

Abstract

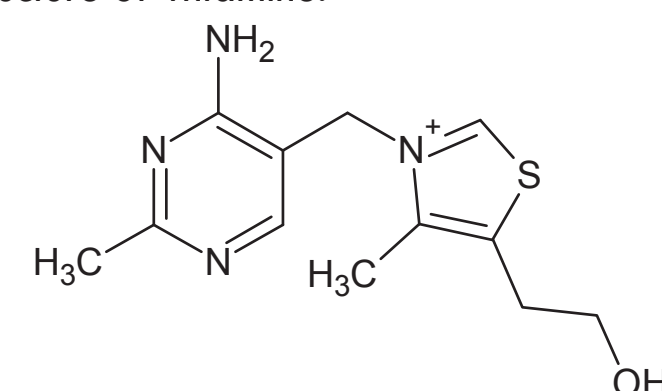
A procedure for the determination of thiamine (vitamin B1) in maize (*Zea mays*) grain, soybean (*Glycine max*) seed and canola (*Brassica napus*) seed utilizing high performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS) was developed and validated. HPLC columns containing a pentaffluorophenyl/propyl (PFP) stationary phase provide an improved means of analyzing polar molecules by HPLC. Water soluble vitamins such as thiamine have been particularly troublesome due to silanol interactions which result in peak tailing. When coupled with tandem mass spectrometry detection, the PFP column becomes even more powerful due to sensitivity and selectivity advantages of MS/MS detection. Grain and seed sub-samples were extracted with aqueous acetic acid/trichloroacetic acid, filtered and diluted with HPLC-MS/MS analysis. Assay precision (relative standard deviation) was 4.77%, 7.82% and 9.10% for maize grain, soybean seed and canola seed, respectively. Mean spiking recovery was 92.5%, 96.5% and 90.1% for maize grain, soybean seed and canola seed, respectively. The validated method provides for rapid, sensitive and selective determination of thiamine in grains and seeds.

Objective

The objective of the research was to develop and validate an analytical method for the determination of thiamine in maize grain, soybean seed and canola seed. Thiamine determination is necessary to meet the requirements of the Organization for Economic Cooperation and Development (OECD) for new varieties of maize, soy and canola. Data for thiamine and numerous other nutritional and compositional components are used to demonstrate substantial equivalency of genetically modified (GM) crops. Substantial equivalency is one of the first steps in the food and feed safety assessment of GM crops.

Chemicals and Reagents

The chemical structure of Thiamine:



- Thiamine Hydrochloride Reference Standard, Sigma-Aldrich, St. Louis, MO.
- Thiamine Stock Standard Solution, 100 µg/mL (cationic form): Prepared by weighing approximately 0.013 g of dried (100-105°C for 2 hours) thiamine hydrochloride into a 100 mL volumetric flask and diluting to volume with 10% acetic acid:4.3% trichloroacetic acid (v/v). After mixing, the solution was transferred to an amber glass bottle and stored refrigerated (ca. 4°C). A correction factor was applied to express the concentration in terms of the cationic form instead of the hydrochloride form.

Chemicals and Reagents (continued)

- Thiamine Intermediate Working Standard, 10 µg/mL: Prepared by diluting the 100 µg/mL stock standard solution 1:10 using 10% acetic acid:4.3% trichloroacetic acid (v/v). The solution was transferred to an amber bottle and stored refrigerated.
- Thiamine Instrument Calibration Standard Solutions: Prepared by dilution of the 10 µg/mL intermediate working standard solution with 0.2% acetic acid:0.086% trichloroacetic acid (v/v) to achieve concentrations ranging from 0.5-60 ng/mL.
- Deionized (DI) water was obtained from a Barnstead NANOPure water system.
- 10% Acetic Acid:4.3% Trichloroacetic Acid (v/v): Combine 100 mL glacial acetic acid (BDH/VWR, Radnor, PA) and 43 mL of trichloroacetic acid (Sigma-Aldrich) in a 1 L volumetric flask filled about half full with DI water. Bring to volume with DI water and mix.

Chemicals and Reagents (continued)

- 0.2% Acetic Acid:0.086% Trichloroacetic Acid (v/v): Transferred 5 mL of 10% acetic acid:4.3% trichloroacetic acid (v/v) to a 250 mL volumetric flask and diluted to volume with DI water.
- 0.1% Acetic Acid:0.043% Trichloroacetic Acid (v/v): Transferred 2.5 mL of 10% acetic acid:4.3% trichloroacetic acid (v/v) to a 250 mL volumetric flask and diluted to volume with DI water.
- 0.1% Formic Acid in DI Water (Mobile Phase A): Transferred 1 mL of formic acid (Fisher Scientific, St. Louis, MO) into a 1 L volumetric flask and diluted to volume with DI water.
- 0.1% Formic Acid in Acetonitrile (Mobile Phase B): Transferred 1 mL of formic acid into a 1 L volumetric flask and diluted to volume with acetonitrile (EMD, Billerica, MA).

