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NATIONAL STANDARD OF THE
PEOPLE'S REPUBLIC OF CHINA

GB 5009.285-2022

National food safety standard - Determination of Vitamin

B₁₂ in foods

食品安全国家标准 食品中维生素 B₁₂ 的测定

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National food safety standard - Determination of Vitamin B₁₂ in foods

1 Scope

This Standard specifies the determination method of vitamin B₁₂ in foods.

Method I of liquid chromatography applies to the determination of vitamin B₁₂ in foods for infants and young children, milk and milk products, meat and meat products.

Method II of liquid chromatography - mass spectrometry applies to the determination of vitamin B₁₂ in foods for infants and young children, milk and milk products, meat and meat products, ready-to-eat cereals, baked foods, jelly, and beverages.

Method III of microbiological method applies to the determination of vitamin B₁₂ in foods for infants and young children, milk and milk products.

Method I – Liquid chromatography

2 Principle

After the sample is enzymatically hydrolyzed, use potassium cyanide (or sodium cyanide) solution to convert cobalamin isomers (hydroxocobalamin, methylcobalamin and 5-deoxyadenosylcobalamin, etc.) to cyanocobalamin. After the sample solution is purified and concentrated by the immunoaffinity column, use the reversed-phase liquid chromatography column for separation, the ultraviolet detector for detection, and the external standard method for quantification.

3 Reagents and materials

Unless otherwise specified, all the reagents are analytical reagents, and the water is grade-1 water which is specified by GB/T 6682.

3.1 Reagents

3.1.1 Anhydrous sodium acetate (CH₃COONa).

3.1.2 Acetic acid (CH₃COOH).

3.1.3 Methanol (CH₃OH): chromatographic grade.

3.1.4 Acetonitrile (CH_3CN): chromatographic grade.

3.1.5 Trifluoroacetic acid (CF_3COOH): chromatographic grade.

3.1.6 Potassium cyanide or sodium cyanide (KCN/NaCN).

3.1.7 Pepsin (CAS number: 9001-75-6, activity ≥ 400 U/mg).

3.1.8 Amylase (activity ≥ 50 U/mg).

3.1.9 Ethanol ($\text{C}_2\text{H}_6\text{O}$).

3.2 Preparation of reagents

3.2.1 Ethanol solution (25%): Weigh 250 mL of ethanol; add water to dilute to 1 000 mL; mix well.

3.2.2 Sodium acetate buffer (0.25 mol/L): Weigh 20.5 g of anhydrous sodium acetate; dissolve in 950 mL of water; use acetic acid to adjust the pH to 4.0 ± 0.1 ; use water to dilute to 1 000 mL.

3.2.3 Potassium cyanide (or sodium cyanide) solution (10 mg/mL): Weigh 1.0 g of potassium cyanide (or sodium cyanide) solid; add an appropriate amount of water to dissolve; use water to dilute to 100 mL.

Note: Potassium cyanide (or sodium cyanide) is a highly toxic chemical; the operator must wear protective equipment and prepare or use potassium cyanide (or sodium cyanide) solution in a fume hood.

3.2.4 Trifluoroacetic acid solution (0.04%, volume ratio): Weigh 500 mL of water; add 200 μL of trifluoroacetic acid; mix well.

3.3 Standard

Vitamin B_{12} (cyanocobalamin) standard substance ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$, CAS number: 68-19-9): purity $\geq 99\%$, or standard substance certified by the nation and granted a certificate of reference material.

3.4 Preparation of standard solutions

3.4.1 Vitamin B_{12} standard stock solution (1 mg/mL): Weigh 10 mg (accurate to 0.01 mg) of standard vitamin B_{12} in a 50 mL beaker; use ethanol solution to dissolve it; then, transfer it to a 10 mL volumetric flask; use ethanol solution to fix the volume to the mark; shake well; transfer to a brown reagent bottle; store at $-20\text{ }^\circ\text{C}$ in the dark. This solution is valid for 6 months.

3.4.2 Vitamin B_{12} standard working solution (10 $\mu\text{g}/\text{mL}$): Draw 1.00 mL of vitamin B_{12} standard stock solution; put it in a 100 mL volumetric flask; use ethanol solution to fix

mL of potassium cyanide (or sodium cyanide) solution in turn; mix well. Put the sample solution into a water bath constant temperature oscillator; at 37 °C, carry out enzymatic hydrolysis for 30 minutes (for meat samples, carry out enzymatic hydrolysis for 10 h ~ 16 h). After enzymolysis, transfer it to a water bath at 100 °C; keep for 30 minutes; then, take it out and cool to room temperature. Transfer the sample solution to a 100 mL volumetric flask; use water to fix the volume to the scale; shake well. Pipette 40 mL of the above solution into a 50 mL centrifuge tube; centrifuge at 10 000 r/min for 10 min; take the supernatant and filter through a glass fiber filter paper for later use.

