AOAC Official Method 2012.16
Pantothenic Acid (Vitamin B_5) in Infant Formula and Adult/Pediatric Nutritional Formula

Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry Method
First Action 2012

[Applicable to the determination of free pantothenic acid (PA) in infant formula and adult/pediatric nutritional formula.]

Caution: Consult Material Safety Data Sheets prior to using chemicals and adhere to the safety precautions provided. Wear personal protective equipment when necessary.

A. Principle

Extraction of PA using a 0.4 M ammonium acetate buffer solution. After filtration, the final solution is subjected to UPLC-MS/MS.

B. Apparatus

(**a**) *Balances*.—With readability of 0.1 mg, capacity 210 g (AG204; Mettler-Toledo, Greifensee, Switzerland); with readability of 0.1 g, capacity 4100 g (PM4800 DeltaRange; Mettler-Toledo, or equivalent).

(**b**) *pH meter*.—Model 691 (Metrohm, Herisau, Switzerland), with readability of 0.01 pH unit, or equivalent.

(**c**) *Homogenizer*.—Polytron PT3000 (drive unit), Aggregate PT-DA 3012 (Kinematics, Lucerne, Switzerland, or equivalent).

(**d**) *Stir plate with magnetic stirrers*.

(**e**) *Filters*.—Syringe filters, 0.22 µm pore size, 33 mm id, Millex-HV PVDF (Millipore, Bedford, MA). Membrane disc filters, 0.22 µm pore size (Millipore, or equivalent).

(**f**) *Filter paper*.—Grade 597½, or equivalent.

(**g**) *UPLC-MS/MS system*.—Acquity UPLC coupled with triple quadrupole detector equipped with electrospray ionization (ESI) source and T3 column (1.7 µm, 100×2.1 mm id; Waters Corp., Milford, MA, or equivalent).

C. Chemicals and Solvents

(**a**) *Standards*.—*Calcium D-pantothenate*.—Sigma (St. Louis, MO), or equivalent. *Calcium pantothenate-[$^{13}C_6$, $^{15}N_2$]*.—IsoSciences (King of Prussia, PA), or equivalent.

(**b**) *Enzyme*.—α-Amylase, Sigma A3176, from porcine pancreas, about 25 U/mg, or equivalent.

(**c**) *Solvents*.—*Acetonitrile*.—LC/MS grade (Honeywell LC015-1; Muskegon, MI, or equivalent). *Water*.—>18 MΩ.

(**d**) *Ammonium acetate*.—ACS grade, >98% Fluka 9690 (Buchs, Switzerland, or equivalent).

(**e**) *Acetic acid*.—ACS grade (Marcon Chemicals, Center Valley, PA; 3121-46, or equivalent).

(**f**) *Formic acid*.—ACS grade (Sigma 695076, or equivalent).

(**g**) *1% Formic acid in water*.—ACS grade (Honeywell LC452-1, or equivalent).

D. Preparation of Standard Solutions and Reagents

(**a**) *PA stock solution (250 µg/mL)*.—Weigh 54.5 mg calcium pantothenate into a 200 mL volumetric flask (take into account the moisture content given in the supplier's certificate) and dilute to volume with water. Store aliquots at –20°C.

(**b**) *PA intermediate solution (10 µg/mL)*.—Transfer 1 mL PA stock solution into a 25 mL volumetric flask and dilute to volume with water. Store aliquots at –20°C.

(**c**) *Calcium pantothenate-[$^{13}C_6$, $^{15}N_2$] internal standard (IS) stock solution (20 µg/mL)*.—Weigh 5.0 mg calcium pantothenate-[$^{13}C_6$, $^{15}N_2$] into a 250 mL volumetric flask and dilute to volume with water. Store aliquots at –20°C.

(**d**) *Preparation of five-level standard curve*.—Transfer appropriate volumes of the PA intermediate solution (10 µg/mL) into 10 mL volumetric flasks to obtain five different concentrations of PA (0.08, 0.16, 0.32, 0.64, and 1.2 µg/mL). Add 500 µL IS stock solution (20 µg/mL) and dilute to volume. Store aliquots of these solutions at –20°C for no longer than 1 month before use.

