BRITISH STANDARD

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Foodstuffs — Determination of d-biotin by HPLC

ICS 67.050

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National foreword

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Foreword

This document (EN 15607:2009) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by November 2009, and conflicting national standards shall be withdrawn at the latest by November 2009.

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1 Scope

This European Standard specifies a method for the determination of the mass fraction of d-biotin by high performance liquid chromatography (HPLC). The method has been validated in an inter-laboratory test on fortified and non-fortifed samples such as cereal breakfast powder, infant milk powder, lyophilized green peas with ham, lyophilized chicken soup and on nutritive orange juice, at levels from 16 µg/100 g to 200 µg/100 g. For further information on the validation data, see Annex B.

NOTE 1 d-biocytin can also be estimated by this method. But none of the samples used for the validation step contained d-biocytin. Nonetheless the recovery rate is more than 90 % for d-biotin and d-biocytin, see [2] and [3].

NOTE 2 The method underestimates the real biotin content when used for samples containing egg.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696:1995, *Water for analytical laboratory use – Specification and test methods (ISO 3696:1987)*

3 Principle

D-biotin is extracted from food after an enzymatic treatment and quantified by HPLC with post-column binding reaction, see [2] and [3].

The complexion of d-biotin with avidin appears to be very specific. On that account, this protein, covalently bound to a fluorescent marker, fluorescein 5-isothiocyanate, can be used as a reagent for a post-column binding of d-biotin, see [4] and [5].

4 Reagents

4.1 General

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and water of at least grade 1 according to EN ISO 3696:1995, or double distilled water.

4.2 Chemicals and solutions

4.2.1 Methanol, HPLC grade, mass fraction $w(CH_3OH) \geq 99{,}8$ %

4.2.2 Sulfuric acid solution, substance concentration $c(H_2SO_4) = 1$ mol/l

4.2.3 Sulfuric acid solution, $c(H_2SO_4) = 1{,}5$ mol/l

4.2.4 Citric acid monohydrate, $w(C_6H_8O_7 \cdot H_2O) \geq 99{,}7$ %

4.2.5 Sodium monohydrogen phosphate dihydrate, $w(Na_2HPO_4 \cdot 2H_2O) \geq 99{,}8$ %

4.2.6 Gluthatione, $w(C_{10}H_{17}N_3O_6S) \geq 98$ %

4.2.7 EDTA sodium salt dihydrate, $w(C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O) \geq 99$ %

4.2.8 Potassium monohydrogen phosphate, $w(K_2HPO_4) \geq 96$ %

4.2.9 Potassium dihydrogen phosphate, $w(KH_2PO_4) \geq 99{,}5$ %

4.2.10 Citrate buffer solution

Dissolve 0,462 g of citric acid monohydrate (4.2.4) and 1,05 g of sodium monohydrogen phosphate dihydrate (4.2.5) in 450 ml of distilled water. Adjust the solution to pH = 5,7 with sulfuric acid solution (4.2.3), and then dilute to 500 ml with distilled water. This solution is stable for 1 day.

4.2.11 Gluthatione solution, mass concentration $\rho(C_{10}H_{17}N_3O_6S) = 10$ g/l

Dissolve 30 mg of gluthatione (4.2.6) in 3 ml of distilled water. This solution is stable for 1 day.

4.2.12 EDTA solution, $\rho(C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O) = 10$ g/l

Dissolve 0,1 g of EDTA (4.2.7) in 10 ml of distilled water. This solution is stable for 1 day.

4.2.13 Potassium monohydrogen phosphate solution, $c(K_2HPO_4) = 0{,}1$ mol/l

Dissolve 17,4 g of potassium monohydrogen phosphate (4.2.8) in 1000 ml of water. This solution is stable for 2 days.

4.2.14 Potassium dihydrogen phosphate solution, $c(KH_2PO_4) = 0{,}1$ mol/l

Dissolve 13,6 g of potassium dihydrogen phosphate (4.2.9) in 1000 ml of water. This solution is stable for 2 days.