Note: For samples with cyanocobalamin as nutritional enhancer and no other forms of cobalamin in the background, potassium cyanide (or sodium cyanide) may not be used during sample extraction.

5.1.3 Purification

Connect the immunoaffinity column to the solid-phase extraction device. After discarding the buffer in the immunoaffinity column, pipette an appropriate amount of the above filtrate (containing 10 ng ~ 500 ng of vitamin B₁₂) to pass through the column; adjust the column passing speed to 2 mL/min ~ 3 mL/min. After the sample solution has completely passed through the column, use 10 mL of water to rinse the immunoaffinity column at a steady flow rate and drain. Place a 10 mL glass test tube under the immunoaffinity column; use 3 mL of methanol to elute in three times; collect all the eluates; use nitrogen flow to blow slowly below 60 °C until nearly dry; use 0.04% trifluoroacetic acid solution to fix the volume to 1.0 mL; vortex for 30 s to dissolve the residue; filter through a 0.22 µm filter; test.

Note: Strictly prohibit the mixture of potassium cyanide (or sodium cyanide) liquid waste and acid. Add sodium hydroxide to adjust pH>10, and then potassium permanganate powder (added as per 3% of the liquid waste mass) to decompose the cyanide; discharge after 24 h.

5.2 Liquid chromatography reference conditions

5.2.1 Chromatographic column: C₁₈ column (column length 150 mm, column inner diameter 4.6 mm, packing particle size 2.5 µm), or equivalent.

5.2.2 Mobile phase: phase A, 0.04% trifluoroacetic acid solution; phase B, acetonitrile.

5.2.3 Gradient elution: 0 min ~ 6.0 min, 90% A; 6.0 min ~ 8.5 min, 90% ~ 0% A; 8.5 min ~ 14.0 min, 90% A.

5.2.4 Flow velocity: 0.8 mL/min.

5.2.5 Column temperature: 40 °C.

5.2.6 Injection volume: 100 µL.

5.2.7 Detection wavelength: 361 nm.

1 000 – conversion coefficient.

Two significant figures shall be kept for the calculation results.

7 Precision

The absolute difference of two independent test results obtained under repeatability cannot exceed 15% of the arithmetic mean value.

8 Others

When the sampling amount is 5.00 g, the detection limit of foods for infants and young children, dairy products, meat and meat products is 0.2 µg/100 g, and the quantification limit is 0.5 µg/100 g.

Method II – Liquid chromatography - mass spectrometry

9 Principle

After the sample is enzymatically hydrolyzed, use potassium cyanide (or sodium cyanide) solution to convert cobalamin isomers (hydroxocobalamin, methylcobalamin and 5-deoxyadenosylcobalamin, etc.) to cyanocobalamin. After the sample solution is purified and concentrated by the immunoaffinity column, use the reversed-phase liquid chromatography column for separation, the tandem mass spectrometry for detection, and the isotope internal standard method for quantification.

10 Reagents and materials

Unless otherwise specified, all the reagents are analytical reagents, and the water is grade-1 water which is specified by GB/T 6682.

10.1 Reagents

10.1.1 Anhydrous sodium acetate (CH₃COONa).

10.1.2 Sodium hydroxide (NaOH).

10.1.3 Acetic acid (CH₃COOH).

10.1.4 Acetonitrile (CH₃CN): chromatographic pure.

10.1.5 Ammonium acetate (CH₃COONH₄): chromatographic pure.

10.1.6 Potassium cyanide or sodium cyanide (KCN/NaCN).

10.1.7 Pepsin (CAS number: 9001-75-6, activity ≥ 400 U/mg).

10.1.8 Amylase (activity ≥ 50 U/mg).

10.1.9 Ethanol (C₂H₆O).

10.2 Preparation of reagents

10.2.1 Ethanol solution (25%): Weigh 250 mL of ethanol; add water to dilute to 1 000 mL; mix well.

10.2.2 Sodium acetate buffer (0.25 mol/L): Weigh 20.5 g of anhydrous sodium acetate; dissolve in 950 mL of water; use acetic acid to adjust the pH to 4.0 ± 0.1 ; use water to dilute to 1 000 mL.

