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National food safety standards -- Determination of pantothenic acid in food

食品安全国家标准　食品中泛酸的测定

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National food safety standards -- Determination of pantothenic acid in food

1 Scope

This Standard specifies the method for the determination of pantothenic acid in food.

Method One of this Standard is applicable to the determination of pantothenic acid in infant formula foods (except infant formula foods for special medical purposes), infant supplementary foods, dairy products, beverages, and nutritional supplements.

Method Two and Method Three of this Standard are applicable to the determination of pantothenic acid in food.

Method One -- Liquid chromatography

2 Principle

After the specimen is extracted with hot water, it is separated by a C_{18} reversed-phase chromatography column, qualitatively determined by retention time, and quantitatively by the external standard method.

3 Reagents and materials

Unless otherwise stated, the reagents used in this method are analytically pure, and the water is grade one water specified in GB/T 6682.

3.1 Reagents

3.1.1 Hydrochloric acid (HCl).

3.1.2 Phosphoric acid (H_3PO_4).

3.1.3 Potassium dihydrogen phosphate (KH_2PO_4).

3.1.4 Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$).

3.1.5 Acetonitrile (CH_3CN): chromatographically pure.

3.1.6 α-amylase: enzyme activity ≥1.5 U/mg.

3.2 Preparation of reagents

3.2.1 Hydrochloric acid (0.1 mol/L): Pipette 8.3 mL of hydrochloric acid into 800 mL of water. Add water to dilute to 1000 mL. Mix well.

3.2.2 Hydrochloric acid (1.0 mol/L): Pipette 8.3 mL of hydrochloric acid into 80 mL of water. Add water to dilute to 100 mL. Mix well.

3.2.3 Zinc sulfate solution (0.5 mol/L): Weigh 14.4 g of zinc sulfate heptahydrate. Add water to dissolve and dilute to 100 mL.

3.2.4 Potassium dihydrogen phosphate solution (0.02 mol/L): Weigh 2.722 g of potassium dihydrogen phosphate. Add 500 mL water to dissolve. Use phosphoric acid to adjust pH to 3.0±0.1. Use water to dilute to 1000 mL. Use a 0.45 μm filter membrane to filter.

3.3 Standard product

D-calcium pantothenate standard product ($C_{18}H_{32}CaN_2O_{10}$, CAS number: 137-08-6): purity ≥99%, or standard product certified by the country and awarded a reference material certificate.

3.4 Preparation of standard solution

3.4.1 Pantothenic acid standard stock solution (500 μg/mL): Accurately weigh 136 mg of D-calcium pantothenate standard (accurate to 0.1 mg). Add water to dissolve and transfer to a 250 mL volumetric flask. Dilute to volume. Mix well. The standard stock solution shall be stored in the dark at -18°C and below. It can be kept for 3 months.

3.4.2 Pantothenic acid standard intermediate solution (100 μg/mL): Accurately pipette 20.0 mL of the standard stock solution into a 100 mL volumetric flask. Add water to dilute to volume. Prepare when needed.

3.4.3 Pantothenic acid standard working solution: Accurately pipette 1.0 mL, 2.0 mL, 4.0 mL, 8.0 mL, 16.0 mL and 32.0 mL of pantothenic acid standard intermediate solution into 100 mL volumetric flasks. Add water and dilute to the mark to obtain standard working solutions of pantothenic acid, of which the concentrations are 1.0 μg/mL, 2.0 μg/mL, 4.0 μg/mL, 8.0 μg/mL, 16.0 μg/mL and 32.0 μg/mL. Prepare when needed.

4 Instruments and equipment

4.1 Balance: resolution is 0.1 mg.

4.2 Constant temperature oscillation water bath: oscillation frequency is 100 r/min±20 r/min.

4.3 Ultrasonic oscillator.

4.4 pH meter: accuracy is ±0.01.

4.5 Centrifuge: rotation speed ≥8000 r/min.

4.6 0.45 μm filter.

4.7 High performance liquid chromatograph: with UV detector or diode array detector.

5 Analysis steps

5.1 Specimen preparation

The solid specimen is crushed and mixed evenly. Carbonated drinks require ultrasonic removal of carbon dioxide, and other liquid specimens need to be shaken well.

