AOAC Official Method 2016.02
Total Biotin in Infant Formula and Adult/Pediatric Nutritional Formulas
Liquid Chromatography Coupled with Immunoaffinity Column Cleanup Extraction
First Action 2016
Final Action 2017

Codex Type II
(AOAC–ISO Method)*

[Applicable for determination of total biotin in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates).]

Caution: Refer to safety data sheets for all chemicals prior to use. Ensure that all appropriate personal protective equipment is used and follow good laboratory practices.

A. Principle/Methodology

The sample is dispersed in phosphate-buffered saline (PBS) and autoclaved at 121 ± 2°C for 25 min. The sample is cooled to room temperature and then diluted to 100 mL in a volumetric flask. The extract is centrifuged and filtered using Whatman glass microfiber filter paper (GE Healthcare Life Sciences). Clear filtrate is collected for cleanup and extraction. A biotin immunoaffinity column (IAC) is mounted onto an SPE manifold. A disposable syringe barrel is connected to the IAC as a reservoir. The buffer in the affinity column is drained, and the sample filtrate is loaded through the reservoir and allowed to flow through by gravity. The column is washed with PBS followed by water. Air is passed through the column to remove residual liquid.

Biotin/biocytin from the column is eluted with methanol and collected in a Reacti-Vial (Cat. No. 13223; Thermo Scientific). The eluent is evaporated to dryness using a heating block set at 85 ± 5°C under a gentle stream of nitrogen, and the sample is reconstituted in 1 mL water. The biotin and biocytin in the reconstituted sample are analyzed simultaneously by HPLC using a PDA detector set at 200 nm. Identification of peaks is based on absolute retention time. Quantification is by multipoint external calibration using peak area responses of the analytes. Spectrum scan (200–350 nm) can be used for purity and identity confirmation as required.

B. Chemicals

(a) *Laboratory water.*—Reagent grade.
(b) *Sodium dihydrogen phosphate dihydrate.*—CAS 13472-35-0.
(c) *Disodium hydrogen phosphate dihydrate.*—CAS 10028-24-7.
(d) *Sodium hydroxide.*—CAS 1310-73-2.
(e) *Methanol.*—HPLC grade (CAS 67-56-1).
(f) *Acetonitrile.*—HPLC grade (CAS 75-05-8).
(g) *o-Phosphoric acid.*—85% (CAS 7664-38-2).
(h) *PBS.*—pH 7.4 (Cat. No 10010031; Life Technologies/Thermo Scientific or equivalent).
(i) *Biotin.*—Purity ≥99% (Cat. No. B4501; Sigma Chemical Co., St. Louis, MO, USA, or equivalent).
(**j**) *Biocytin*.—Purity ≥98% (Cat. No. B4261; Sigma Chemical Co., or equivalent).

C. Reagent Solution Preparation

(a) *Sodium hydroxide, 2 M*.—Weigh 80 g sodium hydroxide, **B**(**d**), into a 1 L volumetric flask. Using water, **B**(**a**), dissolve and dilute to volume.

(b) *Sodium phosphate buffer, 0.15 M*.—Weigh 9.15 g sodium dihydrogen phosphate dihydrate, **B**(**b**), and 16.31 g disodium hydrogen phosphate dihydrate, **B**(**c**), into a 1 L volumetric flask. Using water, **B**(**a**), dissolve and dilute to volume. Adjust the pH to 7 with 2 M sodium hydroxide, **C**(**a**).

(c) *Phosphoric acid, 0.1%.*—In a 1 L volumetric flask, transfer 500 mL water, **B**(**a**). Add 1.2 mL *o*-phosphoric acid, **B**(**g**). Mix and dilute to volume with water.

D. Apparatus

(a) *Whatman glass microfiber filters.*—Cat. No. 1820-125.
(b) *EASI-EXTRACT Biotin IAC Pack.*—P82/P82B (R-Biopharm Rhône Ltd or equivalent).
(c) *SPE manifold.*—With accessories.
(d) *Autoclave.*—Set at 121°C.
(e) *Centrifuge.*—Variable speed.
(f) *Analytical balance.*—Capable of measuring to four decimal places.
(g) *Amber glass screw-cap bottle.*—100 mL.
(h) *Horizontal shaker.*
(i) *Volumetric flasks.*—1 L and 250, 100, and 10 mL.
(j) *Pipettors.*—Calibrated; 10.0, 5.0, and 1.0 mL and 200, 100, and 50 µL.
(k) *Measuring cylinder.*—100 and 50 mL.
(l) *Reacti-Vials.*—Cat. No. 13223 (Thermo Scientific).
(m) *Reacti-Therm™ heating block.*—With nitrogen blow-down (Thermo Scientific).
(n) *Ultrasonic bath.*—Set at 50°C.
(o) *Centrifuge tubes.*—50 mL.
(p) *Vortex mixer.*
(q) *Syringe filter.*—PTFE, 0.45 µm (Advantec Syringe Filters, Cat. No. 13HP045AN; Cole-Parmer, Vernon Hills, IL, USA).
(r) *Disposable syringes.*—10 and 1 mL.
(s) *HPLC vials.*—2 mL with 200 µL glass inserts.

