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**GB**

NATIONAL STANDARD OF THE  
PEOPLE'S REPUBLIC OF CHINA

**GB 5009.259-2023**

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**National Food Safety Standard - Determination of Biotin in  
Foods**

食品安全国家标准 食品中生物素的测定

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# National Food Safety Standard - Determination of Biotin in Foods

## 1 Scope

This Standard specifies the methods for the determination of biotin in foods.

Method 1 - liquid chromatography - tandem mass spectrometry is applicable to the determination of biotin in prepared milk powder and special dietary foods.

Method 2 - microbiological method is applicable to the determination of biotin in foods.

## Method I - Liquid Chromatography - Tandem Mass Spectrometry

## 2 Principle

The specimen is dissolved and extracted, and the starch-containing specimen is subject to enzymatic hydrolysis by amylase, protein precipitation and centrifugal filtration, and separated on a C<sub>18</sub> reversed-phase chromatography column. Adopt the liquid chromatography - tandem mass spectrometry multi-ion reaction monitoring mode for detection, and the isotope dilution internal standard method for quantitative determination.

## 3 Reagents and Materials

Unless it is otherwise specified, the reagents used in this Method are all analytically pure, and the water is Grade-1 water specified in GB/T 6682.

### 3.1 Reagents and Materials

**3.1.1** Formic acid (HCOOH): chromatographically pure.

**3.1.2** Acetonitrile (CH<sub>3</sub>CN): chromatographically pure.

**3.1.3** Ethanol (CH<sub>3</sub>CH<sub>2</sub>OH): chromatographically pure.

**3.1.4** Ammonium formate (HCOONH<sub>4</sub>): chromatographically pure.

**3.1.5** Perchloric acid (HClO<sub>4</sub>): 70% ~ 72%.

**3.1.6** Sodium hydroxide (NaOH): purity ≥ 99.9%.

**3.1.7** Amylase: Taka-amylase, CAS: 9001-19-8, enzyme activity  $\geq 100$  U/mg.

## **3.2 Preparation of Reagents**

**3.2.1** 0.1% formic acid-10 mmol/L ammonium formate aqueous solution: weigh-take 0.63 g of ammonium formate, use 100 mL of water to dissolve it, then, transfer it into a 1,000 mL reagent bottle, add 1 mL of formic acid, use water to dilute to 1,000 mL, shake it well and reserve it for later use.

**3.2.2** Sodium hydroxide solution (2 mol/L): weigh-take 8.00 g of sodium hydroxide in a beaker, add 100 mL of water to dissolve it, shake it well and reserve it for later use.

**3.2.3** Ethanol solution (50%): accurately measure-take 500 mL of ethanol into a 1,000 mL reagent bottle, use water to dilute it to 1,000 mL, shake it well and reserve it for later use.

## **3.3 Reference Material**

**3.3.1** Biotin reference material ( $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$ ): CAS: 58-85-5, purity  $\geq 98\%$ , or a standard substance certified by the state and awarded a reference material certificate.

**3.3.2** Biotin- $\text{D}_4$  ( $\text{C}_{10}\text{D}_4\text{H}_{12}\text{N}_2\text{O}_3\text{S}$ ): CAS: 1217850-77-5, purity  $\geq 98\%$ .

## **3.4 Preparation of Standard Solutions**

**3.4.1** Biotin standard stock solution (100  $\mu\text{g/mL}$ ): in accordance with purity conversion, accurately weigh-take 10.00 mg of biotin reference material (accurate to 0.01 mg), use ethanol solution (50%) to dissolve it and reach a constant volume of 100 mL. Transfer the solution to a brown glass bottle, seal and store it at  $-20^\circ\text{C}$ . It shall remain valid for 3 months.

**3.4.2** Biotin standard intermediate solution (10  $\mu\text{g/mL}$ ): accurately draw-take 5.00 mL of biotin standard stock solution (100  $\mu\text{g/mL}$ ) into a 50 mL volumetric flask; use ethanol solution (50%) to reach a constant volume of 50 mL. Transfer the solution to a brown glass bottle, seal and store it at  $-20^\circ\text{C}$ . It shall remain valid for 3 months.

**3.4.3** Biotin standard intermediate solution (1  $\mu\text{g/mL}$ ): accurately draw-take 1.00 mL of biotin standard intermediate solution (10  $\mu\text{g/mL}$ ) into a 10 mL volumetric flask; use ethanol solution (50%) to reach a constant volume of 10 mL. Transfer the solution to a brown glass bottle, seal and store it at  $-20^\circ\text{C}$ . It shall remain valid for 3 months.