5.2 Specimen extraction

5.2.1 Beverages, nutrient supplements

Accurately weigh or measure an appropriate amount of specimen to the nearest 0.001 g. Generally, the solid specimen is 0.2 g~2 g. The liquid specimen is 10 g~20 g. Place in a 50 mL Erlenmeyer flask. Add about 30 mL of 40℃~50℃ warm water and conduct ultrasonic extraction for 20 min. Transfer to a 50 mL volumetric flask. Dilute to volume with water and mix thoroughly before transferring to a centrifuge tube. Centrifuge at 8000 r/min for 2 min. Pass the supernatant through a 0.45 μm filter membrane. The filtrate is ready to be measured on the machine.

5.2.2 Infant formula food (except infant formula food for special medical purposes), infant supplementary food, dairy products

Accurately weigh an appropriate amount of specimen to the nearest 0.001 g. Generally, the solid specimen is about 5 g. The liquid specimen is about 20 g. Place in a 100 mL Erlenmeyer flask. Add about 30 mL of 40℃~50℃ warm water. Specimens that do not contain starch are directly extracted with ultrasound for 20 min. For starch-containing specimens, add 0.2 g of α-amylase and shake to mix. Cap the bottle. Shake for enzymatic hydrolysis in a water bath at 55°C ±5°C for 30 min. Take out the specimen solution. Cool to room temperature.

Adjust the pH to 5.0 ± 0.1 with 0.1 mol/L hydrochloric acid or 1.0 mol/L hydrochloric acid. Add 5 mL of 0.5 mol/L zinc sulfate solution. Mix thoroughly. Transfer to a 50 mL volumetric flask. Dilute to volume with water, mix thoroughly, and transfer to a centrifuge tube. Centrifuge at 8000 r/min for 2 min. Pass the supernatant through a 0.45 μm filter membrane. The filtrate is ready to be measured on the machine.

5.3 Reference chromatographic conditions for liquid phase

10.1.1 Acetonitrile (CH_3CN): chromatographically pure.

10.1.2 Formic acid (HCOOH): chromatographically pure.

10.1.3 Hydrochloric acid (HCl).

10.1.4 Glacial acetic acid ($C_2H_4O_2$).

10.1.5 Zinc acetate [Zn $(CH_3COO)_2$].

10.1.6 Potassium ferrocyanide [$K_4Fe(CN)_6$].

10.1.7 Trishydroxymethylaminomethane ($C_4H_{11}NO_3$).

10.1.8 Sodium bicarbonate ($NaHCO_3$).

10.1.9 Potassium bicarbonate ($KHCO_3$).

10.1.10 Toluene (C_7H_8).

10.1.11 α-amylase: enzyme activity ≥1.5 U/mg.

10.1.12 Anion exchange resin: Dowex 1×8 particle size is 38 μm~75 μm.

10.1.13 Alkaline phosphatase: derived from bovine intestinal mucosa, EC3.1.3.1, enzyme activity ≥10 U/mg.

10.1.14 Pigeon liver acetone extract dry powder: enzyme activity ≥0.1 U/g.

10.2 Preparation of reagents

10.2.1 Formic acid solution (0.1%): Pipette 1 mL of formic acid. Use water to dilute to 1000 mL. Mix well.

10.2.2 Zinc acetate solution (300 g/L): Weigh 30.0 g of zinc acetate. Use water to dissolve and dilute to 100 mL.

10.2.3 Potassium ferrocyanide solution (150 g/L): Weigh 15.0 g of potassium ferrocyanide. Use water to dissolve and dilute to 100 mL.

10.2.4 Tris buffer: Weigh 121.0 g of trishydroxymethylaminomethane and dissolve it in 500 mL of water. Use glacial acetic acid to adjust pH to 8.1 ±0.2. Add water to dilute to 1000 mL. Mix well. Store in refrigerator at 2℃~8℃.

10.2.5 Sodium bicarbonate solution (0.1 mol/L): Weigh 0.84 g of sodium bicarbonate. Add water to dissolve and dilute to 100 mL. Mix well.