E. Sample Preparation

Note: For weight and loading volumes for the different ranges of product, *see* Table **2016.02A**. A slurry may be used wherever product heterogeneity is expected or not known.

For the slurry, reconstitute 25 g powder with warm water (approximately 50°C) to a total weight of 200 g. Mix thoroughly on a horizontal shaker for 15 min and then sonicate at 50°C for 10 min. Cool to room temperature. For liquid samples, mix well to ensure homogeneity of the sample portion, and weigh the specified quantity.

(a) Weigh sample/slurry into a 100 mL amber glass screw-cap bottle (*see* Table **2016.02A**).
(b) Add 0.15 M sodium phosphate buffer, **C**(**b**), to a volume of 50 mL.
(c) Swirl gently to mix.
(d) Autoclave the sample preparation at 121°C for 25 min.
(e) Cool the sample to room temperature. Quantitatively transfer the extract into a 100 mL volumetric flask and dilute to volume with 0.15 M sodium phosphate buffer, **C**(**b**),mixing well.
(f) Transfer extract into a centrifuge tube and centrifuge the sample at 4000 rpm for 15 min.
(g) Filter the sample using Whatman glass microfiber filter paper and collect the filtrate.
(h) Set up the SPE manifold. Attach the IAC connected to a 10 mL reservoir. Drain off buffer just above the gel.

Table 2016.02A. Sample preparation

Biotin, µg/100 g		Sample preparation				Concn, µg/100 mL	
Minimum	Maximum	Weight, g	Volume, mL	Load, mL	Final, mL	Minimum	Maximum
0.1	0.5	20	100	50	1	1	5
0.5	1.0	10	100	20	1	1	2
1.0	5.0	10	100	10	1	1	5
5.0	50.0	2.0 (Slurry 16 g)	100	10	1	1	10
50.0	100.0	1.0 (Slurry 8 g)	100	10	1	5	10
100.0	400.0	0.5 (Slurry 4 g)	100	5	1	2.5	10

(i) Load the sample filtrate onto the column as per Table **2016.02A**, and initialize the flow with the help of a vacuum pump.

(j) Turn off the vacuum and let the solution pass through the column by gravity at a rate of 1 drop/s.

(k) Wash the column by passing 10 mL PBS through the column, followed by 10 mL water, **B**(**a**). (*Note*: Initialize the flow with the help of vacuum at every step, and then leave it to flow by gravity.)

(l) Remove any residual liquid from the column by introducing a gentle vacuum.

(m) Introduce a Reacti-Vial and elute the analyte under gravity with 2 mL methanol, **B**(**e**). Elute further with an additional 1 mL methanol. Backflush at least three times when eluting; this can be achieved by a gentle up and down motion of the syringe plunger to maximize the elution.

(n) Evaporate the eluent to dryness using a heating block set at 85 ± 5°C, under a gentle nitrogen blow-down.

(o) Remove from the heating block and cool down to room temperature (about 15 min).

(p) Redissolve with 1 mL water, **B**(**a**), and then cap the Reacti-Vials and mix on a vortex mixer for 30 s.

(**q**) Using a syringe filter, filter sample into a clean glass insert for the HPLC analysis.

F. Standard Preparation

(a) *Stock standard biotin (100 µg/mL).*—Weigh 25 mg biotin reference material into a 250 mL amber volumetric flask. Add 150 mL water, **B**(**a**), and sonicate at room temperature for 90 min with occasional shaking. Dilute to volume with water.

(**b**) *Stock standard biocytin (100 µg/mL).*—Weigh 10 mg biotin reference material into a 100 mL amber volumetric flask. Add 60 mL water, **B**(**a**), and sonicate at room temperature for 90 min with occasional shaking. Dilute to volume with water.

(**c**) *Mixed intermediate standard (100 µg/100 mL).*—Dilute 1 mL each of stock standards, **F**(**a**) and **F**(**b**), to 100 mL with water, **B**(**a**).

(d) *Working standards.*—(*1*) *Standard 1 (1.0 µg/100 mL).*—Dilute 100 µL mixed intermediate standard, **F**(**c**), to 10 mL with water, **B**(**a**).

(*2*) *Standard 2 (2.5 µg/100 mL).*—Dilute 250 µL mixed intermediate standard, **F**(**c**), to 10 mL with water, **B**(**a**).

(*3*) *Standard 3 (5.0 µg/100 mL).*—Dilute 500 µL mixed intermediate standard, **F**(**c**), to 10 mL with water, **B**(**a**).

(*4*) *Standard 4 (7.5 µg/100 mL).*—Dilute 750 µL mixed intermediate standard, **F**(**c**), to 10 mL with water, **B**(**a**).

(*5*) *Standard 5 (10 µg/100 mL).*—Dilute 1 mL mixed intermediate standard, **F**(**c**), to 10 mL with water, **B**(**a**).

(*6*) *Standard 6 (20 µg/100 mL).*—Dilute 2 mL mixed intermediate standard, **F**(**c**), to 10 mL with water, **B**(**a**).