**3.4.4** Biotin standard working solution (100 ng/mL): accurately draw-take 1.00 mL of the standard intermediate solution (1  $\mu\text{g/mL}$ ) and use mobile phase A to reach a constant volume of 10 mL. Transfer the solution to a brown glass bottle, seal and store it at  $4^\circ\text{C}$ . It shall remain valid for 1 month.

**3.4.5** Biotin standard working solution (10 ng/mL): accurately draw-take 1.00 mL of biotin standard working solution (100 ng/mL) and use mobile phase A to reach a constant volume of 10 mL. Prepare it right before use.

non-uniform samples, until all of them pass through a 2 mm aperture test sieve. After evenly mixing, divide the samples to 100 g and store in a wide-mouth bottle. Seal it and reserve it for testing. For uniform samples, directly evenly mix them and reserve them for testing.

#### **5.1.2 Semi-solid samples**

The sampling size needs to be greater than 0.5 kg. At least 3 packages (from the same batch) need to be collected. After all samples are homogenized and evenly mixed in a container, store any 100 g of them in a wide-mouth bottle. Seal it and reserve it for testing.

#### **5.1.3 Liquid samples**

The sampling size needs to be greater than 0.5 L. At least 3 packages (from the same batch) need to be collected. After all samples are evenly mixed in a container, store any 100 mL of them in a wide-mouth bottle. Seal it and reserve it for testing.

### **5.2 Pre-treatment of Samples**

#### **5.2.1 Starch-free specimens**

Accurately weigh-take 2 g ~ 5 g (accurate to 0.001 g) of sample and place it in a 50 mL centrifuge tube. Add 750  $\mu$ L of isotope internal standard working solution, then, add 30 mL of warm water (35  $^{\circ}$ C ~ 40  $^{\circ}$ C); oscillate and evenly mix it, and conduct ultrasonic extraction for 15 min. After taking it out, quickly cool it to room temperature, and use perchloric acid to adjust pH to about 1.6. At 4  $^{\circ}$ C, at 8,500 r/min, centrifuge for 10 min. After filtering through glass fiber, use sodium hydroxide solution to adjust pH to  $4.6 \pm 0.1$ . Transfer the sample solution to a 50 mL volumetric flask, use water to reach a constant volume to the scale and evenly mix it. Then, transfer-take 1.5 mL of the extracting solution to a 2 mL centrifuge tube. At 10,000 r/min, centrifuge for 10 min. Filter the sample solution through a 0.22  $\mu$ m water-based filter membrane and analyze it on the machine.

#### **5.2.2 Starch-containing specimens**

Accurately weigh-take 2 g ~ 5 g (accurate to 0.001 g) of sample and place it in a 50 mL centrifuge tube. Add 1% of the sample amount of amylase and 750  $\mu$ L of isotope internal standard working solution, then, add 30 mL of warm water (35  $^{\circ}$ C ~ 40  $^{\circ}$ C), oscillate and evenly mix it. Place it in a 50  $^{\circ}$ C ~ 60  $^{\circ}$ C incubator for about 30 min, take it out and perform ultrasonic extraction for 15 minutes. After taking it out, quickly cool it to room temperature, and use perchloric acid to adjust pH to about 1.6. At 4  $^{\circ}$ C, at 8,500 r/min, centrifuge for 10 min. After filtering through glass fiber, use sodium hydroxide solution to adjust pH to  $4.6 \pm 0.1$ . Transfer the sample solution to a 50 mL volumetric flask, use water to reach a constant volume to the scale and evenly mix it. Then, transfer-take 1.5 mL of the extracting solution to a 2 mL centrifuge tube. At 10,000 r/min, centrifuge for 10 min. Filter the sample solution through a 0.22  $\mu$ m water-based filter membrane and analyze it on the machine.

### **5.3 Reference Conditions of Instrument Determination**

$X$ ---the content of biotin in the specimen, expressed in ( $\mu\text{g}/100\text{ g}$ );

$\rho$ ---the mass concentration of biotin in the specimen calculated based on the standard curve, expressed in ( $\text{ng}/\text{mL}$ );

$V$ ---the final constant volume of the specimen solution, expressed in ( $\text{mL}$ );

$m$ ---the mass of the specimen, expressed in ( $\text{g}$ );

100---the conversion factor for the specimen weight per 100 g;

1,000---the conversion factor for converting ng into  $\mu\text{g}$  in the specimen.

