Quantitative Determination of Vitamin C in Maize Grain and Soybean Seed by High Performance Liquid Chromatography-Tandem Mass Spectrometry

Joe Warnick (jwarnick@eplbas.com) and Fred Claussen (claussen@eplbas.com)

EPL BIO ANALYTICAL SERVICES

Abstract

Vitamin C, or L-ascorbic acid, is an essential nutrient for humans and other animal species. Vitamin C has not typically been quantitated in maize grain and soybean. However, measureable amounts were found in both matrices as part of the validation. A new procedure for analysis of maize grain and soybean seed utilizing High Performance Liquid Chromatography with Tandem Mass Spectroscopy (HPLC-MS/MS) was developed and validated. The method precision was 3.65% (relative standard deviation) and the overall mean spiking recovery was 116% for soybean seed. Precision for maize grain was 6.32% and the spiking recovery was 109%.

Objective

The objective of the research was to develop and validate an analytical method for the determination of vitamin C in maize grain and soybean seed. The analytical procedure was based on the extraction procedure described by Walker, et. al. (1).

Conclusion

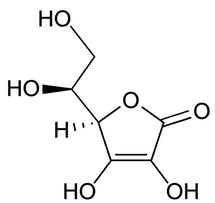
A method for the extraction and HPLC-MS/MS analysis of vitamin C in maize grain and soybean seed was developed and validated. The method demonstrated acceptable accuracy, precision, selectivity and linear range. Intrinsic vitamin C was found at quantifiable levels in the seed tissue of both plant species.

Reference

(1) Walker, P.G. et al. A high-throughput monolithic HPLC method for rapid vitamin C phenotyping of berry fruit. Phytochem. Anal. 2006. 17: 284-290.

Chemicals and Reagents

The chemical structure of vitamin C (L-Ascorbic Acid)



L-Ascorbic Acid Reference Standard, Sigma-Aldrich, St. Louis, MO.

Stock Standard Solution, 1,000 µg/mL: Prepared by weighing approximately 0.010 g of the neat reference standard into a 10 mL volumetric flask and diluting to volume with 5% meta-phosphoric acid with 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). After mixing, the solution was transferred to an amber glass bottle and stored refrigerated (ca. 4°) for up to 14 days. TCEP is a crucial anti-oxidant which prevents conversion of ascorbic acid to its reduced dehydroascorbic acid form.

Additional working and instrument calibration solutions were prepared by serial dilution of the stock standard with 5% meta-phosphoric acid with 5 mM TCEP.

Equipment

- Centrifugal mill grinder, Retsch, Haan, Germany
- Analytical balance (stock standard preparation)
- Top-loading balance (sample weighing)
- Centrifuge
- Centrifuge tubes, plastic, 15 mL
- Platform shaker with orbital rotation
- Syringes, 3 cc, disposable
- Syringe filters, Nylon, 0.2 µm
- HPLC autosampler vials, glass
- HPLC column, PFPP, 150 mm x 2.1 mm x 3 µm, Restek, Bellefonte, PA
- HPLC, 2695 Separations Module, Waters Corporation, Milford, MA
- Mass spectrometer detector, Quattro micro™ API, Waters Corporation, Milford, MA

Test Matrices

The method was validated using commercial varieties of maize grain and soybean seed. Samples were ground and homogenized using a centrifugal mill grinder to pass a 0.75 mm sieve. Ground seed was stored in a freezer (ca. -20ºC) when not needed in the laboratory.

Extraction Procedure

- Weighed 0.5 g test portion of ground maize grain or soybean seed into a 15 mL plastic centrifuge tube.
- Soybean Seed: Laboratory fortified test portions were spiked with 0.2 mL of a 100 µg/mL vitamin C working standard solution.
- Maize Grain: Laboratory fortified test portions were spiked with 0.2 mL of a 10 µg/mL vitamin C working standard solution.
- Vitamin C was extracted with 10 mL of 5% meta-phosphoric acid with 5 mM TCEP for 1 hour by shaking at 300 rpm.
- Samples were centrifuged at 3,500 rpm for 10 minutes.
- Supernatant extracts were filtered through a 0.2 µm Nylon syringe filter into a glass vial.
- Maize grain extracts were analyzed without further dilution.
- For soybean seed, 100 µL of filtered extract were mixed with 900 µL of 5% meta-phosphoric acid with 5 mM TCEP in an HPLC autosampler vial to achieve a dilution factor of 10.