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
- Transfer pipets
- Pipettors, fixed and adjustable volume
- Centrifuge
- Centrifuge tubes, plastic, 15 mL

Equipment

- Platform shaker with orbital rotation
- Syringes, 3cc, plastic
- Syringe filters, Nylon, 0.45 µm
- HPLC autosampler vials, 1.5 mL, glass, snap or screw caps
- HPLC column, PFP, 100 mm x 2.1 mm x 3 µm, Ultra II, Restek, Bellefonte, PA
- HPLC, 2695 Separations Module, Waters Corporation, Milford, MA
- Instrument Data System, Masslynx Version 4.1
- Mass spectrometer detector, Quattro micro™ API, Waters Corporation, Milford, MA
- Centrifugal mill grinder, Retsch, Haan, Germany

Test Matrices

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

Extraction and Cleanup Procedures

- Weighed 1 g (± 0.05 g) test portions of ground maize grain or soybean and 0.25 g (± 0.03 g) test portions of canola seed into 15 mL plastic centrifuge tubes.
- Laboratory fortified test portions were spiked with appropriate aliquots of the 100 µg/mL thiamine stock standard solution.
- Thiamine was extracted with 10 mL of 10% acetic acid:4.3% trichloroacetic acid (v/v) for 1 hour by shaking at 350 rpm via a mechanical platform orbital shaker.
- Samples were centrifuged at 3,000 rpm for 10 minutes.

Extraction and Cleanup Procedures

- Supernatant extracts were filtered through a 0.45µm Nylon syringe filters into glass vials.
- For soybean seed and canola seed, 10 µL of filtered extract were mixed with 990 µL of 0.1% acetic acid:0.043% trichloroacetic acid (v/v) in an HPLC autosampler vial to achieve a dilution factor of 100. Samples were then analyzed by HPLC-MS/MS.
- For maize grain, 20 µL of filtered extract were mixed with 980 µL of 0.1% acetic acid:0.043% trichloroacetic acid (v/v) in an HPLC autosampler vial to achieve a dilution factor of 50. Samples were then analyzed by HPLC-MS/MS.

HPLC-MS/MS Parameters

- Flow Rate: 0.3 mL/min.
- Injection Volume: 10 µL
- Column Temp.: 40°C
- MS Source: Electrospray
- MS Polarity: Positive
- MS Mode: Multiple Reaction Monitoring (MRM)
- Precursor>Product Ions: m/z 265>122 (Figure 1)
- Dwell Time: 0.05-0.1 second
- Cone Voltage: 15-20
- Collision Energy: 10-12
- HPLC Gradient:
 - Mobile Phase A: 0.1% Formic Acid in DI Water
 - Mobile Phase B: 0.1% Formic Acid in Acetonitrile

HPLC-MS/MS Parameters

Maize Grain Mobile Phase Linear Gradient Program

Time (min.)	Flow Rate (mL/min.)	%A	%B
0.00	0.3	75	25
1.00	0.3	75	25
1.10	0.3	100	0
6.00	0.3	100	0
6.10	0.5	75	25
9.00	0.5	75	25
9.10	0.3	75	25
10.00	0.3	75	25

Soybean Seed: Isocratic, 87% A/13% B for 5 minutes.
Canola Seed: Isocratic, 95% A/5% B for 6 minutes.

HPLC-MS/MS Parameters

- Calibration: Linear, external standards, 1/x weighting
- Calibration Ranges: 1.8-20 ng/mL (maize grain), 1-60 ng/mL (soybean seed), 0.5-30 ng/mL (canola seed)
- Expected Retention Time: 7.2 minutes (maize grain), 2.0 minutes (soybean seed), 4.8 minutes (canola seed). See Figures 2-7.

Results/Discussion

Method Validation: Thiamine content was expressed on a dry weight basis (DB). Repeatability (Table 1) was the relative standard deviation (RSD) obtained by a single analyst for a single set of seven test portions. Accuracy (Table 2) was based on spike and recovery of three laboratory fortified test portions and was determined in the same batch with the repeatability test portions.

Results/Discussion

Table 1. Repeatability

Matrix	Mean (n=7) (mg/kg DB)	Std. Dev.	RSD (%)	Range (mg/kg DB)
Maize Grain	3.17	0.151	4.77	2.92-3.35
Soybean Seed	1.67	0.131	7.82	1.49-1.80
Canola Seed	12.1	1.10	9.10	10.4-14.0

Results/Discussion

Table 2. Accuracy

Matrix	Approximate Spike Level (mg/kg DB)	Mean (n=3) Recovery (%)	Recovery Range (%)
Maize Grain	3.0	92.5	77.9-106
Soybean Seed	5.7	96.5	89.3-107
Canola Seed	21.8	90.1	73.6-98.6

Results/Discussion

Linearity: A regression calibration curve produced during method validation from canola seed appears in Figure 8. The detector response was found to be linear ($r^2 > 0.99$) over a concentration range of 0.5-60 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.

