Quantitative Determination of Biotin in Feed by Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

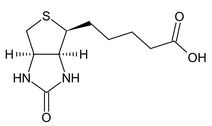
Biotin is an essential micronutrient for mammals and is classified as a B-complex water soluble vitamin. Mammalian cells are unable to biosynthesize biotin, and therefore it becomes an essential component in the diet. An analytical procedure for the determination of biotin in a commercial laboratory rodent feed/diet was developed and validated. The validated method included hydrolysis and extraction of diet sub-samples with 3N sulfuric acid at 121°C for 30 minutes. Following adjustment of the extract pH to 3-6 and dilution with water, the sample solutions were filtered and analyzed by Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS). The chromatographic separation was carried out on a C18 column designed for increased retention of polar solutes utilizing an aqueous ammonium formate/methanol mobile phase gradient. Biotin was detected using electrospray ionization (ESI) in positive ion mode and quantified by multiple reaction monitoring (MRM). An internal standard, d2-biotin, was used to compensate for detector source suppression and losses during sample workup. Assay precision was 11% (relative standard deviation), and the mean fortification recovery was 96%.

Objective

The objective of the research was to develop and validate an analytical method for the determination of biotin in a commercial rodent diet. Research diet samples must be certified for nutritional content to support mammalian feeding trials with biotech crops. Biotin is one of several water soluble vitamin assays required. The developed analytical procedure was based on procedures described by Holler *et. al.* (1) and an application note provided by Waters Corporation (2).

Chemicals and Reagents

The chemical structure of biotin:



Chemicals and Reagents

- Biotin Analytical Standard, >99%, Sigma-Aldrich, St. Louis, MO.
- d_2-Biotin Internal Standard (Ring-6, 6-d_2), ≥97%, 98% deuterium isotopic purity, Sigma-Aldrich, St. Louis, MO.
- Individual stock standard solutions of biotin and d2-biotin were prepared in deionized (DI) water, each at a target concentration of 100 µg/mL.
- Subsequent dilutions of the stock solutions were prepared in DI water 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.
- Ammonium Formate, Sigma-Aldrich, St. Louis, MO
- Formic acid, 88%, ACS, Fisher Scientific, St. Louis, MO
- Methanol, HPLC grade, EMD, Billerica, MA
- Sodium Hydroxide, Avantor, Center Valley, PA
- Sulfuric Acid, VWR, Radnor, PA
- Deionized (DI) water obtained from a PURELAB flex™ water system, ELGA, Marlow, UK

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)
- Autoclave, Sterilmatic™, Market Forge Industries, Inc., Everett, MA
- Anaerobic Culture Tubes, 18 x 150 mm with rubber stoppers and crimp-top aluminum seals and crimping tool, VWR, Radnor, PA
- Class A volumetric glassware
- Transfer pipets
- Pipetters, fixed and adjustable volume
- Centrifuge, Model 5810, Eppendorf, Hamburg, Germany
- Centrifuge tubes, plastic, 50 mL, VWR, Radnor, PA

Equipment

- Vortex mixer, VWR, Radnor, PA
- UHPLC System, Acquity UPLC™, Waters Corporation, Milford, MA
- Mass spectrometer detector, TQD™, Waters Corporation, Milford, MA
- UPLC™ autosampler vials, 1.5 mL, glass, snap caps, Waters Corporation, Milford, MA
- UPLC™ column, HSS T3 C18, 100 x 2.1 mm x 1.7 µm, Waters Corporation, Milford, MA
- Instrument Data System, Masslynx™ Version 4.1, Waters Corporation, Milford, MA
- Syringe filters, 0.2 µm, Nylon, VWR, Radnor, PA
- Syringes, 3 cc, plastic, VWR, Radnor, PA
- pH Test Strips, VWR, Radnor, PA

Test Matrix

The method was validated using a commercial rodent diet (Purina Product #5002, Certified Rodent Diet, Purina Mills, Inc., St. Louis, MO). The test matrix material was stored in a freezer (ca. -20°C) when not needed in the laboratory.