The results shall retain 3 significant figures.

## 7 Precision

The absolute difference between the results of two independent determinations obtained under repeatability conditions shall not exceed 15% of the arithmetic mean.

## 8 Others

When the sampling size is 5.0 g and the constant volume is 50 mL, the detection limit of this Method is  $0.300\text{ }\mu\text{g}/100\text{ g}$ , and the quantitation limit is  $1.00\text{ }\mu\text{g}/100\text{ g}$ .

# Method II - Microbiological Method

## 9 Principle

Biotin is an essential nutrient for the growth of *Lactiplantibacillus plantarum*. In the biotin determination culture medium, the growth of *Lactiplantibacillus plantarum* is correlated with the biotin content. In accordance with the standard curve of biotin content and absorbance, calculate the biotin content in the specimen.

## 10 Reagents and Materials

Unless it is otherwise specified, the reagents used in this Method are all analytically pure, and the water is Grade-2 or Grade-1 water specified in GB/T 6682.

### 10.1 Strain

*Lactiplantibacillus plantarum* [the former *Lactobacillus plantarum*] ATCC 8014, or equivalent strain.

## 10.2 Culture Media

**10.2.1** Lactobacillus agar culture medium: see B.1 in Appendix B.

**10.2.2** Lactobacillus broth culture medium: see B.2 in Appendix B.

**10.2.3** Medium for biotin determination: see B.3 in Appendix B.

**NOTE:** commercially available synthetic media can be used and operated in accordance with the instructions.

## 10.3 Reagents

**10.3.1** Absolute ethanol ( $C_2H_5OH$ ).

**10.3.2** Sulfuric acid ( $H_2SO_4$ ): 95% ~ 98%.

**10.3.3** Sodium hydroxide ( $NaOH$ ).

**10.3.4** Sodium chloride ( $NaCl$ ).

## 10.4 Preparation of Reagents

**10.4.1** Ethanol solution (50%): measure-take 500 mL of absolute ethanol, add it to water and reach a constant volume of 1,000 mL.

**10.4.2** Sulfuric acid solution A (3.0 mol/L): measure-take 163.2 mL of sulfuric acid, add it to water and reach a constant volume of 1,000 mL.

**10.4.3** Sulfuric acid solution B (1.0 mol/L): measure-take 54.4 mL of sulfuric acid, add it to water and reach a constant volume of 1,000 mL.

**10.4.4** Sulfuric acid solution C (0.5 mol/L): measure-take 27.2 mL of sulfuric acid, add it to water and reach a constant volume of 1,000 mL.

**10.4.5** Sodium hydroxide solution A (10 mol/L): weigh-take 400 g of sodium hydroxide, add it to water and reach a constant volume of 1,000 mL.

**10.4.6** Sodium hydroxide solution B (0.1 mol/L): draw-take 10 mL of sodium hydroxide solution A (10 mol/L), add water to reach a constant volume of 1,000 mL.

**10.4.7** Sterile physiological saline: weigh-take 8.5 g of sodium chloride and dissolve it in 1,000 mL of distilled water, divide it into stoppered test tubes, with 10 mL in each tube. At 121 °C, perform autoclaved sterilization for 15 min.

**NOTE:** when preparing sulfuric acid solution, prepare in a fume hood, wear gloves and pay attention to safety. Slowly inject concentrated sulfuric acid into water and keep stirring it.

## 10.5 Reference Material

**11.8** Refrigerator: 2 °C ~ 8 °C.

**11.9** Sterile microplate.

**11.10** Quantitative filter paper: with a diameter of 90 mm.

**11.11** Test tube: 18 mm × 180 mm.

**11.12** Volumetric flask: with a capacity of 100 mL, 250 mL and 500 mL.

**11.13** One-mark pipette: 1 mL, 5 mL and 10 mL.

**11.14** Graduated pipette: 5 mL (with a scale of 0.1 mL).

**11.15** Glass funnel: with a diameter of 100 mm.

**11.16** Conical flask: with a capacity of 250 mL.

**11.17** Beaker: with a capacity of 100 mL.

**11.18** Dispenser: 0 mL ~ 10 mL.

**11.19** Micropipette: 1,000 µL and 200 µL.

**11.20** Sterile centrifuge tube: 1.5 mL.

**11.21** Syringe filter: with an aperture of 0.22 µm.

**NOTE:** the cleaned glassware and metal appliances shall be dried at 200 °C ~ 250 °C for 1 h ~ 2 h.

## 12 Analytical Procedures

### 12.1 Preparation of Test Bacterial Suspension

**12.1.1** Use an inoculating needle to pierce the strain of *Lactiplantibacillus plantarum* to the lactobacillus agar culture medium, at 36 °C ± 1 °C, culture for 20 h ~ 24 h. After taking it out, put it in the refrigerator and preserve it. It shall remain valid for 1 month. Propagate at least once a month and store it as a reserve strain.