10.2.6 Alkaline phosphatase solution: Weigh 0.2 g of alkaline phosphatase into a mortar. Add water in small amounts and grind until it is dissolved. Use water to dilute to 10

mL. Prepare when needed. Store in a refrigerator at 2℃~8℃ to pre-cool.

10.2.7 Hydrochloric acid solution (1 mol/L): Pipette 83 mL of hydrochloric acid into 800 mL of water. Add water to dilute to 1000 mL. Mix well.

10.2.8 Potassium bicarbonate solution (0.02 mol/L): Weigh 2 g of potassium bicarbonate. Add water to dissolve and dilute to 1000 mL. Mix well.

10.2.9 Resin activation: Weigh 100 g of anion exchange resin into a 2 L Erlenmeyer flask. Add 1 L of 1 mol/L hydrochloric acid solution and shake thoroughly on an oscillator for 10 min. Filter through a Buchner funnel lined with filter paper. Transfer the anion exchange resin back to the Erlenmeyer flask. Then add 1 L of 1 mol/L hydrochloric acid solution, shake repeatedly for 10 min, and filter. Add 1 L of water to the anion exchange resin and shake for 10 min. filter. Repeat washing with water 10 times. Transfer to the Erlenmeyer flask. Add a small amount of water to the anion exchange resin. Mix well. Add Tris buffer dropwise to the anion exchange resin. Adjust pH to 8.0±0.1. Store in a refrigerator at 2℃~8℃. Use within 2 d.

10.2.10 Pigeon liver extract: At the day before preparing this reagent, place the container in a refrigerator at 2℃~8℃ overnight. Weigh 30 g of pigeon liver acetone extract dry powder and put it into a pre-cooled mortar. Add 300 mL of 0.02 mol/L potassium bicarbonate solution in portions under ice bath conditions. Grind to a homogeneous suspension. Transfer the suspension to a pre-cooled capped centrifuge tube. Cover tightly, shake thoroughly, and place in a -20°C refrigerator for 10 min. After taking out, centrifuge at 3000 r/min for 5 min. Transfer the supernatant to a 500 mL pre-cooled jar. Add partially activated resin (approximately 150 g) to the supernatant. Place in an ice water bath and shake for 5 min. Pour into a pre-cooled centrifuge tube and centrifuge at 3000 r/min for 5 min. Transfer the supernatant to another 500 mL pre-cooled jar. At -20℃, let stand for 10 mins. Then add the remaining activated resin (about 150 g). Place in an ice bath and shake for 5 min. Pour into a centrifuge tube. Centrifuge at 3000 r/min for 5 min. Collect the supernatant. At -20℃, let stand for 10 min. Aliquot into pre-cooled stoppered test tubes. Store frozen at -20°C. It can be stored for 1 year. Defrost in the refrigerator at 2℃~8℃ before use.

10.3 Standard product

10.3.1 D-calcium pantothenate standard ($C_{18}H_{32}CaN_2O_{10}$, CAS No.: 137-08-6): purity ≥99%, or a standard certified by the country and awarded a reference material certificate.

10.3.2 $^{13}C_6$, $^{15}N_2$-Calcium Pantothenate ($^{13}C_6C_{12}H_{32}Ca^{15}N_2O_{10}$, CAS No.: 356786-94-2): Purity ≥97%.

10.4 Preparation of standard solution

10.4.1 Pantothenic acid standard stock solution (500 μg/mL): Accurately weigh 136 mg of D-calcium pantothenate standard (accurate to 0.1 mg). Add water to dissolve and transfer to a 250 mL volumetric flask. Dilute to volume. Mix well. Standard stock

mL of Tris buffer and 30 mL of water. Shake to mix. Hydrolyze under high pressure conditions at 121°C for 20 min. Cool to room temperature. Add 0.1 mL of sodium bicarbonate solution, 0.4 mL of alkaline phosphatase solution, and 0.2 mL of pigeon liver extract in sequence. After mixing, add 100 μL of toluene and stopper. Conduct constant temperature oscillation at 37℃ ±1℃ with an amplitude of 100 r/min ± 20 r/min for 8 h~10 h. Transfer to a 100 mL volumetric flask. Add water to the mark. Filter.