4.2.15 Phosphate buffer solution pH = 6,0

Mix 4.2.13 and 4.2.14 in such a proportion that the final solution has a pH of 6,0 (e.g. 30 parts per volume of 4.2.13 and 70 parts per volume of 4.2.14). This solution is stable for 1 week at room temperature.

4.2.16 Phosphate buffer solution pH = 7,0

Mix 4.2.13 and 4.2.14 in such a proportion that the final solution has a pH of 7,0 (e.g. 40 parts per volume of 4.2.13 and 60 parts per volume of 4.2.14). This solution is stable for 1 week at room temperature.

4.2.17 Papain powder, (CAS 9001-73-4), enzyme activity is 15 nkat/mg[1] with substrate N-benzoyl-L-arginine ethyl ester (BAEE) at pH = 6,2 and $t = 25\ ^{o}C$. 15 nkat/mg corresponds to 1 U/mg.

4.2.18 Papain solution, ρ(papain) = 20 g/l

4.2.18.1 General

Dissolve 1 g of papain powder (4.2.17) in 50 ml of citrate buffer solution (4.2.10). This solution is stable for 5 days at 4 °C.

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1 Katal (symbol "kat") is a derived SI unit of enzyme activity. One katal is that catalytic activity which will raise the rate of reaction by one mol/s in a specified assay system.

4.2.18.2 Activity check of papain

The activity of papain can be checked by making a second extract (see 6.2) with a double amount of enzyme. Verify that the level of d-biotin calculated is the same and not higher.

NOTE For the interlaboratory study, the papain powder from VWR International GmbH, Hilpertstraße 20a, 64295 Darmstadt, ref. nr. 26.146.180 has been used [2].

4.2.19 Avidin fluoresceine isothiocyanate (Avidin-FITC), labelled, 80 % protein, 2 mol to 4 mol FITC per mol of avidin

4.2.20 Stock solution reagent for post-column binding reaction, ρ(avidin-FITC) = 50 mg/ml

Dissolve 2,5 mg of avidin-FITC (4.2.19) in 50 ml of phosphate buffer solution pH = 7,0 (4.2.16). This solution is stable for 2 weeks at 4 °C.

4.2.21 Reagent for post-column binding reaction, ρ(avidin-FITC) = 2 mg/ml

Add 600 ml of phosphate buffer solution pH = 7,0 (4.2.16) to 25 ml of the stock solution (4.2.20). Filter this solution through a 0,45 µm filter (5.5). This solution is stable for 8 hours, screened from light.

4.2.22 HPLC mobile phase

Mix 80 parts per volume of phosphate buffer solution pH = 6,0 (4.2.15) with 20 parts per volume of methanol (4.2.1). Filter this solution through a 0,45 µm filter (5.5).

4.2.23 Taka-diastase from *Aspergillus Oryzae*, enzyme activity is 1 500 nkat/mg (1 500 nkat/mg corresponds to 100 U/mg), suitable for samples with a high starch content.

4.3 Standard substances

4.3.1 General

D-biotin and d-biocytin can be obtained from various suppliers. The baseline separation of d-biotin and d-biocytin shall be verified. So it is necessary to prepare a standard solution.

The biotin content of the standard can be confirmed according to the European Pharmacopoeia procedure [6].

4.3.2 d-biotin, $w(C_{10}H_{16}N_2O_3S) \geq 99$ %

4.3.3 d-biocytin, $w(C_{16}H_{28}N_4O_4S) \geq 98$ %

4.4 Stock solutions

4.4.1 d-biotin, $\rho(C_{10}H_{16}N_2O_3S) = 100$ µg/ml

Dissolve an amount of the d-biotin standard substance (4.3.2), approximately 10 mg to the nearest 0,1 mg in 100 ml of distilled water. It may take 4 h to 5 h to dissolve. This solution is stable for 2 months at –18 °C.