**12.1.2** Inoculate the reserve strain to the lactobacillus agar culture medium, at 36 °C ± 1 °C, culture for 20 h ~ 24 h to activate the strain and use it for the preparation of the inoculum solutions. The reserve strain that has been stored for more than several weeks cannot be immediately used to prepare the inoculum solutions. Before the test, continuously propagate for 2 ~ 3 generations to ensure the viability of the strain.

**12.1.3** Sub-cultivate the activated strain within 24 h to sterilized lactobacillus broth, at 36 °C ± 1 °C, culture it for 16 h ~ 20 h. After taking it out, centrifuge the bacterial suspension and discard the supernatant. Add 10 mL of physiological saline, use a vortex mixer to oscillate the

suspension. At 3,000 r/min ~ 5,000 r/min, centrifuge for 5 minutes, and discard the supernatant. After repeating the above-mentioned operation for 2 ~ 3 times of cleaning, add 10 mL of physiological saline and thoroughly mix it. Draw-take an appropriate amount of the bacterial suspension into 10 mL of physiological saline and evenly mix it to prepare a test bacterial suspension.

**12.1.4** Use physiological saline as a blank, use a spectrophotometer to determine the transmittance  $T$  (%) of the test bacterial suspension at a wavelength of 550 nm, and adjust the amount of the above-mentioned bacterial solution added, so that the transmittance of the test bacterial suspension is between 60% and 80%.

## 12.2 Specimen Extraction

**NOTE:** lumpy and granular specimens need to be crushed; powdered specimens, such as: milk powder and rice flour, need to be evenly mixed; fruits and vegetables, meat, eggs, fish and animal offal, etc., need to be made into chyme; semi-solid foods need to be homogenized and evenly mixed; liquid specimens shall be shaken and mixed before use.

**12.2.1** Solid specimens: accurately weigh-take the specimen (accurate to 0.001 g) and place it in a 250 mL conical flask. Specifically speaking, 2 g ~ 5 g of fresh fruit and vegetable specimens; 0.2 g ~ 1 g of cereals, beans, nuts, offal, raw meat and dried specimens; 1 g ~ 3 g of special dietary foods; 2 g ~ 3 g of milk powder and rice flour specimens; 0.2 g ~ 1 g of general nutrient supplements or other foods.

**12.2.2** Liquid beverages or liquid and semi-liquid specimens: weigh-take 5 g ~ 10 g of specimen (or use a one-mark pipette to draw-take an appropriate volume) and place it in a 250 mL conical flask. Special sports beverages do not need to be processed after sample weighing. After weighing the sample, directly reach a constant volume of 100 mL ( $V$ ); in accordance with 12.2.4, dilute it.

**12.2.3** In accordance with the specimen matrix, add 50 mL of sulfuric acid solution: for fortified foods, such as: special dietary foods and prepared milk powder, add sulfuric acid solution C (0.5 mol/L); for plant-derived foods, add sulfuric acid solution B (1.0 mol/L); for animal-derived foods, add sulfuric acid solution A (3.0 mol/L).

Put the above-mentioned mixture into a pressure steam sterilizer, at 121 °C, hydrolyze it for 30 min. Take it out and quickly cool it to room temperature in a water bath. Use sodium hydroxide solution A and sodium hydroxide solution B to adjust pH to  $4.5 \pm 0.2$ , transfer it to a 250 mL volumetric flask, and use water to reach a constant volume to the scale ( $V_1$ ). Use quantitative filter paper to filter it. The first 10 mL of filtrate shall be discarded. Draw-take 5 mL ( $V_2$ ) of the filtrate into a 100 mL beaker and use sodium hydroxide solution B to adjust pH to  $6.8 \pm 0.2$ , transfer it to a 100 mL volumetric flask, and use water to dilute to the scale ( $V$ ).