10.2.3 Potassium cyanide (or sodium cyanide) solution (10 mg/mL): Weigh 1.0 g of potassium cyanide (or sodium cyanide) solid; add an appropriate amount of water to dissolve; use water to dilute to 100 mL.

Note: Potassium cyanide (or sodium cyanide) is a highly toxic chemical; the operator must wear protective equipment and prepare or use potassium cyanide (or sodium cyanide) solution in a fume hood.

10.2.4 Sodium hydroxide solution (1 mol/L): Weigh 4.0 g of sodium hydroxide; add an appropriate amount of water to dissolve; use water to dilute to 100 mL.

10.2.5 Ammonium acetate solution (2.5 mmol/L): Weigh 0.19 g of ammonium acetate; add an appropriate amount of water to dissolve; use water to dilute to 1 000 mL.

10.2.6 Acetonitrile solution (90%, volume ratio): Weigh 100 mL of water and add it to 900 mL of acetonitrile; mix well; ultrasonically degas.

10.3 Standard substance

10.3.1 Vitamin B₁₂ (cyanocobalamin) standard substance (C₆₃H₈₈CoN₁₄O₁₄P, CAS number: 68-19-9): purity $\geq 99\%$, or standard substance certified by the nation and granted a certificate of reference material.

10.3.2 Vitamin B₁₂ isotope internal standard solution (¹³C₇-C₆₃H₈₈CoN₁₄O₁₄P): 1 µg/mL methanol solution.

10.4 Preparation of standard solutions

10.4.1 Vitamin B₁₂ standard stock solution (1 mg/mL): Weigh 10 mg (accurate to 0.01 mg) of standard vitamin B₁₂ in a 50 mL beaker; use ethanol solution to dissolve it; then, transfer it to a 10 mL volumetric flask; use ethanol solution to fix the volume to the mark; shake well; transfer to a brown reagent bottle; store at -20 °C in the dark. This solution is valid for 6 months.

12 Analysis steps

Note: Avoid ultraviolet light during the operation, and operate as far away from light as possible.

12.1 Sample pretreatment

12.1.1 Sample preparation

Crush and grind the solid sample, or use a meat grinder to make it into chyme; homogenize and mix. Shake and mix liquid samples before measurement.

12.1.2 Extraction

12.1.2.1 Foods for infants and young children, dairy products, meat and meat products, baked foods, ready-to-eat cereals

Weigh 1 g ~ 5 g (accurate to 0.01 g) of the well-mixed sample into a 50 mL centrifuge tube; add 100 μ L of isotope internal standard working solution, 25 mL of sodium acetate buffer, 0.04 g of pepsin, 0.01 g of amylase and 2 mL of potassium cyanide (or sodium cyanide) in turn; mix well. Put the sample solution into a water bath constant temperature oscillator; at 37 °C, shake and carry out enzymatic hydrolysis for 30 minutes (for meat samples, carry out enzymatic hydrolysis for 10 h ~ 16 h). After enzymolysis, transfer it to a water bath at 100 °C; keep for 30 minutes; then, take it out and cool to room temperature. Centrifuge at 10 000 r/min for 10 min; take the supernatant and filter through a glass fiber filter paper for later use.

12.1.2.2 Beverages

Weigh 25 g (accurate to 0.01 g) of the well-mixed sample into a 50 mL centrifuge tube; add 100 μ L of the isotope internal standard working solution; use sodium hydroxide to adjust the pH to 5 ~ 7 (carbonated beverages need to be degassed in an ultrasonic oscillator for 10 minutes before pH adjustment). Centrifuge at 10 000 r/min for 10 min; take the supernatant and filter it through a glass fiber filter paper for later use.

12.1.2.3 Jelly

Weigh 5 g (accurate to 0.01 g) of the mixed sample into a 50 mL centrifuge tube; add 100 μ L of isotope internal standard working solution; add 40 mL of water; vortex and mix; dissolve the sample in a 70 °C water bath; sonicate in a water bath at 50 °C for 20 min; use sodium hydroxide solution to adjust the pH to 5 ~ 7; centrifuge at 10 000 r/min for 10 min; filter the supernatant through a glass fiber filter paper for later use.

Note: For samples with cyanocobalamin as nutritional enhancer and no other forms of cobalamin in the background, potassium cyanide (or sodium cyanide) may not be used during sample extraction.