Analysis of AACCI Fortified Cereal Proficiency Sample: Additional method performance data was obtained by analysis of AACCI fortified cereal proficiency sample VMP-4 (Table 3). The calculated z value of -0.72 indicates an acceptable level of method performance.

Results/Discussion

Table 3. Summary of AACCI Proficiency Sample Results (Fortified Cereal VMP-4)

Mean (mg/100 g) (n=14)	Std. Dev.	Range (mg/100 g)	EPL Result (mg/100 g)	Calculated z Value
1.43	0.32	1.02-1.99	1.21	-0.72

Thiamine concentrations expressed on a fresh weight basis.

Conclusions

A method for the extraction and HPLC-MS/MS analysis of thiamine in maize grain, soybean seed and canola seed was developed and validated. The method demonstrated acceptable accuracy, precision, selectivity and linear range. Proficiency sample results indicate acceptable method performance on an inter-laboratory basis.

Figure 1. Thiamine Product Ion Spectrum

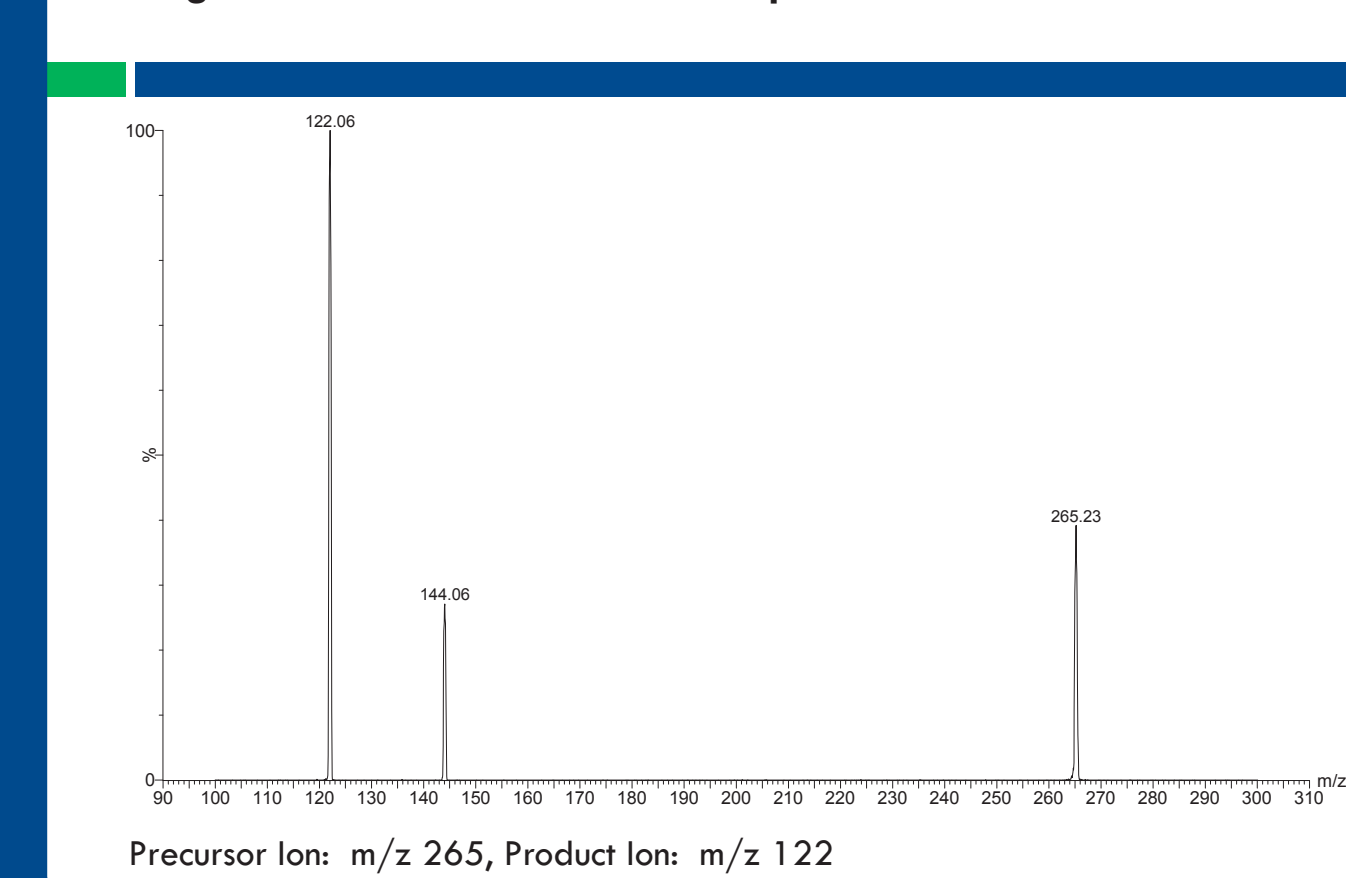


Figure 2. HPLC-MS/MS Chromatogram: Calibration Standard (Maize Grain Analysis)