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2 This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.4.2 d-biocytin, $\rho(C_{16}H_{28}N_4O_4S)$ = 100 µg/ml

Dissolve an amount of the d-biocytin standard substance (4.3.3), approximately 10 mg to the nearest 0,1 mg in 100 ml of distilled water. This solution is stable for 2 months at –18 °C.

4.5 Standard solutions

4.5.1 d-biotin solution, $\rho(C_{10}H_{16}N_2O_3S)$ = 0,05 µg/ml to 0,30 µg/ml

Prepare for example a solution with 1 ml of the stock solution (4.4.1) in 10 ml of distilled water. Then prepare six calibration solutions (0,5 ml, 1,0 ml, 1,5 ml, 2,0 ml, 2,5 ml and 3 ml) in 100 ml of distilled water. These solutions are stable for 1 day.

4.5.2 d-biocytin solution, $\rho(C_{16}H_{28}N_4O_4S)$ = 0,30 µg/ml

Prepare for example a solution with 1 ml of the stock solution (4.4.2) in 10 ml of distilled water. Then prepare a standard solution with 3 ml in 100 ml of distilled water. Solution is stable for 1 day.

5 Apparatus

5.1 General

Usual laboratory apparatus and glassware, and the following.

5.2 Oven

Capable of maintaining a temperature of 37 °C ± 2 °C.

5.3 HPLC system

Consisting of a pump, sample injecting device, fluorescence detector with excitation wavelength set at 490 nm and emission wavelength set at 520 nm, and an evaluation system such as an integrator.

5.4 Analytical reverse-phase separating column, e.g. LiChrospher® 100 RP-18 endcapped [3]

The column shall ensure a baseline resolution of the analytes concerned with the following characteristics:

a) a length of 250 mm;

b) an inner diameter of 4,0 mm;

c) a particle size of 5 µm.

Other particle sizes or column dimensions than specified in this European Standard may be used. Separation parameters shall be adapted to such other materials to guarantee equivalent results.

3 LiChrospher® 100 RP-18 endcapped is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.5 Filter device

Large and small scale filter devices to filter HPLC mobile phases and sample solutions respectively, e.g. of 0,45 µm pore size is appropriate.

NOTE Filtering of the mobile phase as well as of the sample test solution through a membrane filter prior to use or injection can increase longevity of the columns.

5.6 Post-column reactor derivatization system

Consisting of a suitable reagent delivery system, a T-type connecting tube followed by a knitted open-tubular (KOT) reactor with a length of 10 m made of polytetrafluoroethylene (PTFE) tubing with an inner diameter of 0,5 mm and a helix diameter of 14 mm prepared according to for example [7], (KOT2 model). Knitted open-tubular reactors can commercially be obtained from for example Supelco [4)] or MicroSolv Tech [4].

6 Procedure

6.1 Sample preparation

Homogenize the test sample. Grind coarse material with an appropriate mill and mix again. Measures such as pre-cooling shall be taken to avoid exposing to high temperature for long periods of time.

6.2 Extraction

Weigh an appropriate amount of the test sample to the nearest mg, e.g. 0,5 g to 10 g (equivalent from 2 µg to 15 µg of d-biotin in the test portion), in a conical flask. Add 300 µl of the gluthatione solution (4.2.11), 300 µl of the EDTA solution (4.2.12), 30 ml of the citrate buffer solution (4.2.10) and 3 ml of the papain solution (4.2.18). If the sample contains high amounts of starch, add 100 mg of taka-diastase (4.2.23). Incubate the solution overnight at 37 °C in an oven with continuous stirring. After cooling, transfer the solution to a volumetric flask and dilute to 50 ml with distilled water. Mix the solution and filter through a paper filter. Filter again through a 0,45 µm (5.5) filter before injection.

NOTE Filtering of the mobile phase as well as the test sample solution through a membrane filter prior to use or injection can increase the longevity of the column.