HPLC-MS/MS Parameters

- Injection Volume: 5 µL
- Column Temp.: 30˚C
- MS Source: Electrospray
- MS Polarity: Negative
- MS Mode: Multiple Reaction Monitoring (MRM)
- Precursor>Product Ions: m/z 175>115
- Dwell Time: 1.0 second
- Cone Voltage: 22
- Collision Energy: 11
- HPLC Conditions:

Mobile Phase A: 0.1% Formic Acid in DI Water
Mobile Phase B: 0.1% Formic Acid in Acetonitrile
Isocratic Conditions at 99% A and 1% B at 0.2 mL/minute for 10 minutes.

- Calibration: Linear, external standards, 1/x weighting
- Calibration Range: 25 - 1,000 ng/mL

Results/Discussion

Method Validation: Vitamin C content was expressed on a dry weight basis (DB). Repeatability precision (Table 1) was the relative standard deviation (RSD) obtained by a single analyst. Repeatability was based on intrinsic vitamin C content of soy and maize seeds. Accuracy (Table 2) was based on spike and recovery and was determined concurrently with the repeatability experiment. Seven replicate unfortified (repeatability) and seven replicate fortified test portions (accuracy) were analyzed. Target fortification levels were approximately 1 times (1x) the intrinsic level of vitamin C in unfortified seed test portions. Average recovery acceptance criteria were set at 75 - 120%. Minimum acceptable RSD values were defined as less than or equal to 15%. The recovery and RSD values for soybean seed and maize grain met the established accuracy and precision acceptance criteria.

Table 1. Repeatablility

Matrix	Mean (n=7) (mg/kg DB)	Std. Dev. (%)	RSD (%)	Range (mg/kg DB)
Soybean Seed	45.2	1.65	3.65	43.0-48.1
Maize Grain	39.5	0.250	6.32	3.67-4.34

Table 2. Accuracy

Matrix	Spike Level (mg/kg DB)	Mean (n=7) Recovery (%)	Recovery Range (%)
Soybean Seed	44	116	113-121
Maize Grain	4.4	109	99-116

- *Linearity:* The detector response was found to be linear (r2>0.99) over a concentration range of 25 - 1,000 ng/mL.
- *Selectivity:* No interfering peaks were observed in the matrix chromatograms using the highly selective MRM program. Spike recovery data indicates that no significant interferences were present in the matrix extracts.

Figure 1. HPLC-MS/MS Chromatogram: 250 ng/mL Calibration Standard

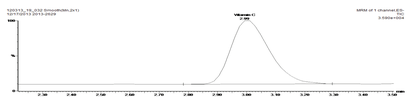


Figure 2. HPLC-MS/MS Chromatogram: Method Blank (0.0 ng/mL Found)

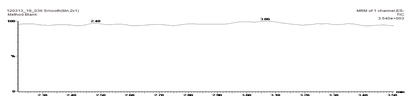


Figure 3. HPLC-MS/MS Chromatogram: Unfortified Soybean Seed (201 ng/mL Found)

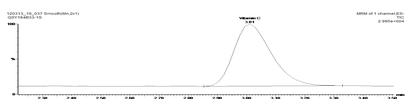


Figure 4. HPLC-MS/MS Chromatogram: Fortified Soybean Seed (435 ng/mL Found)

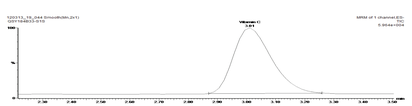


Figure 5. HPLC-MS/MS Chromatogram: Unfortified Maize Grain (187 ng/mL Found)

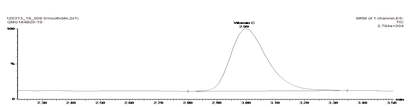


Figure 6. HPLC-MS/MS Chromatogram: Fortified Maize Grain (406 ng/mL Found)

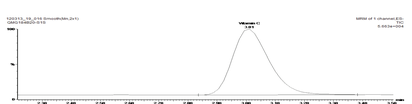
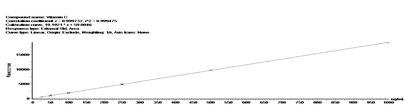



Figure 7. Vitamin C Calibration Curve 25 – 1,000 ng/ mL