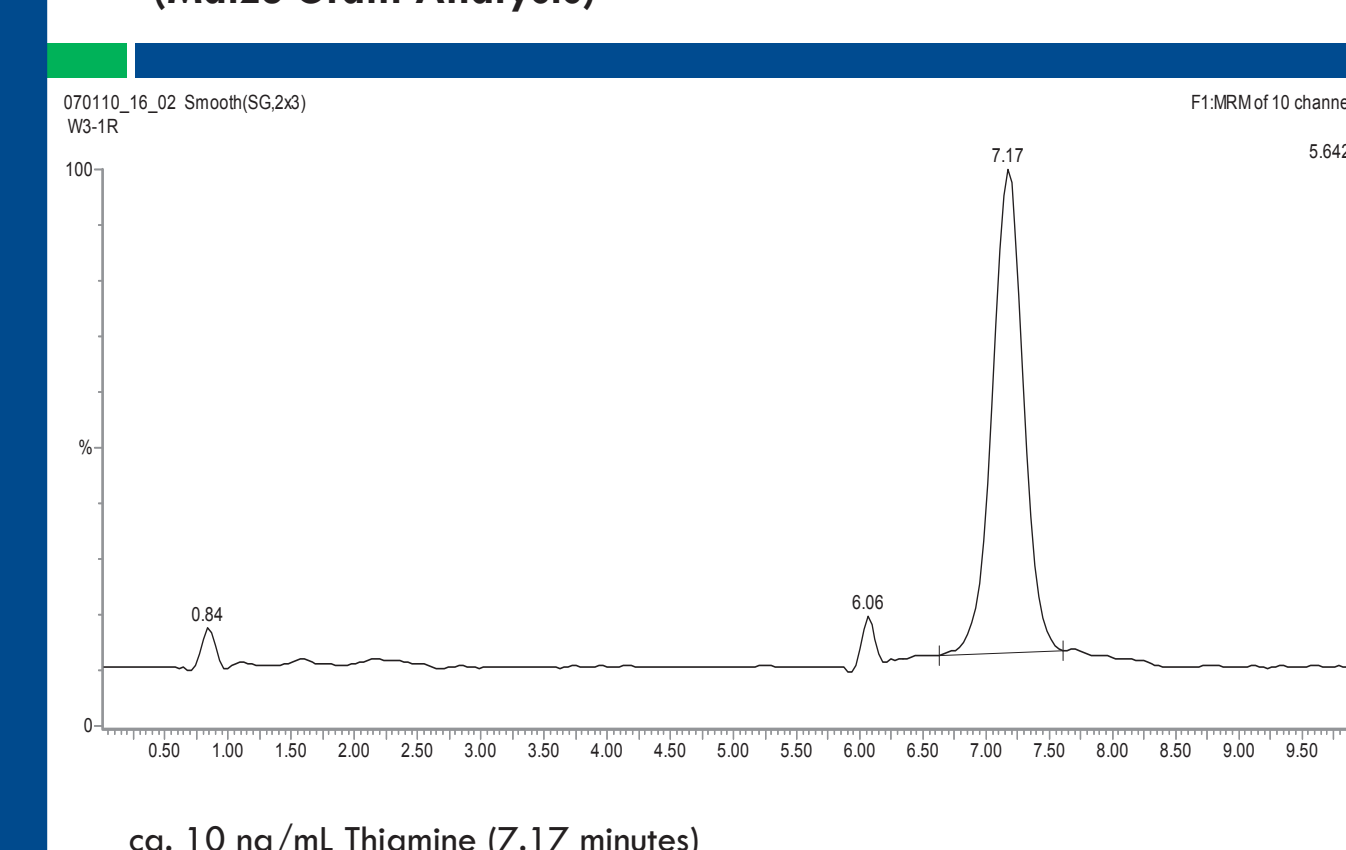


Figure 3. HPLC-MS/MS Chromatogram: Maize Grain Extract

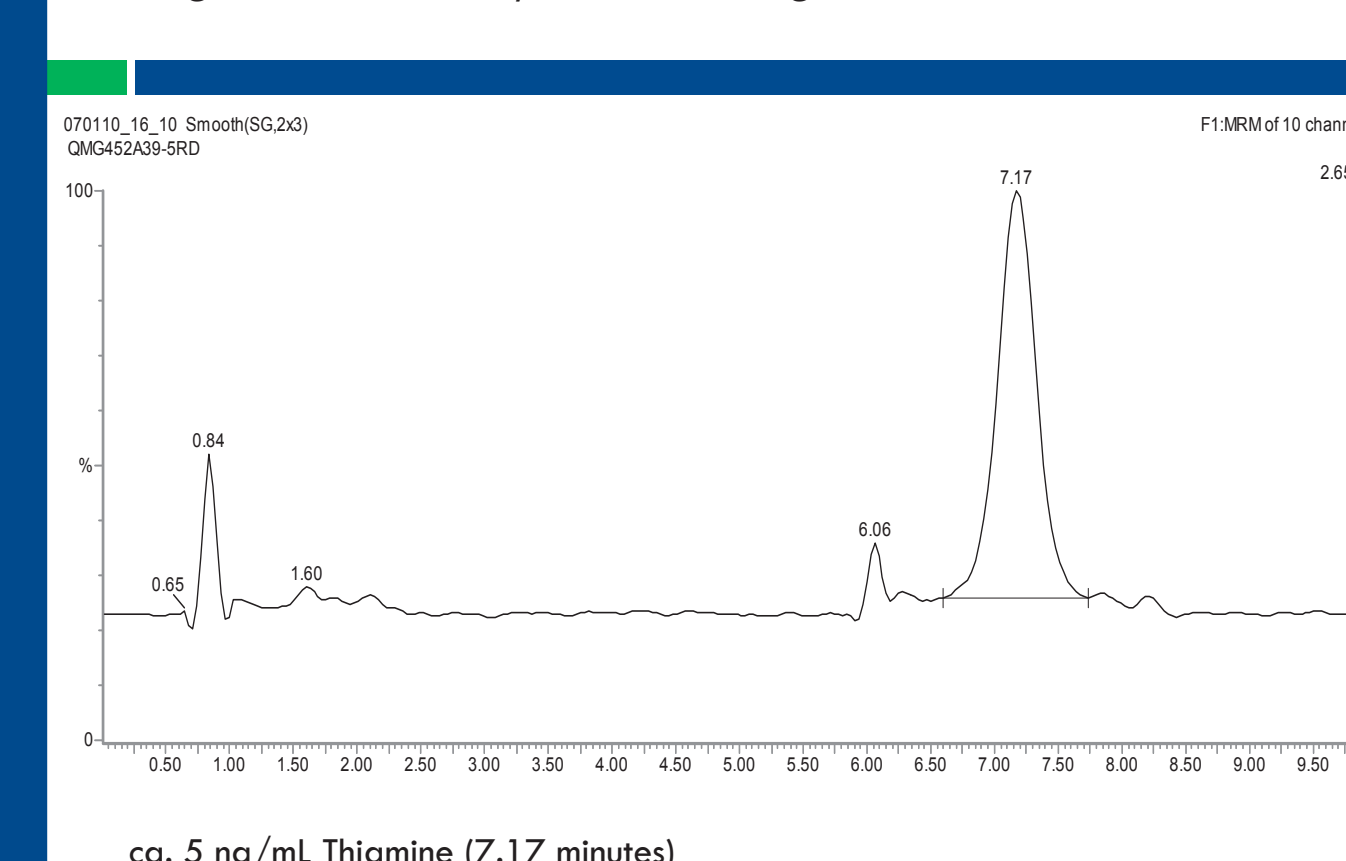


Figure 4. HPLC-MS/MS Chromatogram: Calibration Standard (Soybean Seed Analysis)