6.3 Chromatography

Inject equal volumes of the calibration solutions and the sample solutions into the HPLC-system. Identify d-biotin by comparison of the retention time of the individual peak in the chromatograms obtained with the sample test solution, and with the standard test solution. Adding the standard substance to the sample test solution can also perform peak identification.

Due to limited stability of the binding reagent (4.2.21) check it during chromatography runs with regular injections of the standard solution.

The separation and the quantification were proven to be satisfactory if the following experimental conditions are followed:

Stationary phase: LiChrospher® 100 RP-18 endcapped, 5 µm, 250 mm x 4,0 mm;

4 This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can show to lead to the same results.

Mobile phase:	8 parts per volume of phosphate buffer solution pH = 6 (4.2.15) and 2 parts per volume of methanol (4.2.1);
Flow rate:	0,4 ml/min;
Injection volume:	30 µl;
Fluorescence detection:	excitation wavelength: 490 nm; emission wavelength: 520 nm;
Flow rate post-column derivatization reagent:	1 ml/min

NOTE This procedure can also be used to estimate d-biocytin.

7 Calculation

To carry out a determination by external calibration, integrate the peak areas or peak heights and use a second degree calibration curve.

Calculate the mass fraction, w, of d-biotin in µg/100 g of the sample using Equation (1):

$$w = \frac{\rho \times V_e}{m_s} \times 100 \quad (1)$$

where

ρ is the mass concentration of d-biotin in the sample test solution (6.2), in microgram per millilitre calculated with the second degree calibration curve;

V_e is the volume of the test solution (6.2), in millilitre;

m_s is the sample mass, in gram;

100 is the factor to calculate the content per 100 g.

8 Precision

8.1 General

The precision data of this HPLC method for the determination of d-biotin were established according to ISO 5725-2, see bibliography, [1], in 2000 by a French collaborative study organised by the CGd'UMA (Commission Générale d'Unification des Méthodes d'Analyses), see [3]. The calibration curves used by all the participants were calculated with three calibration points. The study provided the statistical information shown in Annex B.

8.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5 % of the cases.

The values for d-biotin are:

Cereal breakfast powder:	$\bar{x}$ = 197 µg/100 g	r = 25,1 µg/100 g
Infant milk formula:	$\bar{x}$ = 16,0 µg/100 g	r = 5,24 µg/100 g

Fortified orange juice:	$\bar{x}$ = 40,7 µg/100 g	*r* = 2,51 µg/100 g
Lyophilized mashed green peas with ham:	$\bar{x}$ = 88,9 µg/100 g	*r* = 8,99 µg/100 g
Lyophilized chicken soup:	$\bar{x}$ = 168 µg/100 g	*r* = 19,4 µg/100 g

8.3 Reproducibility

The absolute difference between two single test results obtained on identical material reported by two laboratories will exceed the reproducibility limit *R* in not more than 5 % of the cases.

The values for d-biotin are:

Cereal breakfast powder:	$\bar{x}$ = 197 µg/100 g	*R* = 96,7 µg/100 g
Infant milk formula:	$\bar{x}$ = 16,0 µg/100 g	*R* = 13,5 µg/100 g
Fortified orange juice:	$\bar{x}$ = 40,7 µg/100 g	*R* = 22,8 µg/100 g
Lyophilized mashed green peas with ham:	$\bar{x}$ = 88,9 µg/100 g	*R* = 44,1 µg/100 g
Lyophilized chicken soup:	$\bar{x}$ = 168 µg/100 g	*R* = 69,5 µg/100 g

9 Test report

The test report shall contain at least the following data:

a) all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) a reference to this European Standard;

c) the date and type of sampling procedure (if known);

d) the date of sample receipt;

e) the date of test;

