
**Fortified milk powders, infant
formula and adult nutritionals —
Determination of total biotin by
liquid chromatography coupled with
immunoaffinity column clean-up
extraction**

*Poudres de lait fortifié, formules infantiles et produits nutritionnels
pour adultes — Détermination de la teneur en biotine totale
par chromatographie liquide après une purification sur colonne
d'immunoaffinité*



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, in collaboration with AOAC INTERNATIONAL. It is being published by ISO and separately by AOAC INTERNATIONAL. The method described in this document is equivalent to the AOAC Official Method 2016.02: *Determination of Total Biotin by Liquid Chromatography Coupled with Immunoaffinity Column Cleanup Extraction: Multilaboratory Testing, Final Action 2016.02*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Fortified milk powders, infant formula and adult nutritionals — Determination of total biotin by liquid chromatography coupled with immunoaffinity column clean-up extraction

WARNING — The use of this method can involve hazardous materials, operations and equipment. This method does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this method to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This document specifies a method for the quantitative determination of biotin and/or biocytin in fortified milk powders, infant formula and adult nutritionals in solid (i.e. powders) or liquid (i.e. ready-to-feed liquids and liquid concentrates) forms using liquid chromatography coupled with immunoaffinity column clean-up extraction.

Precision data from an interlaboratory study is given in [Annex B](#). A comparison between data obtained with the method in this document and EN 15607 is given in [Annex C](#).

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

adult nutritional

nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein

3.2

infant formula

breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: Codex Standard 72-1981]

4 Principle

The sample is dispersed in sodium phosphate buffer and autoclaved at $121\text{ °C} \pm 2\text{ °C}$ 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 a glass microfibre filter. Clear filtrate is collected for clean-up and

extraction. A biotin immunoaffinity column is mounted onto a solid phase extraction (SPE) manifold. A disposable syringe barrel is connected to the immunoaffinity column 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 phosphate-buffered saline (PBS) followed by water. Air is passed through the column to remove residual liquid.

Biotin and/or biocytin from the column is eluted with methanol and collected in a vial. The eluate is evaporated to dryness using a heating block set at $85\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ under a gentle stream of nitrogen and the sample is re-constituted in 1 ml of water. The biotin and biocytin in the reconstituted sample are analysed simultaneously by HPLC using a photodiode array (PDA) 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. A spectrum scan (200 nm to 350 nm) can be used for the purity and identity confirmation as required.

5 Reagents and materials

5.1 General

During the analysis, unless otherwise stated, only use reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

5.1.1 Laboratory reagent grade water.

5.1.2 Sodium dihydrogen phosphate dihydrate (CAS # 13472-35-0).

5.1.3 Disodium hydrogen phosphate dihydrate (CAS # 10028-24-7).

5.1.4 Sodium hydroxide (CAS #1310-73-2).

5.1.5 Methanol, HPLC grade (CAS # 67-56-1).

5.1.6 Acetonitrile, HPLC grade (CAS # 75-05-8).

5.1.7 Ortho-phosphoric acid, mass fraction $w(\text{H}_3\text{PO}_4) = 85\%$ (CAS # 7664-38-2).

5.1.8 PBS, pH = 7,4 (Thermo Scientific¹⁾ or equivalent).

5.1.9 Biotin, purity $\geq 99\%$ (Cat. No. B4501 Sigma Chemical¹⁾ or equivalent).

5.1.10 Biocytin, purity $\geq 98\%$ (Cat. No. B4261 Sigma Chemical¹⁾ or equivalent).

5.2 Reagent preparation

5.2.1 Sodium hydroxide, substance concentration $c = 2\text{ mol/l}$.

Weigh 80 g of sodium hydroxide into a 1 l volumetric flask, dissolve and make up to the mark with water.

¹⁾ This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

5.2.2 Sodium phosphate buffer, $c = 0,15$ mol/l.

Weigh 9,15 g of sodium dihydrogen phosphate dihydrate and 16,31 g of disodium hydrogen phosphate dihydrate into a 1 l volumetric flask, dissolve and make up to the mark with water. Adjust the pH to 7 with 2 mol/l sodium hydroxide.

5.2.3 Phosphoric acid, $w = 0,1$ %.

Into a 1 l volumetric flask, transfer 500 ml water. Add 1,2 ml of ortho-phosphoric acid. Mix and make up to the mark with water.