Extraction Procedures

- Weighed 1.0 g (±0.05 g) of ground rodent feed into an anaerobic culture tube.
- Laboratory fortified feed sub-samples were spiked with 0.25 mL of a 1 µg/mL biotin working standard solution.
- All samples were spiked with 0.25 mL of a 1 µg/mL internal standard working solution.
- 10 mL of 3N sulfuric acid were added.
- The tube was stoppered and secured with an aluminum crimp-top enclosure.
- The contents of the tube were vortex mixed for about 10 seconds.
- Samples were autoclaved at 121°C for 30 minutes.

Extraction Procedures

- After cooling to room temperature, the tube contents were quantitatively transferred to a 50 mL plastic centrifuge tube using multiple DI water rinses.
- The pH of the sample solution was adjusted to fall within the range of pH 3-6 using 6N sodium hydroxide. pH strips were used to verify the pH.
- The final volume was adjusted to 25 mL with DI water.
- The sample was centrifuged for 10 minutes at 3,000 rpm.
- Approximately 1-1.5 mL of the supernatant was filtered through a 0.2 µm syringe filter into a UPLC™ autosampler vial.

UHPLC-MS/MS Parameters

- Flow Rate: 0.3 mL/min.
- Injection Volume: 3 µL
- Colum Temp.: 30°C
- MS Source: Electrospray
- MS Polarity: Positive
- MS Mode: Multiple Reaction Monitoring (MRM)
- Expected Retention Times (Figures 1a-1c): 11-12 minutes
- HPLC Gradient: Linear

UHPLC-MS/MS Parameters

Time (min.)	%A	%B
0.00	100	0
2.00	100	0
12.00	65	35
13.00	65	35
13.10	10	90
14.00	10	90
14.10	100	0
16.00	100	0

A: 10 mM Ammonium Formate, 0.1% Formic Acid in DI Water
B: 10 mM Ammonium Formate, 0.1% Formic Acid in Methanol

Mobile phase flow was diverted to waste from 0-11.3 minutes and again from 12.1 to 16 minutes. Flow diversion was used to prevent fouling of the MS source cone.

UHPLC-MS/MS Parameters

Compound	Ion (m/z)		Dwell Time (s)	Cone Voltage	Collision Energy
	Precursor	Product			
Biotin	ca. 245	ca. 227	0.1-0.2	20	15
d_2-Biotin	ca. 247	ca. 229	0.1-0.2	20	15

For each analysis sequence, an internal standard calibration curve was generated over a biotin concentration range of 1-50 ng/mL. Each calibrant solution contained approximately 10 ng/mL of d_2-biotin internal standard. A linear regression calibration curve was constructed using peak area ratios (y-axis) and the standard solution concentrations (x-axis). Sample solution concentrations were calculated using the straight line equation from the calibration curve. A regression weighting function of 1/x was used.

Results/Discussion

Method Validation

Biotin content was determined on a fresh weight basis. The relative standard deviation (RSD) was calculated to determine method precision (Table 1). Two analysts prepared and assayed seven replicate sub-samples of rodent feed on two different days. Multiple analyst precision was defined as the overall RSD for the two data sets. Fortification and recovery was used to determine the accuracy of the method. Two analysts prepared and assayed three laboratory fortified sub-samples of rodent feed on two different days with the seven sub-samples used to determine precision. A single fortification level at a concentration approximately equal to the expected level in the unfortified diet was used (0.025 mg/100 g). In addition, excellent agreement was achieved between the overall mean result (0.0247 mg/100 g) and the certified biotin content (0.025 mg/100 g) (3).