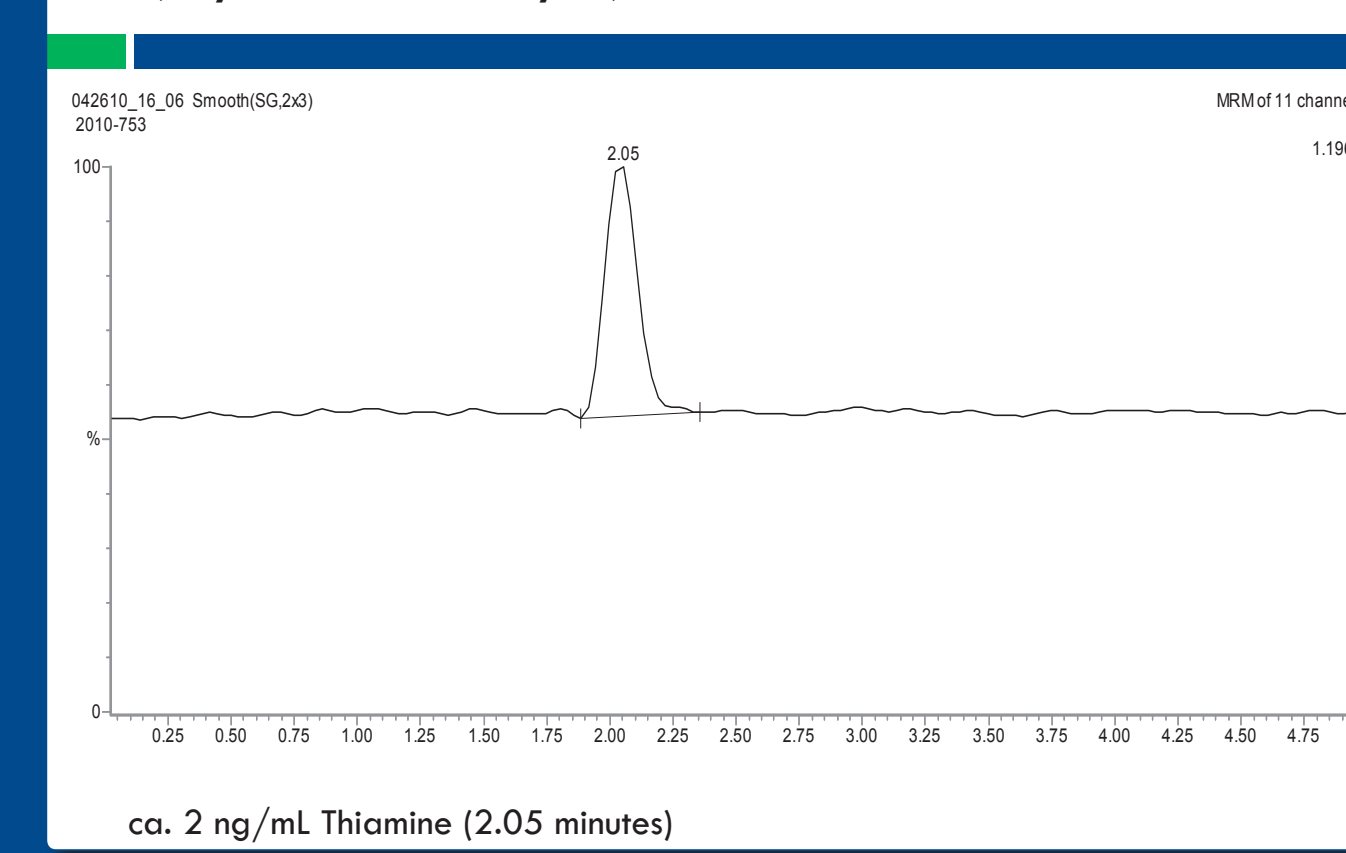


Figure 5. HPLC-MS/MS Chromatogram: Soybean Seed Extract