Pipette 10.0 mL of the above liquid into a 50 mL centrifuge tube. Add 100 μL of internal standard stock solution. Add water to 20 mL. Vortex for 10 s. Add 0.4 mL of zinc acetate solution and potassium ferrocyanide solution, respectively. Add water to 25 mL. Vortex for 10 s. After letting it stand for 10 to 30 min, centrifuge at 8000 r/min for 2 min. The supernatant is passed through a 0.22 μm nylon filter and injected for analysis.

12.1.2 Infant formula food, infant supplementary food, formula food for special medical purposes, dairy products, beverages, nutrient supplements, jelly

Accurately weigh 5 g of solid specimen (accurate to 0.01 g). Add water to 50 g (accurate to 0.01 g). Add 0.2 g of α-amylase. Shake to mix. Cap the bottle. Shake for 30 min in a water bath at 55°C ±5°C. Weigh 0.5 g ~ 5.0 g of the above liquid (accurate to 0.1 mg) and place it in a 50 mL centrifuge tube. After mixing the liquid specimen, directly weigh 0.5 g~5.0 g (accurate to 0.1 mg) and place it in a 50 mL centrifuge tube.

Add 100 μL of internal standard stock solution. Add water to 20 mL. Vortex for 10 s. Add 0.4 mL of zinc acetate solution and potassium ferrocyanide solution, respectively. Add water to 25 mL. Vortex for 10 s. After letting it stand for 10 to 30 min, centrifuge at 8000 r/min for 2 min. The supernatant is passed through a 0.22 μm nylon filter and injected for analysis.

12.2 Reference conditions for instruments

12.2.1 Reference chromatographic conditions for liquid phase

The liquid phase reference chromatography conditions are as follows:

a) Chromatographic column: C_{18}, 1.8 μm, 100 mm × 2.1 mm, or equivalent.

b) Column temperature: 35℃.

c) Flow rate: 0.3 mL/min.

d) Injection volume: 2 μL.

e) Mobile phase: Phase A is 0.1% formic acid solution; phase B is acetonitrile. The liquid chromatography gradient elution conditions are shown in Table 1.

Table 1 -- Gradient elution conditions for liquid chromatography

17.1 Reagents

17.1.1 Hydrochloric acid (HCl).

17.1.2 Glacial acetic acid ($C_2H_4O_2$).

17.1.3 Sodium hydroxide (NaOH).

17.1.4 Sodium chloride (NaCl).

17.1.5 Sodium bicarbonate ($NaHCO_3$).

17.1.6 Potassium bicarbonate ($KHCO_3$).

17.1.7 Sodium acetate trihydrate ($C_2H_3O_2Na \cdot 3H_2O$).

17.1.8 Trishydroxymethylaminomethane ($C_4H_{11}NO_3$).

17.1.9 Toluene (C_7H_8).

17.1.10 Anion exchange resin Dowex1×8: particle size 38 μm~75 μm.

17.1.11 α-amylase: enzyme activity ≥1.5 U/mg.

17.1.12 Papain: enzyme activity ≥10 U/mg.

17.1.13 Alkaline phosphatase: derived from bovine intestinal mucosa, EC 3.1.3.1, enzyme activity ≥10 U/mg.

17.1.14 Pigeon liver acetone extract dry powder: enzyme activity ≥0.1 U/g.

17.2 Preparation of reagents

17.2.1 Physiological saline: Weigh 9.0 g of sodium chloride. Add water to dissolve and dilute to 1000 mL. Mix well. Autoclave at 121°C for 10 min before use and set aside.

17.2.2 Hydrochloric acid solution: Take 100 mL of hydrochloric acid and mix with 50 times of water.

17.2.3 Acetic acid solution (0.2 mol/L): Pipette 1.2 mL of glacial acetic acid. Dilute to 100 mL with water. Mix well.

17.2.4 Sodium acetate solution (0.2 mol/L): Weigh 2.7 g of sodium acetate trihydrate. Add water to dissolve and dilute to 100 mL. Mix well.