5.2.4 Mobile phase A, 0,1 % phosphoric acid in water.

5.2.5 Mobile phase B, 100 % acetonitrile.

5.2.6 Mobile phase C, 80 % acetonitrile.

5.3 Standard preparation

5.3.1 Stock standard biotin, mass concentration $\rho = 100$ µg/ml.

Weigh 25 mg biotin standard in a 250 ml amber volumetric flask. Add 150 ml water and sonicate at room temperature for 90 min with occasional shaking. Make up to volume with water.

5.3.2 Stock standard biocytin, $\rho = 100$ µg/ml.

Weigh 10 mg biocytin standard in a 100 ml amber volumetric flask. Add 60 ml water and sonicate at room temperature for 90 min with occasional shaking. Make up to volume with water.

5.3.3 Mixed intermediate standard, $\rho = 100$ µg/100 ml.

Dilute 1 ml each of stock standards to 100 ml with water.

5.3.4 Calibration standard 1, $\rho = 1,0$ µg/100 ml.

Dilute 100 µl mixed intermediate standard to 10 ml with water.

5.3.5 Calibration standard 2, $\rho = 2,5$ µg/100 ml.

Dilute 250 µl mixed intermediate standard to 10 ml with water.

5.3.6 Calibration standard 3, $\rho = 5,0$ µg/100 ml.

Dilute 500 µl mixed intermediate standard to 10 ml with water.

5.3.7 Calibration standard 4, $\rho = 7,5$ µg/100 ml.

Dilute 750 µl mixed intermediate standard to 10 ml with water.

5.3.8 Calibration standard 5, $\rho = 10$ µg/100 ml.

Dilute 1 ml mixed intermediate standard to 10 ml with water.

5.3.9 Calibration standard 6, $\rho = 20$ µg/100 ml.

Dilute 2 ml mixed intermediate standard to 10 ml with water.

5.4 Calculation of concentration

The concentrations given in 5.3 are indicative only. Calculate the actual concentrations of biotin and biocytin in each calibration standard, in µg/100 ml, using Formula (1). Calibration standards should be bracketed at the beginning and at the end of an analytical run.

$$\rho_{(\text{biotin/biocytin})} = \frac{(m_1 \times P \times 10 \times V_{is})}{(V \times 10)} \quad (1)$$

where

m_1 is the mass of biotin or biocytin, in mg;

P is the percentage purity from the certificate of analysis or verified by USP/BP/Ph Eur monographs;

V_{is} is the volume of mixed intermediate standard used for the calibration standard, in ml;

V is the volume of stock standard, $V = 250$ ml for biotin and $V = 100$ ml for biocytin.

6 Apparatus

Usual laboratory glassware and equipment and, in particular, the following.

6.1 HPLC system, consisting of a PDA detector, low pressure gradient pump system, a sample injector unit, a degasser unit, and a column oven.

6.2 Column, Kinetex Phenyl-Hexyl (Cat. No. 00F-4495-E0, Phenomenex²⁾), 150 mm × 4,6 mm × 2,6 µm × 10 nm.

6.3 Glass microfibre filters (Cat. No. 1820-125, Whatman^{®2)}).

6.4 Immunoaffinity column pack (R-Biopharm Rhone EASI-EXTRACT[®] BIOTIN P82/P82B²⁾ or equivalent).

6.5 SPE manifold, with accessories.

6.6 Autoclave, set at 121 °C.

6.7 Centrifuge, variable speed.

6.8 Analytical balance, four decimal places.

6.9 Amber glass screw-cap bottle, 100 ml.

6.10 Horizontal shaker.

6.11 Volumetric flasks, 1 l, 250 ml, 100 ml and 10 ml.

6.12 Pipettors, calibrated, 10,0 ml, 5,0 ml, 1,0 ml, 200 µl, 100 µl and 50 µl.

2) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

6.13 Measuring cylinder, 1000 ml, 100 ml and 50 ml.

6.14 Reaction vial, Reacti-Vials (Cat. No. 13223, Thermo Scientific²⁾).

6.15 Heating block, Reacti-therm, with nitrogen blow down (Thermo Scientific²⁾).

6.16 Ultrasonic bath, set at 50 °C and room temperature.

6.17 Centrifuge tubes, 50 ml.

6.18 Vortex mixer.

6.19 Syringe filter, polytetrafluoroethylene (PTFE) 0,45 µm.

6.20 Disposable syringes, 10 ml and 1 ml.

6.21 HPLC vials, 2 ml with 200 µl glass inserts.

7 Procedure

7.1 Sample preparation

7.1.1 For mass and loading volumes for the different ranges of product, see [Table 1](#). A slurry may be used wherever product homogeneity is suspected or unknown.