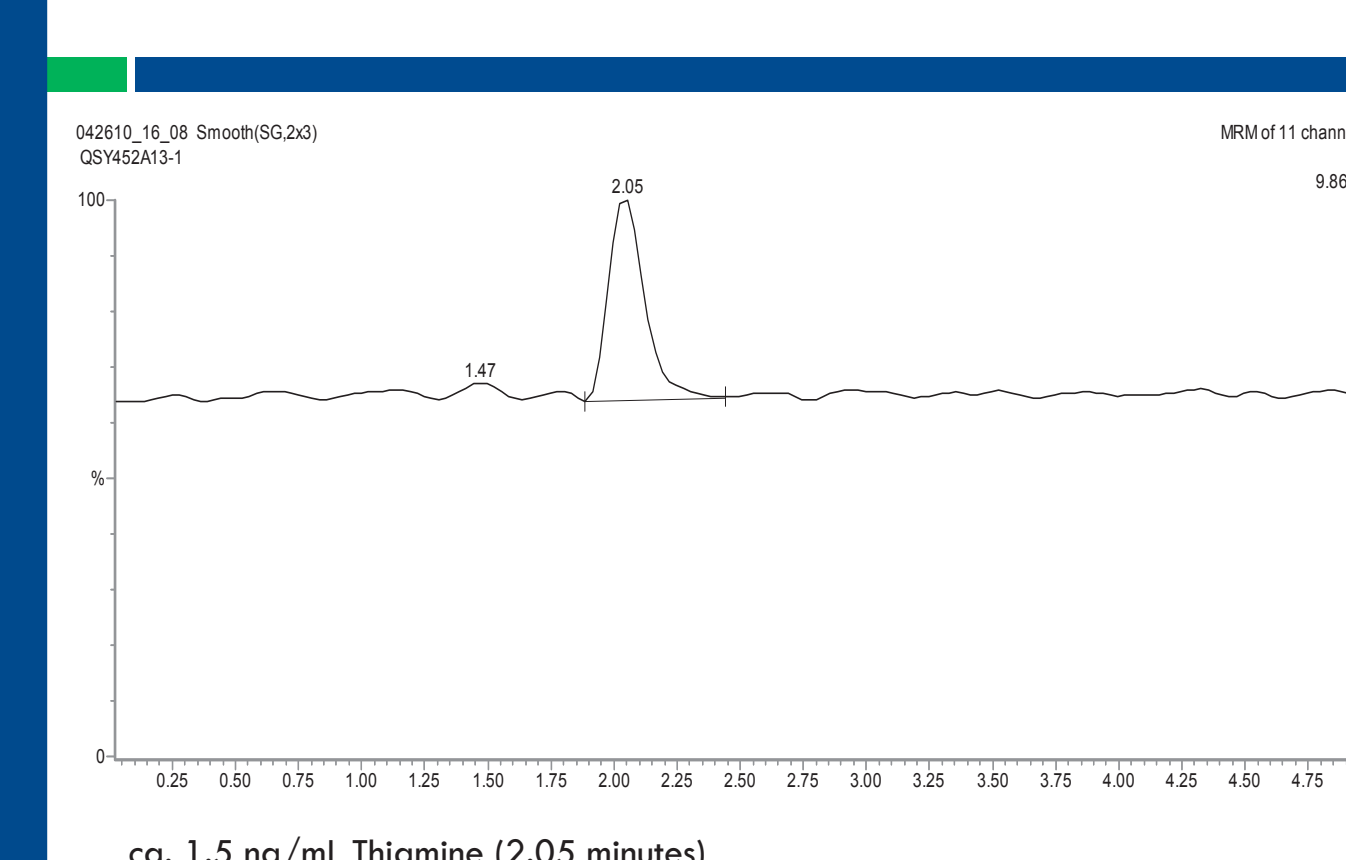


Figure 6. HPLC-MS/MS Chromatogram: Calibration Standard (Canola Seed Analysis)