12.1.3 Purification

Connect the immunoaffinity column to the solid-phase extraction device. After discarding the buffer in the immunoaffinity column, transfer all the filtrate through the column, and adjust the passing speed through the column to be 2 mL/min ~ 3 mL/min. After the sample solution has completely passed through the column, use 10 mL of water to rinse the immunoaffinity column at a steady flow rate and drain. Place a 10 mL glass test tube under the immunoaffinity column; use 3 mL of methanol to elute in three times; collect all the eluates; use nitrogen flow to blow slowly below 60 °C until nearly dry; use ammonium acetate solution to fix the volume to 1.0 mL; vortex for 30 s to dissolve the residue; filter through a 0.22 µm filter; test.

Note: Strictly prohibit the mixture of potassium cyanide (or sodium cyanide) liquid waste and acid. Add sodium hydroxide to adjust pH > 10, and then potassium permanganate powder (added as per 3% of the liquid waste mass) to decompose the cyanide; discharge after 24 h.

12.2 Apparatus reference conditions

12.2.1 Liquid chromatography reference conditions

12.2.1.1 Chromatographic column: C₁₈ column (column length 100 mm, column inner diameter 2.1 mm, packing particle size 1.7 µm), or equivalent.

12.2.1.2 Mobile phase: phase A, ammonium acetate solution (2.5 mmol/L); phase B, acetonitrile solution (90%, volume ratio).

12.2.1.3 Gradient elution: 0 min ~ 0.5 min, 93% A; 0.5 min ~ 2.0 min, 93% ~ 85% A; 2.0 min ~ 2.5 min, 85% ~ 10% A; 2.5 min ~ 3.0 min, 10% A; 3.0 min ~ 3.5 min, 10% ~ 93% A; 3.5 min ~ 6.0 min, 93% A.

12.2.1.4 Flow rate: 0.3 mL/min.

12.2.1.5 Column temperature: 40 °C.

12.2.1.6 Injection volume: 10 µL.

12.2.2 Mass spectrometry reference conditions

12.2.2.1 Ionization mode: ESI⁺.

12.2.2.2 Ion source temperature: 350 °C.

12.2.2.3 Cone hole backflushing gas flow: 50 L/h.

12.2.2.4 Desolvation gas temperature: 650 °C.

12.2.2.5 Desolvation gas flow rate: 900 L/h.

Method III – Microbiological method

16 Principle

Vitamin B₁₂ is an essential nutrient for the growth of *Lactobacillus leichmannii*. Under certain conditions, the growth of *Lactobacillus leichmannii* has a corresponding relationship with the content of vitamin B₁₂. According to the standard working curve of vitamin B₁₂ content and light transmittance (or absorbance value), calculate the content of vitamin B₁₂ in the sample.

17 Reagents and materials

Unless otherwise specified, all the reagents in this method are analytical reagents, and the water used is grade-2 water specified by GB/T 6682.

17.1 Reagents

17.1.1 Sodium chloride (NaCl).

17.1.2 Anhydrous disodium hydrogen phosphate (Na₂HPO₄).

17.1.3 Anhydrous sodium metabisulfite (Na₂S₂O₅).

17.1.4 Citric acid monohydrate (C₆H₈O₇·H₂O).

17.1.5 Sodium hydroxide (NaOH).

17.1.6 Hydrochloric acid (HCl).

17.1.7 Ethanol (C₂H₆O).

17.2 Strains

Lactobacillus leichmannii ATCC 7830 or an equivalent strain.

17.3 Standard

Vitamin B₁₂ (cyanocobalamin) standard substance (C₆₃H₈₈CoN₁₄O₁₄P, CAS number: 68-19-9): purity ≥99%, or standard substance certified by the nation and granted a certificate of reference material.

17.4 Preparation of reagents

17.4.1 Ethanol solution (25%): Weigh 250 mL of ethanol; add water to dilute to 1 000 mL; mix well.

19 Test procedures

19.1 Preparation of test bacteria solution

19.1.1 Strain recovery: After activating the freeze-dried strain of *Lactobacillus leichmannii* (ACC 7830), inoculate it on the *Lactobacillus* agar medium, and culture at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18 h ~ 24 h. Then, transfer 2 ~ 3 generations to enhance vitality. Store in a refrigerator at $2^{\circ}\text{C} \sim 8^{\circ}\text{C}$ for later use. Transfer every 15 days; the number of passages shall not exceed 15 times.

19.1.2 Bacterial suspension preparation: inoculate the activated strains into the *Lactobacillus* broth medium; culture at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18 h ~ 24 h; centrifuge at 2 000 r/min for 2 min ~ 5 min; discard the supernatant; add 10 mL of 9 g/L sodium chloride solution; mix well; then, centrifuge for another 2 ~ 5 minutes, as before; discard the supernatant; add 10 mL of 9 g/L sodium chloride solution; mix well.