For the slurry, reconstitute 25 g of powder (m_1) with warm water (~50 °C) to a total mass of 200 g (m_2). Mix thoroughly on a horizontal shaker for 20 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.

7.1.2 Weigh the sample/slurry (m_3) into a 100 ml amber glass screw-cap bottle. See [Table 1](#).

7.1.3 Add 0,15 mol/l sodium phosphate buffer to an approximate volume of 50 ml.

7.1.4 Swirl gently to mix.

7.1.5 Autoclave the sample preparation at 121 °C for 25 min.

7.1.6 Cool the sample to room temperature. Quantitatively transfer the extract into a 100 ml volumetric flask and make up to the mark with 0,15 mol/l sodium phosphate buffer, mix well.

7.1.7 Transfer the extract into a centrifuge tube and centrifuge the sample at 4 000 rpm for 15 min.

7.1.8 Filter the sample using the glass microfibre filter paper ([6.3](#)) and collect the filtrate.

7.1.9 Set up the SPE manifold ([6.5](#)). Attach the immunoaffinity column (IAC) connected to a 10 ml reservoir. Drain off buffer just above the gel.

7.1.10 Load the sample filtrate onto the column in accordance with [Table 1](#) and initialize the flow with the help of a vacuum pump.

7.1.11 Turn off the vacuum and let the solution pass through the column by gravity at a rate of one drop per second.

7.1.12 Wash the column by passing 10 ml of PBS (5.1.8) through the column, followed by 10 ml of water. Initialize the flow with the help of vacuum at every step and then leave it to flow by gravity.

7.1.13 Remove any residual liquid from the column by introducing a gentle vacuum.

7.1.14 Introduce a reaction vial (6.14) and elute the analyte under gravity with 2 ml methanol. Elute further with an additional 1 ml of 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.

7.1.15 Evaporate the eluate to dryness using a heating block (6.15) set at $85\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ under a gentle nitrogen blow down.

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

7.1.17 Re-dissolve with 1 ml water and then cap the reaction vial (6.14) and vortex for 30 s. Filter by using a syringe filter into a clean glass insert in a HPLC vial for the HPLC analysis.

Table 1 — Sample mass, dilution and loading volume

Biotin $\mu\text{g}/100\text{g}$		Sample preparation				Conc $\mu\text{g}/100\text{ ml}$	
Min.	Max.	Mass (g)	Volume (ml)	Load (ml)	Final	Min.	Max.
0,1	0,5	20	100	50	1 ml	1	5
0,5	1,0	10	100	20	1 ml	1	2
1,0	5,0	10	100	10	1 ml	1	5
5,0	50,0	2,0 (Slurry 16 g)	100	10	1 ml	1	10
50,0	100,0	1,0 (Slurry 8 g)	100	10	1 ml	5	10
100,0	400,0	0,5 (Slurry 4 g)	100	5	1 ml	2,5	10

7.2 Chromatography

7.2.1 Set-up the HPLC system with the following configuration. Examples of chromatograms of a calibration standard and an infant formula sample used in the interlaboratory study are given in Annex A.

7.2.2 Mobile phase A, 0,1 % phosphoric acid.

7.2.3 Mobile phase B, 100 % acetonitrile.

7.2.4 Mobile phase C, 80 % acetonitrile.

7.2.5 Column, see 6.2.

7.2.6 Column temperature, $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$.

7.2.7 Retention times, biocytin is 4,5 min to 5,5 min and biotin is 16 min to 17 min.

7.2.8 Run time, 27 min.

7.2.9 Detector, a PDA detector operating at 200 nm (spectrum scan 200 nm to 350 nm).

7.2.10 Injection volume, 100 µl.

7.2.11 Form low pressure gradients by mixing the three mobile phases, A, B and C, using the procedure given in [Table 2](#).