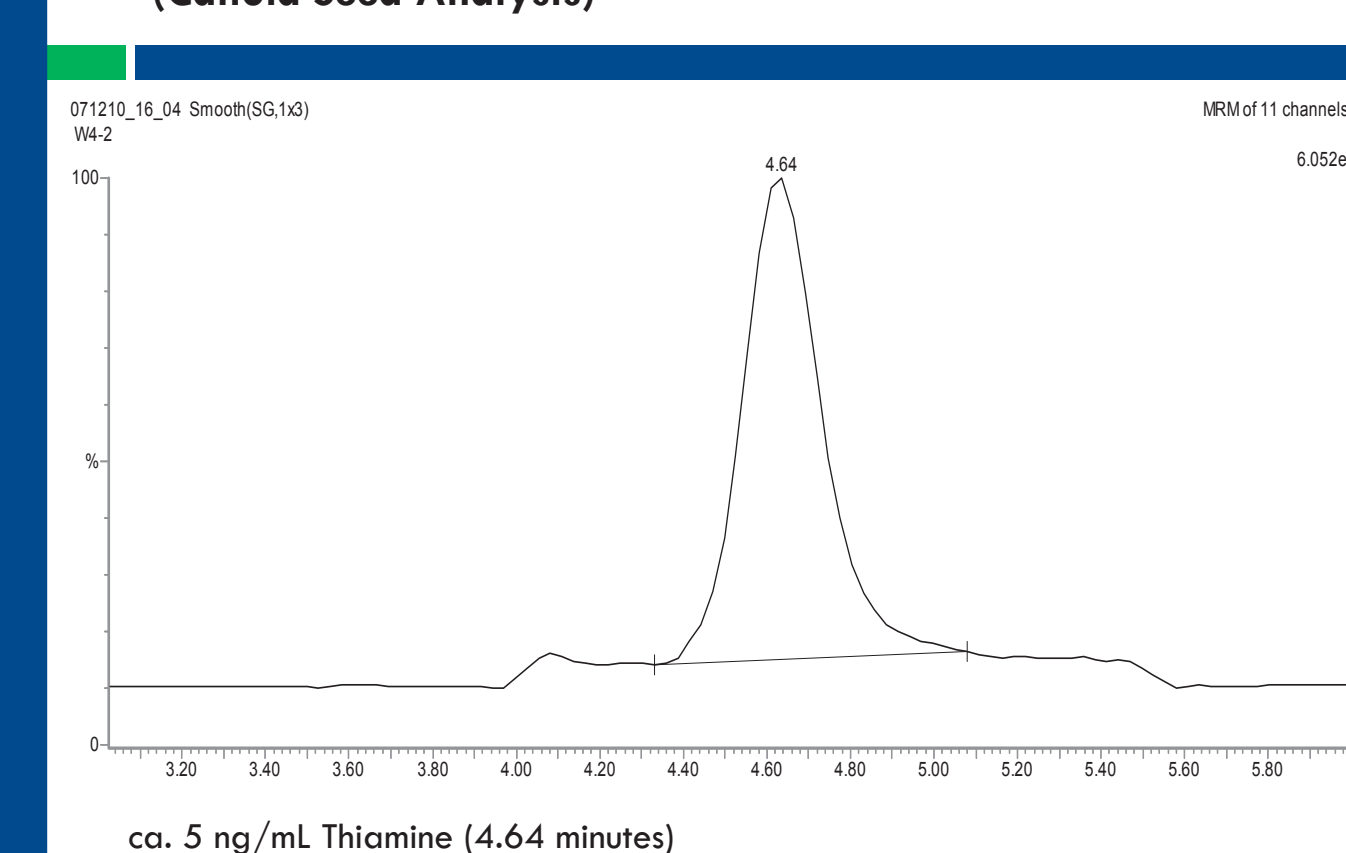


Figure 7. HPLC-MS/MS Chromatogram: Canola Seed Extract