17.2.5 Sodium hydroxide solution (2 mol/L): Weigh 8 g of sodium hydroxide. Add water to dissolve and dilute to 100 mL. Mix well.

17.2.6 Acetate buffer (1.0 mol/L, pH3.8): Take 58 mL of glacial acetic acid and add it to 800 mL of water. Adjust pH to 3.8±0.1 with 2 mol/L sodium hydroxide. Add water

to 1000 mL. Mix well.

17.2.7 Acetate buffer (1.0 mol/L, pH 4.5): Take 58 mL of glacial acetic acid and add it to 800 mL of water. Adjust pH to 4.5±0.1 with 2 mol/L sodium hydroxide. Add water to 1000 mL. Mix well.

17.2.8 Tris buffer: Same as 10.2.4.

17.2.9 Sodium bicarbonate solution (0.1 mol/L): Same as 10.2.5.

17.2.10 Alkaline phosphatase solution: Same as 10.2.6.

17.2.11 Pigeon liver extract: Same as 10.2.10.

17.3 Culture medium

17.3.1 Lactobacillus agar medium: See B.1 in Annex B.

17.3.2 Culture medium for pantothenic acid determination: See B.2 in Annex B.

17.4 Preparation of culture medium

See Annex B.

17.5 Standard product

D-calcium pantothenate standard product ($C_{18}H_{32}CaN_2O_{10}$, CAS No.: 137-08-6): purity ≥99%, or standard product certified by the country and awarded a reference material certificate.

17.6 Preparation of standard solution

17.6.1 Pantothenic acid standard stock solution (400 μg/mL): Accurately weigh 435 mg of D-calcium pantothenate (accurate to 0.1 mg). Add water to dissolve and transfer to a 1000 mL volumetric flask. Add 10 mL of 0.2 mol/L acetic acid solution and 100 mL of 0.2 mol/L sodium acetate solution. Bring to volume with water. Store in a brown bottle. Add 200 μL of toluene. It can be stored in the refrigerator at 2℃~4℃ for 2 years.

17.6.2 Pantothenic acid standard intermediate solution (1.00 μg/mL): Accurately pipette 2.5 mL of pantothenic acid standard stock solution into a 1000 mL volumetric flask. Add 10 mL of 0.2 mol/L acetic acid solution and 100 mL of 0.2 mol/L sodium acetate solution. Bring to volume with water. Add 200 μL of toluene. It can be stored in the refrigerator at 2℃~8℃ for 1 year.

17.6.3 Pantothenic acid standard working solution (20.0 ng/mL): Accurately pipette 2.00 mL of pantothenic acid standard intermediate solution and place it in a 100 mL volumetric flask. Bring to volume with water. Mix well. Prepare when needed.

19.4 Strain activation

At 2 to 3 days before the test, inoculate the latest saved strain into a sterile agar tube. Culture at 36℃±1℃ for 20 h~24 h to activate the strain for preparation of inoculum.

NOTE: If the new strain is stored for more than 2 weeks, it shall be continuously propagated for 2 to 3 generations before testing to ensure bacterial viability.

19.5 Preparation of inoculum

At the day before the test, take 2 mL of pantothenic acid standard working solution (20 ng/mL) and 4 mL of culture medium for pantothenic acid determination and mix them well. Distribute them into 2 centrifuge tubes of 5 mL. Put the tampon in place. Autoclave at 121°C for 15 min. Cool to room temperature. This shall be the seed culture solution.

Use an inoculation loop to inoculate the activated strain into the seed culture. Incubate at 36℃±1℃ for 20 h~24 h. Remove the centrifuge tube. Centrifuge at 3000 r/min for 10 min. Discard the supernatant. Under aseptic operation, add approximately 3 mL of pre-sterile saline. Wash the pellet by shaking. Centrifuge at 3000 r/min for 10 min. Discard the supernatant. Repeat washing once with saline. Add approximately 3 mL of sterile saline. Shake to mix. Prepare inoculation solution. Use immediately.