19.1.3 Test bacteria solution: Absorb an appropriate amount of bacterial suspension into 10 mL of 9 g/L sodium chloride solution; mix well to prepare a test bacteria solution. Use a spectrophotometer; use the 9 g/L sodium chloride solution as a blank; detect the light transmittance of the test bacteria solution at a wavelength of 550 nm; make the light transmittance of the test bacterial solution 60% ~ 80%.

19.2 Sample pretreatment

19.2.1 Sample preparation

Crush and grind the solid sample; homogenize and mix. Shake and mix the liquid sample before testing. Refrigerate all prepared samples and measure within one week.

19.2.2 Sample extraction

Weigh a certain amount of sample (accurate to 0.000 1 g, the vitamin B₁₂ contained in the sample is 50 ng ~ 100 ng) into a 250 mL autoclave bottle; mix it with 10 mL of the extraction solution; then, add 150 mL of water; shake well; place in a pressure sterilizer for hydrolysis at 121°C for 10 min. After cooling, use hydrochloric acid solution (1 mol/L) to adjust the pH to 4.5 ± 0.2 ; transfer it to a 250 mL volumetric flask; adjust the volume to the mark. Mix well and filter. Pipette 5 mL of the filtrate; add 20 mL ~ 30 mL of water; use sodium hydroxide (1 mol/L) to adjust the pH to 6.8 ± 0.2 ; transfer it to a 100 mL volumetric flask; adjust the volume to the mark; use it as the sample extract.

If necessary, further adjust the dilution ratio (represented by f), so that the mass concentration of vitamin B₁₂ in the sample extract is 0.01 ng/mL ~ 0.02 ng/mL, and the mass concentration of sodium metabisulfite is less than 0.03 mg/mL.

19.3 Preparation of the standard curve

19.8.2 Use the uninoculated blank test tube (S1) as a blank; adjust the light transmittance of the spectrophotometer to 100% (or the absorbance to 0); read the reading of the inoculated blank test tube (S2). Then, take the inoculated blank test tube (S2) as a blank; adjust the transmittance to 100% (or the absorbance to 0); read the transmittance (or absorbance) of each other test tube in turn.

19.8.3 After fully mixing the culture in each test tube with a vortex shaker (or adding a drop of antifoaming agent), immediately transfer the culture solution into a cuvette for measurement, with a wavelength of 550 nm. After the reading is stable for 30 s, read the transmittance; the stabilization time of each test tube shall be the same. The relative standard deviation of the absorbance values of the 3 tubes at each standard curve concentration point shall be less than 15%. If the relative standard deviation of the absorbance of the 3 test tubes is greater than or equal to 15%, discard the standard curve concentration point (the number of standard curve concentration points discarded shall not exceed one). Take the vitamin B₁₂ concentration in the standard series tubes as the abscissa and the light transmittance as the ordinate to draw a standard curve.

19.8.4 Calculate the concentration of vitamin B₁₂ in the to-be-tested solution from the standard working curve according to the transmittance of the to-be-tested solution; calculate the content of vitamin B₁₂ in the sample according to the dilution factor and the weighing sample. Discard the test value whose light transmittance exceeds the range of the standard curve tubes S3 ~ S10.

19.8.5 Use the light transmittance of each test tube to calculate the concentration of vitamin B₁₂ per milliliter of each numbered test solution, and the average concentration of vitamin B₁₂ in the test solution of this number. The concentration measured in each test tube shall not exceed this 15% of the average value, or shall be discarded. If the number of tubes that meet this requirement is less than 2/3 of the total number of tubes for the to-be-tested solution of all four numbers, the data used to calculate the content of the sample is insufficient and needs to be re-examined. If the number of tubes that meet the requirements exceeds 2/3 of the original number of tubes, recalculate the average value of vitamin B₁₂ content per milliliter of test solution in the valid numbered sample tube, and calculate the total average value of all numbered sample tubes from this average value as ρ . Use Formula (3) to calculate the content X of vitamin B₁₂ in the sample.

Note: Draw a standard curve to read both transmittance (T%) and absorbance (A).

20 Description of the analysis results

The content of vitamin B₁₂ (calculated as cyanocobalamin) in the sample is calculated according to Formula (3):

$$X = \frac{\rho \times f \times 100}{m \times 1\,000} \dots\dots\dots (3)$$

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