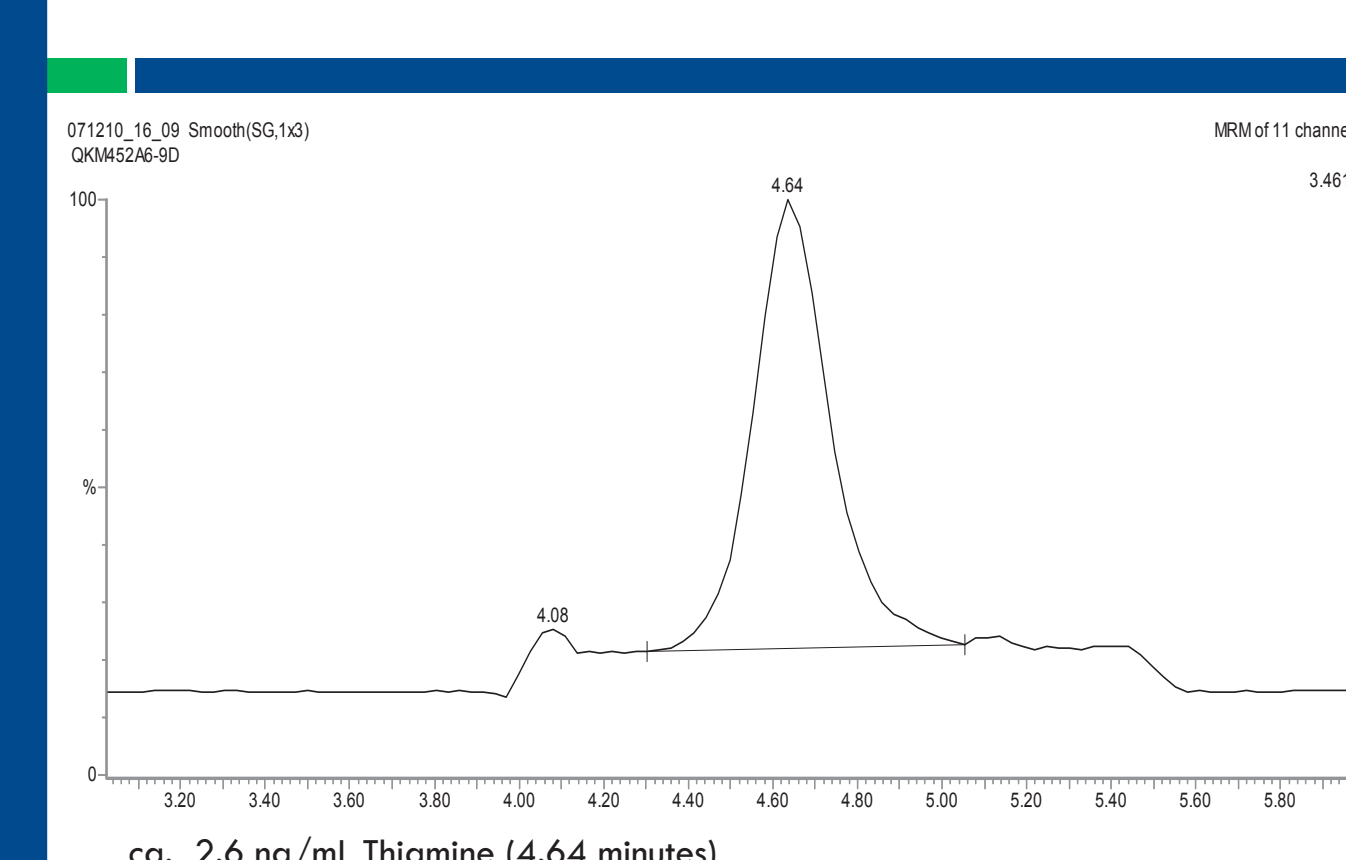


Figure 8. Example HPLC-MS/MS Calibration Curve: Canola Seed (0.5-30 ng/mL)