Table 2 — Gradient programme

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

7.3 Quality control

7.3.1 Check system suitability by injecting standard 3 five times. RSD should be ≤ 2 %.

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

7.3.4 The six-point calibration should give a correlation coefficient $\geq 0,997$.

7.3.5 Test one in five samples in duplicate. The duplicates should be within the method repeatability.

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

7.3.5 The identification of the biotin peak is based on the absolute retention time. A spectrum scan can be used for peak purity confirmation if required.

7.3.6 Perform three high level recoveries with every new batch of immunoaffinity columns.

8 Calculations

The chromatography software will automatically calculate the concentration of the sample, ρ_b , in µg/100g, provided the concentration of the standards (in µg/100 ml), the sample mass (m) and the dilution are entered correctly. A manual calculation can also be performed using [Formula \(2\)](#):

$$\rho_b = \frac{(S \times D)}{(a \times m)} \quad (2)$$

where

- S is the sample area;
- D is the dilution, 10 ($= 100 \times 1 / 10$) (sample made up to 100 ml, 10 ml used for IAC clean-up to a final volume of 1 ml with water for HPLC analysis; the dilution will be 20 if 5 ml is used for IAC clean-up);
- a is the valid slope calculation based on the concentration on the x-axis and the peak area on the y-axis;
- m is the sample mass (powder equivalent), in g.

Calculate the powder equivalent of the sample mass m , in g, using [Formula \(3\)](#) for reconstituted powder samples:

$$m = \frac{(m_1 \times m_3)}{m_2} \quad (3)$$

where

- m_1 is the mass of powder sample, in g;
- m_2 is the mass of powder and water or total slurry mass, in g;
- m_3 is the mass of slurry taken for analysis, in g.

For ready to feed liquid samples, use the sample mass used for extraction for the calculation.

Report the results to three significant figures, using microgram-per-100-g units or convert to other units as required.

9 Precision

9.1 General

Details of the interlaboratory test of the precision of the method are summarized in [Annex B](#). The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and/or matrices other than those given in [Annex B](#).

9.2 Repeatability

The difference between the results of duplicate portions of the same sample tested on the same day/batch should not exceed 6 % of the mean result.

9.3 Reproducibility

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

10 Uncertainty of measurement

Uncertainty of the method was calculated as 10 %, using an appropriate statistical procedure (square root of the sum of squares of the errors expressed as a percentage).

11 Limit of quantitation

The limit of quantitation ($x_{LOQ} = 0,1 \mu\text{g}/100 \text{ g}$) was calculated based on the lowest working standard concentration and the dilution factor using [Formula \(4\)](#):

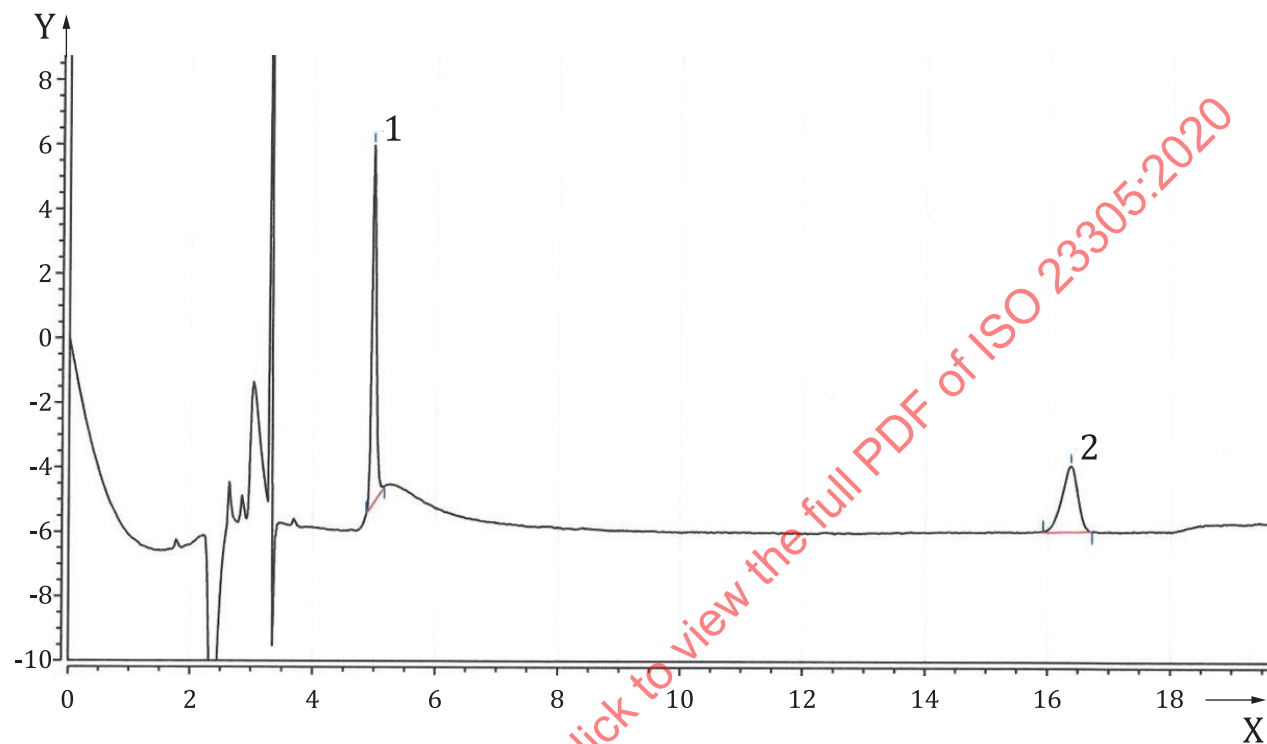
$$x_{LOQ} = \frac{(1 \times 100)}{(20 \times 50)} \quad (4)$$