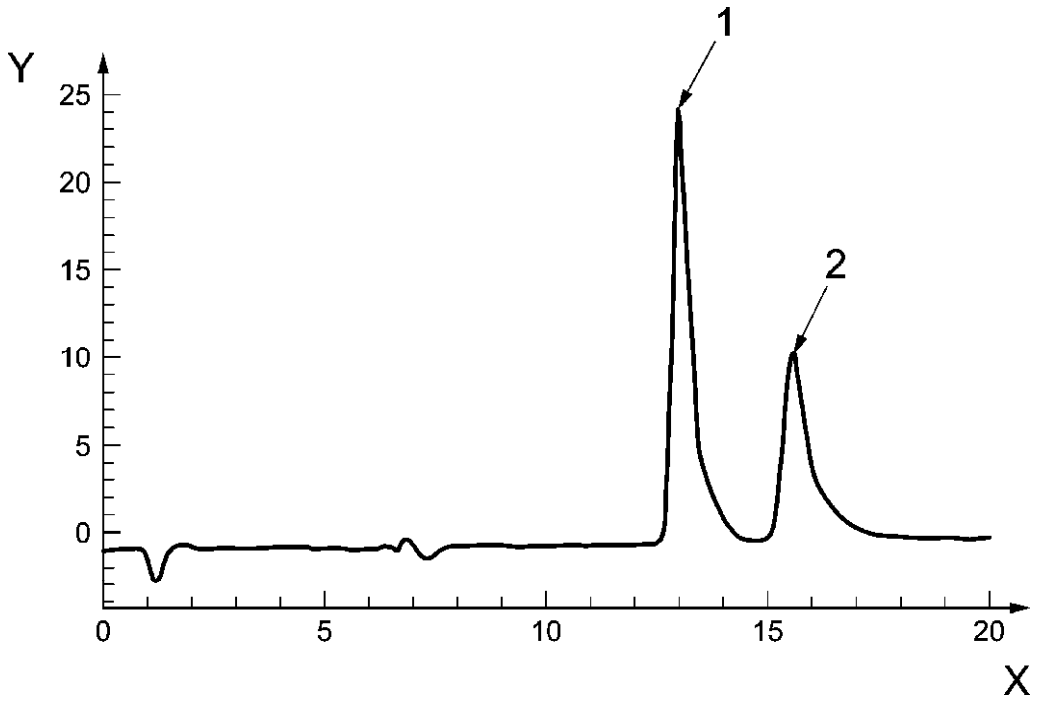
f) the test results and the units in which they have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional which might have effected the results.

Annex A
(informative)

Typical chromatogram



Key

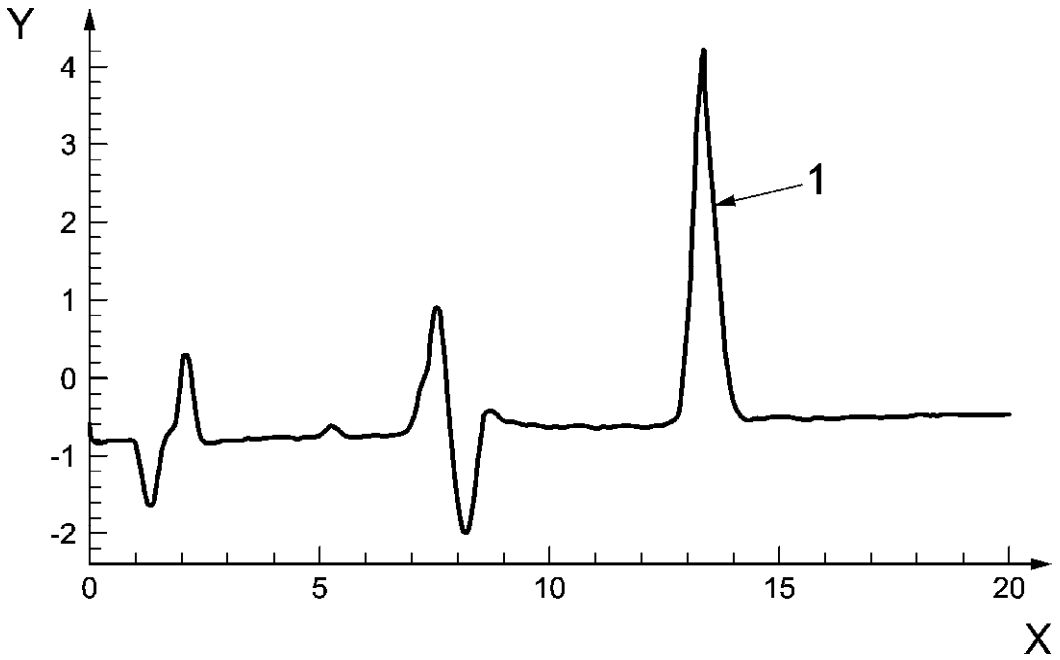
X Time in min
Y Fluorescence intensity
1 d-biotin
2 d-biocytin