Results/Discussion

Table 1. Precision

Analyst/Day	Mean (mg/100 g)	Std. Dev.	RSD (%)	Range (mg/kg DB)
Analyst 1/Day 1 (n=7)	0.0226	0.00155	6.85	0.0208-0.0247
Analyst 2/Day 2 (n=7)	0.0268	0.00187	6.97	0.0245-0.0290
Overall (n=14)	0.0247	0.00274	11.1	0.0208-0.0290

Results/Discussion

Table 2. Accuracy

Analyst/Day	Mean Recovery (%)	Recovery Range (%)
Analyst 1/Day 1 (n=3)	91.7	85.9-94.7
Analyst 2/Day 2 (n=3)	100	94.3-105
Overall (n=6)	96.1	85.9-105

Results/Discussion

Limit of Quantitation (LOQ):

The method LOQ was established at 0.0025 g/100 g. The LOQ was based on the analyte peak signal to noise ratio (S/N) for feed extracts. S/N at the LOQ was approximately 10.

Linearity:

The linear concentration range was 1-50 ng/mL (Figure 4). Coefficient of determination (r^2) values for the regression curves exceeded 0.995.

Interferences:

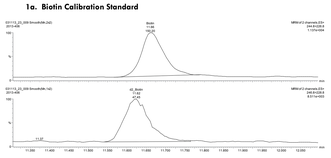
The use of d2-biotin as an internal standard provided adequate compensation for ESI source suppression. No co-eluting peaks were observed in the MRM chromatograms of rodent feed extracts.

Conclusions

A method for the extraction and UHPLC-MS/MS analysis of biotin in rodent feed was developed and validated. The method demonstrated acceptable accuracy, precision, sensitivity, selectivity and linear range. The validated method is now used for routine analysis of rodent feed to support biotech crop feeding trials.

Figure 1. UHPLC-MS/MS Chromatograms

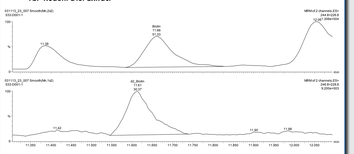
1a. Biotin Calibration Standard



10 ng/mL Biotin, 10 ng/mL d2-Biotin Internal Standard

Figure 1. UHPLC-MS/MS Chromatograms

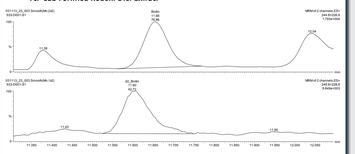
1b. Rodent Diet Extract



9.7 ng/mL Biotin, 10 ng/mL d2-Biotin Internal Standard

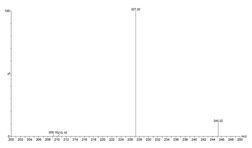
Figure 1. UHPLC-MS/MS Chromatograms

1c. Lab Fortified Rodent Diet Extract



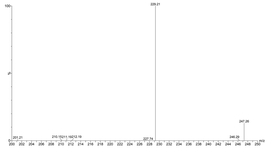
Fortification Level: 0.025 mg/100 g
21.5 ng/mL Biotin, 10 ng/mL d2-Biotin Internal Standard

Figure 2. Product Ion Spectrum of Biotin



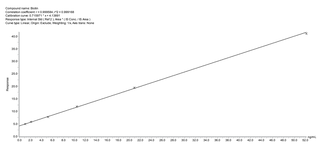
Precursor Ion: m/z 245, Product Ion: m/z 227 (-OH)

Figure 3. Product Ion Spectrum of d_2-Biotin



Precursor Ion: m/z 247, Product Ion: m/z 229 (-OH)

Figure 4. Biotin Linear Range



1-50 ng/mL

References

1) Holler, U.; Wachtel, F.; Wehrli, C.; Fizet, C. Quantification of biotin in feed, food, tablets and premixes using HPLC-MS/MS. *Journal of Chromatography B*. 2006. 831: 8-16.

2) Cotolin, D.; Welbin, C.; Mazzeo, J. UPLC-MS Monitoring of Water-Soluble Vitamin Bs in Cell Culture Media in Minutes. Waters Application Note. 2011.

3) Anonymous. Summary of Nutritional Analyses, Product #5002-Certified Rodent Diet. Purina Mills, Inc.. 2003.