where

- 1 is 1 $\mu\text{g}/100 \text{ ml}$, the lowest standard;
- 100 is the volume, in ml;
- 20 is 20 g of sample;
- 50 is the volume loaded on the immunoaffinity column, in ml;
- 1 is the final volume, in ml.

Annex A (informative)

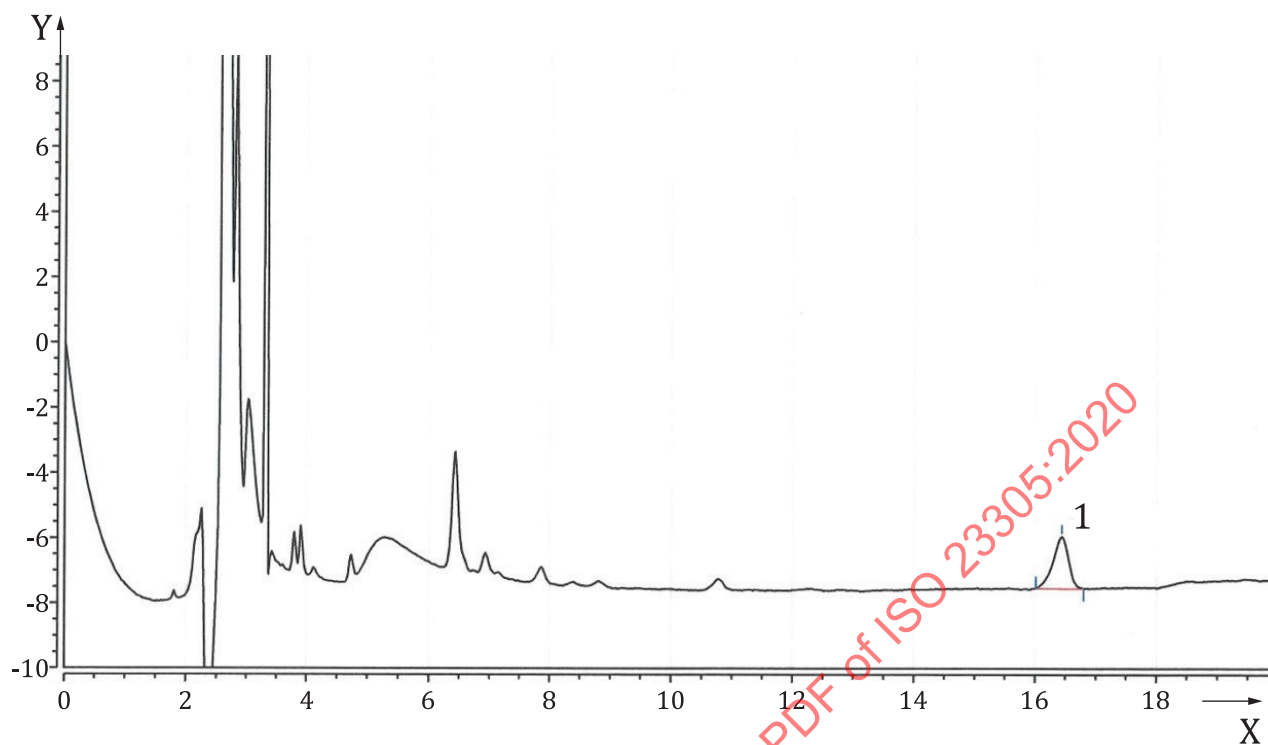
Example chromatograms



Key

- 1 biocytin, at 4,973 min
- 2 biotin, at 16,377 min
- X time, min
- Y absorbance, arbitrary units

Figure A.1 — Chromatogram calibration standard

**Key**

- 1 biotin, at 16,400 min
- X time, min
- Y absorbance, arbitrary units

Figure A.2 — Chromatogram of an infant formula interlaboratory study sample (8^h)

Annex B (informative)

Precision data

The data given in [Table B.1](#) were obtained in an interlaboratory study and published in 2018^[1] in accordance with ISO 5725-2^[2] and the AOAC-IUPAC Harmonized Protocol for collaborative study procedures, to assess precision characteristics of a method of analysis^[3]. The study was performed based on requirements given in Reference ^[4].

Table B.1 — Precision data for biotin

Samples	1 ^a	2 ^b	3 ^c	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h	9 ⁱ	10 ^j	11 ^k	12 ^l
Year of interlaboratory test	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017
Number of laboratories	9	9	9	9	9	9	9	9	9	9	9	9
Number of non-compliant laboratories	0	0	0	0	0	0	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	9	9	9	9	9	9	9	9	9	9	9	9
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0	0	0
Number of accepted results	18	18	18	18	18	18	18	18	18	18	18	18
Mean value, $\bar{x}$ (µg/100 g)	34,01	79,20	4,07	71,46	26,97	44,75	196,5	258,7	166,8	10,12	42,90	52,68