**12.2.4** Dilution: in accordance with the biotin content in the specimen, use water to appropriately dilute the extracting solution ( $f$ ), so that the mass concentration of biotin in the specimen extracting solution after dilution is 0.1 ng/mL ~ 0.2 ng/mL.

cooling, the sterilization test tubes shall not be too close to the inner wall of the sterilizer, and the test tubes shall not be placed too densely, so as to avoid affecting air circulation.

#### **12.3.1.4 Inoculation**

Under sterile conditions, respectively add 1 drop (50  $\mu\text{L}$  ~ 100  $\mu\text{L}$ ) of the test bacterial solution to each of the above-mentioned tubes (except the sample blank tube and standard curve uninoculated blank tube UN), cover them, and thoroughly oscillate to evenly mix all the test tubes.

#### **12.3.1.5 Culture**

Put the test tubes into a constant-temperature incubator, at  $36\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ , culture for 18 h ~ 24 h.

#### **12.3.1.6 Determination**

After the culture is completed, visually inspect each test tube. The culture solution in the uninoculated blank test tube (UN) shall be clear. There shall be a gradient difference in the concentration of the culture solution in the standard curve series of tubes and the specimen series of tubes. If the uninoculated blank test tube (UN) is turbid, then, the determination shall be deemed as invalid.

**12.3.1.6.1** Use the uninoculated blank test tube (UN) as a blank, adjust the spectrophotometer absorbance ( $A$ ) to 0%, and read the reading of the inoculated blank test tube (IN). Then, use the inoculated blank test tube (IN) as the blank and adjust the absorbance to 0%.

**12.3.1.6.2** Use a vortex mixer to thoroughly mix each test tube, then, immediately transfer the culture solution into a cuvette, at a wavelength of 550 nm, determine the absorbance. After the reading is stable, read the absorbance value. The stabilization time of each test tube shall be the same. Successively read the absorbance of other test tubes. Culture tubes whose absorbance exceeds the range of standard curve series of tubes S1 ~ S8 shall be discarded.

Take the concentration of the biotin reference material as the x-coordinate and the absorbance as the y-coordinate to draw a standard curve.

**12.3.1.6.3** The absorbance of the sample blank tube shall be read together. If the absorbance of the sample blank tube is greater than 0.02, the absorbance value of a culture tube added with 1 mL of specimen extracting solution shall be subtracted by 1/5 of the absorbance value of the sample blank tube; the absorbance value of a culture tube added with 2 mL of specimen extracting solution shall be subtracted by 2/5 of the absorbance value of the sample blank tube, and so on, which is used as the basis for calculation results.

#### **12.3.2 Microplate culture method**

##### **12.3.2.1 Standard curve series of centrifuge tubes**

Filter and sterilize the biotin standard curve working solution into a sterile centrifuge tube under

$X$ ---the content of biotin in the specimen, expressed in ( $\mu\text{g}/100\text{ g}$ ) or ( $\mu\text{g}/100\text{ mL}$ );

$\rho$ ---the total average value of mass concentration of biotin in the specimen extracting solution obtained through calculation, expressed in ( $\text{ng}/\text{mL}$ );

$V$ ---the constant volume of the specimen extracting solution, expressed in ( $\text{mL}$ );

$m$ ---the mass or volume of the specimen, expressed in ( $\text{g}$ ) or ( $\text{mL}$ );

$V_1$ ---the constant volume before filtration, expressed in ( $\text{mL}$ );

$V_2$ ---the volume of the filtrate drawn after filtration, expressed in ( $\text{mL}$ );

$f$ ---the dilution factor of the specimen extracting solution;

100---conversion factor;

1,000---conversion factor.

The results shall retain 3 significant figures.

**NOTE 1:** for samples that do not require extraction, remove  $V_1$  and  $V_2$  from the Formula.

**NOTE 2:** if the biotin content in the sample is relatively high, it can be appropriately further diluted;  
if the content is relatively low, the sampling size of the sample can be appropriately increased, or the dilution factor can be reduced.

## 14 Precision

The absolute difference between the results of two independent determinations obtained under repeatability conditions shall not exceed 20% of the arithmetic mean.

## 15 Others

**15.1** For samples that need to be extracted: when the sampling size is 2 g, the quantitation limit of this Standard is  $2.5\text{ }\mu\text{g}/100\text{ g}$ .

**15.2** For samples that do not need to be extracted, for example, special-purpose beverages: when the sampling size is 10 g, the quantitation limit is  $0.1\text{ }\mu\text{g}/100\text{ g}$ .

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