Figure A.1 — Example of a HPLC separation of d-biotin and d-biocytin standards using post-column derivatization

Experimental conditions for Figure A.1 are:

Stationary phase:	LiChrospher® 100 RP-18 endcapped, 5 µm, 250 mm x 4,0 mm;
Mobile phase:	8 parts per volume of phosphate buffer solution pH = 6 (4.2.15) and 2 parts per volume of methanol (4.2.1);
Flow rate:	0,4 ml/min;
Injection volume:	30 µl;
Fluorescence detection:	excitation wavelength: 490 nm; emission wavelength: 520 nm;

Flow rate for reagent (post-column derivatization): 1 ml/min



Key

X Time in min
Y Fluorescence intensity
1 d-biotin

Figure A.2 — Typical chromatogram of d-biotin in an infant milk formula sample using post-column derivatization

Experimental conditions for Figure A.2 are:

Stationary phase:	LiChrospher® 100 RP-18 endcapped, 5 µm, 250 mm x 4,0 mm;
Mobile phase:	8 parts per volume of phosphate buffer solution pH = 6 (4.2.15) and 2 parts per volume of methanol (4.2.1);
Flow rate:	0,4 ml/min;
Injection volume:	30 µl;
Fluorescence detection:	excitation wavelength: 490 nm; emission wavelength: 520 nm;
Flow rate for reagent (post-column derivatization):	1 ml/min

Annex B
(informative)

Precision data

The following data were obtained in an interlaboratory study organized by CGd'UMA (Commission Générale d'Unification des Méthodes d'Analyses) in 2000, see [3] and [8]. It was organized in accordance with ISO 5725-2 [1]. The calibration curves used by all the participants were calculated with 3 calibration points.

Table B.1 — Precision data for cereal breakfast (powder), infant milk powder, fortified orange juice, lyophilized mashed green peas with ham and lyophilized chicken soup

Samples	**Cereal breakfast powder**	**Infant milk powder**	**Fortified orange juice**	**Lyophilized mashed green peas with ham**	**Lyophilized chicken soup**
Year of interlaboratory test	2000	2000	2000	2000	2000
Number of laboratories	10	10	10	10	10
Number of samples	2	2	2	2	2
Number of laboratories retained after eliminating outliers	10	9	10	10	10
Number of outliers	0	1	0	0	0
Number of accepted results	20	18	20	20	20
Mean value, $\bar{x}$, µg/100g	197	16,0	40,7	88,9	168
Repeatability standard deviation s_r, µg/100g	8,85	1,85	0,89	3,18	6,84
Repeatability relative standard deviation, RSD_r, %	4,5	11,6	2,2	3,6	4,1
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/100g	25,1	5,24	2,51	8,99	19,4
Reproducibility standard deviation s_R, µg/100g	34,2	4,76	8,05	15,6	24,6
Reproducibility relative standard deviation, RSD_R, %	17,4	29,8	19,8	17,5	14,6
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/100g	96,7	13,5	22,8	44,1	69,5
Horrat values [8]	1,2	1,4	1,1	1,1	1,0

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