Repeatability standard deviation, s_r	1,88	2,09	0,22	2,77	1,83	2,58	4,68	7,26	11,74	0,58	0,97	2,71
Reproducibility standard deviation, s_R	3,22	4,07	0,31	5,54	2,36	3,11	5,97	18,19	11,74	0,67	2,45	3,10
Coefficient of variation of repeatability, $C_{v,r}$ %	5,53	2,64	5,52	3,88	6,79	5,77	2,38	2,81	7,03	5,74	2,26	5,14

Key

a: infant formula powder partially hydrolysed milk based, b: infant elemental powder, c: infant formula RTF milk based, d: adult nutritional RTF high fat, e: infant formula powder milk based, f: infant formula powder soy based, g: NIST SRM 1849a, h: adult nutritional powder low fat, i: child formula powder, j: toddler formula powder milk based, k: infant formula powder milk based, l: adult nutritional RTF high protein.

Table B.1 (continued)

Samples	1 ^a	2 ^b	3 ^c	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h	9 ⁱ	10 ^j	11 ^k	12 ^l
Coefficient of variation of reproducibility, $C_{V,R}$, %	9,48	5,14	7,59	7,75	8,76	6,96	3,04	7,03	7,03	6,65	5,72	5,89
Repeatability limit, r ($r = 2,8 \times s_r$)	5,3	5,9	0,62	7,8	5,1	7,2	13,1	20,3	32,9	1,6	2,7	7,6
Reproducibility limit, R ($R = 2,8 \times s_R$)	9,0	11,4	0,87	15,5	6,6	8,7	16,7	50,9	32,9	1,9	6,9	8,7
HorRat value ^[5]	0,50	0,31	0,29	0,46	0,45	0,39	0,21	0,51	0,47	0,29	0,31	0,33
Key a: infant formula powder partially hydrolysed milk based, b: infant elemental powder, c: infant formula RTF milk based, d: adult nutritional RTF high fat, e: infant formula powder milk based, f: infant formula powder soy based, g: NIST SRM 1849a, h: adult nutritional powder low fat, i: child formula powder, j: toddler formula powder milk based, k: infant formula powder milk based, l: adult nutritional RTF high protein.												