(**e**) *Ammonium acetate, 400 mmol/L, pH 3.8 (used for sample extraction)*.—Into a 500 mL beaker, add 30.8±0.10 g ammonium acetate. Add about 300 mL water and stir to dissolve with a magnetic stirrer. Adjust to pH 3.8±0.1, carefully adding glacial acetic acid (about 150 mL is needed). Transfer into a 1000 mL volumetric flask and make up to volume with water. This solution is stable for 1 month at 4°C.

E. Sample Preparation and Extraction

(**a**) *Preparation of food samples*.—Weigh a 25.0 g sample portion of homogeneous solid samples (i.e., powdered infant formula or nutritionals). Add 200 mL water at 40°C before mixing until a homogeneous suspension is obtained. A homogenizer can be used when necessary.

Note: If the product contains starch, add 50 mg α-amylase and incubate for 15 min at 40°C to decrease viscosity and facilitate handling. Mix liquid samples well to ensure homogeneity and continue directly to extraction.

(**b**) *Extraction*.—Weigh a 15.0 g aliquot of homogenized sample suspension (corresponding to 5.0 g sample portion) or 20.0 g liquid sample into a 50 mL volumetric flask. Add a 25 mL volume of a 0.4 M ammonium acetate solution, pH 3.8. Dilute the sample extract to volume with water. Add a stir bar and stir for 10 min. Filter a 20 mL portion through folded paper (grade 597½). Run chromatographic analysis.

F. Analysis

(**a**) *Chromatographic analysis*.—Transfer a 1 mL aliquot of the filtrate obtained in *Sample Preparation and Extraction* (**b**) into a 15 mL polypropylene tube (e.g., Falcon tube) containing 500 µL of the IS stock solution. Dilute the solution to 10.0 ± 1.0 mL with water cap and mix. Filter through a 0.22 µm syringe filter. Inject into the UHPLC-MS/MS system.

(**b**) *UPLC conditions*.—Injection volume, 2 µL; column temperature, 30°C; flow rate, 0.45 mL/min; mobile phase A, 0.1% (v/v) formic acid in water; and mobile phase B, acetonitrile.

The initial mobile phase composition was 92% A and 8% B. The gradient program was a 0 to 2.2 min ramp from 92 to 80% phase A; 2.2 to 2.4 min ramp from 80 to 50% phase A; 50% A hold from 2.4 to 4.0 min; back to the initial mobile phase composition at 4.1 min, and hold until 7.0 min. The HPLC flow was directed into the MS detector only between 0 and 2 min to prevent source fouling as much as possible.

(**c**) *MS/MS conditions*.—Positive ESI; capillary voltage, 2.2 kV; cone, 25 V; extractor, 3.0 V; source temperature, 140°C; desolvation temperature, 350°C; and cone gas flow, 700 L/h.

MS was run in the single-reaction monitoring mode. The transitions monitored between 0 to 2.1 min were *m/z* 220.2→90.1 for PA, and *m/z* 224.2→94.1 for the isotope-labeled IS. The collision energy was set at 14 V. The dwell time for each monitored transition was 0.1 s.

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(**d**) *Identification*.—MS detection in the single-reaction monitoring mode included simultaneous detection of molecular ions corresponding to PA and labeled IS. The selected mass transitions were *m/z* 220.2 → 90.1 and *m/z* 224.2 → 94.1, respectively.

(**e**) *Quantitation*.—Calculate for each standard the peak area ratio between PA and IS. Establish a 5-point calibration curve (ranging from 0.16 to 0.24 ng on column) by plotting peak area ratio versus PA concentration. Calculate the linear regression. It is recommended to use a weighed regression curve (1/x). Calculate the slope (S) and the intercept (I). Calculate the PA concentration, w, in (mg/100 g) using the following equation:

$$w = \frac{(A - I) \times V_1 \times V_3 \times 100}{S \times m \times V_2 \times 1000}$$

where A = peak area ratio PA/IS in the test solution; I = intercept of the calibration curve; S = slope of the calibration curve; V_1 = volume of the of sample extract, in mL (= 50); V_2 = volume of the filtrate pipetted, in mL (= 1); V_3 = final volume of the of the test solution, in mL (= 10 ± 1); m = mass of the test portion, in g; 100 = conversion to 100 g basis; and 1000 = conversion from μg to mg.

References: *J. AOAC Int*. **95**, 143(2012)

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