NOTE: In order to ensure the number of bacterial colonies in the inoculum solution, an additional seed culture solution can be prepared. Inoculate and wash in the same way. Add saline and mix well. Normal saline is used as a control. The light transmittance of the suspension is measured using a spectrophotometer at a wavelength of 550 nm. Adjust the volume of the inoculation solution according to the light transmittance of this tube so that the light transmittance is in the range of 60%~80%.

20 Analysis steps

NOTE: Avoid direct sunlight and ultraviolet rays during all operations.

20.1 Specimen preparation

Specimens of cereals, potatoes, beans, milk powder, etc. need to be crushed, ground, and sieved (sieve plate aperture 0.3 mm~0.5 mm). Meat, eggs, nuts, etc. are made into chyme using a homogenizer. Mix fruits, vegetables, semi-solid foods, etc. with a homogenizer. Shake and mix liquid Specimens before use. They can be stored in the refrigerator at 4°C for 1 week. They can be stored in -20°C refrigerator for half a year. An appropriate amount of water can be added during the specimen homogenization process. Weigh the specimen mass before and after adding water. Calculate the mass conversion factor.

20.2 Specimen extraction

20.2.1 Enzymatic hydrolysis

The pantothenic acid content in food specimens such as cereals and potatoes, meat and eggs, fruits and vegetables, bacteria and algae, beans and nuts shall be determined by enzymatic hydrolysis.

Hydrolysis: Accurately weigh an appropriate amount of specimen (m), accurate to 0.001 g. Generally, 1 g~5 g of solid and semi-solid cereals, potatoes, meat, eggs, beans, bacteria and algae and their products are weighed. Weigh 5 g~10 g of foods with high water content such as fresh fruits and vegetables. The weighing amount can be adjusted according to the pantothenic acid content of the specimen. Transfer to a 100 mL Erlenmeyer flask. Add 10 mL of Tris buffer and 30 mL of water. Shake to mix. Hydrolyze under high pressure conditions at 121°C for 15 min~20 min.

Enzymatic hydrolysis: Cool to room temperature. Add 0.1 mL of sodium bicarbonate solution, 0.4 mL of alkaline phosphatase solution, and 0.2 mL of pigeon liver extract in sequence. Mix carefully. If the starch content in the specimen is high, resulting in a thicker specimen solution, an additional 20 mg~40 mg of α-amylase can be added. Add 50 μL of toluene. Plug. Shake the water bath at 37°C±1°C with an amplitude of 80 r/min ± 20 r/min for 8 h~10 h.

Distillation, sedimentation, filtration: Transfer the specimen enzymatic solution to a 100 mL volumetric flask. Bring water to volume (V). Filter. Accurately pipette an appropriate amount of filtrate (1.0 mL~10.0 mL, V_1) to the bottom of a 25 mL graduated test tube with a stopper. Add water to 15 mL. If the filtrate is clear and no precipitation occurs when adding glacial acetic acid dropwise, directly adjust the pH to 6.8±0.2 with 1.0 mol/L acetic acid buffer. Dilute to 25 mL (V_2) with water. If the filtrate is turbid or precipitation occurs when glacial acetic acid is added dropwise, adjust the pH to 4.5 ±0.1 with glacial acetic acid. Add water to make the volume to 25 mL (V_2). After filtration, accurately transfer an appropriate amount of filtrate (5.0 mL~10.0 mL, V_3) to another 25 mL graduated test tube with a stopper. Adjust the pH to 6.8±0.2 with Tris buffer or dilute sodium hydroxide solution. Dilute to 25 mL (V_4) with water.

Take another test tube and add buffer, sodium bicarbonate solution, alkaline phosphatase solution, pigeon liver extract, and α-amylase (when used) according to the sample extraction step. After shaking the water bath, adjust the pH and then filter to constant volume to serve as enzyme blank solution.

20.2.2 Direct extraction method

Infant formulas, supplementary foods for infants, formulas for special medical purposes, dairy products, beverages, nutrient supplements, and jelly shall adopt the direct extraction method.

Accurately weigh an appropriate amount of specimen (m) to the nearest 0.001 g. Generally, take 0.2 g~2 g of solid specimen, 1 g~10 g or 1 mL~10 mL of liquid specimen. Transfer them into a 100 mL Erlenmeyer flask. Add 10 mL of acetic acid

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