Seger, C.; Langle, T.; Pernfuss, B.; Stuppner, H.; Strasser, H. High-performance liquid chromatography-diode array detection assay for the detection and quantification of the *Beauveria* metabolite oosporein from potato tubers. *Journal of Chromatography A*. 2005. 1092: 254-257.