Note: The concentrations given in **F** are indicative only; calculate the actual concentrations of biotin and biocytin in each calibration standard using the following formula:

$$\text{Biotin/biocytin, µg/100 mL} = (W1 \times P \times 10 \times \text{Vis}) \div (V \times 10)$$

where $W1$ = weight of biotin or biocytin (mg); P = percentage purity from the certificate of analysis or verified by United States Pharmacopeia/British Pharmacopoeia/European Pharmacopoeia monographs; Vis = volume of mixed intermediate standard used for the calibration standard (mL); and V = volume of stock standard (250 mL for biotin and 100 mL for biocytin).

G. Chromatographic Conditions

(a) *Mobile phase A.*—0.1% Phosphoric acid.

(b) *Mobile phase B.*—100% Acetonitrile.

(c) *Mobile phase C.*—80% Acetonitrile.

(d) *Column.*—Kinetex Phenyl-Hexyl, 150 × 4.6 mm × 2.6 µm × 100 Å (Cat. No. 00F-4495-E0; Phenomenex, Torrance, CA, USA).

(e) *Column temperature.*—25 ± 2°C.

(f) *Retention times.*—Biocytin, 4.5 to 5.5 min; biotin, 16 to 17 min.

(g) *Run time.*—27 min.

(h) *Detector.*—PDA detector operating at 200 nm (spectrum scan 200–350 nm).

(i) *Injection volume.*—100 µL.

(**j**) *Gradient program.*—*See* Table **2016.02B**.

H. QC

(a) Check system suitability by injecting Standard 3 five times. RSD should be ≤2%.

(b) Run the calibration standards at the beginning and end of the sequence (slope drift ≤2%).

(c) The six-point calibration should give a correlation coefficient ≥0.997.

Table 2016.02B. Gradient program

Time, min	Flow rate, mL/min	Mobile phase A, %	Mobile phase B, %	Mobile phase C, %
0.0	0.6	90	10	0
18.0	0.6	90	10	0
18.5	0.8	0	0	100
24.0	0.8	0	0	100
24.5	0.6	90	10	0
27.0	0.6	90	10	0

(d) Test one in five samples in duplicate. The duplicates should be within the method repeatability.

(e) Inject one of the calibration standards after every five sample injections.

(f) Analyze a reference sample (e.g., National Institute of Standards and Technology Standard Reference Material 1849a) in duplicate.

(g) Identification of biotin peak is based on absolute retention time. Spectrum scan can be used for peak purity confirmation if required.

(**h**) Perform three high-level recoveries with every new batch of IACs.

I. Calculation and Reporting

The chromatography software will automatically calculate the concentration of the sample (µg/100 g), provided the concentration of the standards (µg/100 mL), sample weight (g), and dilution are entered correctly.

Manual calculation can also be performed by using the following equation:

$$\text{Biotin or Biocytin}\,(\mu\text{g}/100\ \text{g}) = \frac{(\text{Sample Area} \times \text{Dilution})}{(\text{Slope} \times \text{Sample weight in grams})}$$

where dilution = 10 (i.e., 100 × 1 ÷ 10): sample is diluted with water to 100 mL, with 10 mL used for IAC cleanup, to a final volume of 1 mL for HPLC analysis (the dilution will be 20 if 5 mL is used for IAC cleanup); slope = the valid slope calculation based on concentration on the *x*-axis and peak area on the *y*-axis; and sample weight = the calculated powder equivalent (g) using the following equation for reconstituted powder samples:

$$\text{Sample weight (powder equivalent) in grams} = (W1 \times W2)/W3$$

where $W1$ = weight of powder sample (g); $W2$ = weight of powder and water or total slurry weight (g); and $W3$ = weight of slurry taken for analysis (g).

For ready-to-feed liquid samples, the sample weight used for extraction is used for the calculation.

Report results to three significant figures (i.e., µg/100 g), or convert to other units as required.

J. Repeatability

The difference between the results of duplicate portions of the same sample tested at the same sequence should not exceed 6% of the mean result.

K. Reproducibility

The difference between the results of duplicate determinations tested on different days should not exceed 12% of the mean result.

L. Uncertainty of Measurement

Uncertainty of the method is calculated as 7%, using appropriate statistical procedure (i.e., square root of the sum of squares of the errors, expressed as a percentage).

M. LOQ

LOQ was calculated based on the lowest working standard and the dilution factor as follows:

$$\text{LOQ} = (1 \times 100)/(20 \times 50) = 0.1\ \mu\text{g}/100\ \text{g}\ (1\ \text{ppb})$$

where 1 = the lowest working standard concentration of 1 µg/100 mL; 100 = volume (mL); 20 = 20 g sample; 50 = volume (mL) loaded on the IAC; and 1 = final volume (mL).

References: *J. AOAC Int*. **99**, 1110(2016)
DOI: 10.5740/jaoacint.16-0155 (First Action)

J. AOAC Int. **101**, 831(2018)
DOI: 10.5740/jaoacint.17-0242 (Final Action)

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J. AOAC Int. **98**, 1044(2015)
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* Method adopted by Codex as a Type II Standard, by AOAC as an *Official Method of Analysis*℠, and by the International Organization for Standardization as ISO 23305:2018.

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