National Standard of the People's Republic of China

GB 5413.17 – 2010

National food safety standard

Determination of pantothenic acid in foods for infants and young children, milk and milk products

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Preface

The 1st Method in this Standard is identical to AOAC (Association of Official Analytical Chemists) Official Method 945.74 Pantothenic Acid in Vitamin Preparations.

This Standard will replace GB/T 5413.17-1997 “Milk powder and formula foods for infant and young children--Determination of pantothenic acid”.

Compared with GB/T 5413.17-1997, the main amendments of the 1 st Method described in this Standard are as follows:

- The preparation method of tris buffer was added;

- The determination of wavelength was assured;

- The verbal description of drawing a standard curve was added;

The main amendments of the Method 2 are as follows;

- The chromatographic column has been changed;

- The mobile phase has been changed;

- The treatment method for hydrolysis of starch-containing specimen was added;

Appendix A of this Standard is informative.

The versions replaced by this standard are:

-GB 5413—1985 and GB/T 5413.17—1997.

National food safety standard

Determination of fatty acids in foods for infants and young children, milk and milk products

1. Scope

This standard provides the determination of pantothenic acid in infant foods and dairy.

This standard applies to determination of pantothenic acid in infant foods and dairy.

2. Normative Reference

The following normative documents contain provision which, through reference in this text, constitute provisions of this national standard. For dated reference, subsequent amendments to, or revisions of, any of these publications do not apply to this standard; but parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. For undated references, the latest edition of the normative document referred to applies.

The 1st Method: Microbiological Methods

3. Principle

Pantothenate Medium is a pantothenic acid/pantothenatefree dehydrated medium containing all other nutrients and vitamins essential for the cultivation of Lactobacillus plantarum ATCC 8014. The addition of calcium pantothenate in specified increasing concentrations gives a growth response that can be measured turbidimetrically or titrimetrically.

4. Reagents and Materials

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

4.1 0.9% physiological saline

Dissolve 9.0 g sodium chloride in the 1000 mL water, place the solution respectively in the test tubes with stopper, 10 mL each, and then sterilized 15 min under 121□. Prepare it weekly.

4.2 Standard Calcium Pantothenate

4.3 Acetic acid solution (0.2mol/L)

pipette 12 mL glacial acetic acid and dilute with distilled water to 1000 mL.

4.4 Toluene (C_7H_8)

4.5 Sodium acetate: *c*(NaAc) is 0.2mol/L.

Dissolve 16.4g anhydrous sodium acetate in the water and dilute to 1000 mL.

4.6 Bacterial strain

Lactobacillus plantarum, ATCC 8014

4.7 Culture Medium

4.7.1 Lactobacillus agar culture medium

Mix 15g phtolytic peptone, 5g yeast extract, 10g glucose, 100mL tomato juice, 2g potassium dihydrogen phosphate ,1g sorbitan monooleate and 10g agar, add the distilled water to 1000 mL, and adjust the pH value to 6.8±0.2 (20□～25□).

4.7.2 Lactobacillus broth culture medium

Mix 15g phtolytic peptone, 5g yeast extract, 10g glucose, 100mL tomato juice, 2g potassium dihydrogen phosphate, 1g sorbitan monooleate in the distilled water to 1000 mL and adjust the pH value to 6.8±0.2 (20□～25□)

4.7.3 Culture medium for determination of pantothenic acid

Mix 40g glucose, 20g sodium acetate, 10g Vitamin-free acid hydrolyzed casein, 1g dipotassium hydrogen phosphate, 1g potassium dihydrogen phosphate, 0.4g L-cystine, 0.1g L-tryptophan, 0.4g magnesium sulfate, 20mg sodium chloride, 20 mg ferrous sulphate, 20 mg manganous sulfate, 20 mg adenine Sulfate, 20mg guanine hydrochloride, 20mg uracil, 400μg carotene, 200μg thiamine hydrochloride, 0.8μg biotin, 200μg p-aminobenzoic acid, 1mg nicotinic acid, 800 μg pyridoxine hydrochloride, and 0.1 g orbitan monooleate, add distilled water 1000 mL, and adjust the pH value to 6.7±0.1(20℃～25℃).

4.8 Tris buffer

Weigh 24.2g Trizma Base in the beaker, and add 200 mL water to dissolve it.

4.9 Hydrochloric acid solution (0.1 mol/L)

Dissolve 8.3 mL hydrochloric acid to 1000 mL.

4.10 Standard solution

4.10.1 Pantothenic acid standard stock solution (40μg/mL)

Dissolve 45 mg～55 mg dried calcium pantothenate (4.2) in 500 mL purified water, 10 mL 0.2N acetic acid (4.3)and 100 mL 0.2N sodium acetate (4.5). Dilute with additional water to make calcium pantothenate concentration 43.47 μg/mL (should equal 40 μg/mL pantothenic

acid). Add 0.5 mL toluene (4.4) to above solution then kept in 2 □～4 □ refrigerate. Use it in 4 month.

4.10.2 Pantothenic acid intermediate solution (1μg/mL)

Dilute further by adding 25 mL of this solution (4.10.1) to 500 mL purified water, 10 mL 0.2N acetic acid (4.3) and 100 mL 0.2N sodium acetate (4.5). Dilute this solution to 1 liter with purified water to make a stock solution containing 1 μg pantothenic acid per mL. Add 0.5 mL toluene (4.4) to above solution then kept in 2 □～4 □ refrigerate. Use it in one month.

4.10.3 Pantothenic acid standard work solution (10 ng/mL, 5 ng/mL)

The standard solution is made by diluting 5 mL of the intermediate solution (4.10.2) to 500mL and 1000 mL with purified water to obtain a solution containing 10 ng and 5 ng pantothenic acid per mL. Prepare it before use.

5. Apparatus and Equipments

5.1 Spectrophotometer

5.2 pH meter: with an accuracy of 0.01

5.3 Vortex oscillator

5.4 Analytical balance: sense quantity 0.1 mg

5.5 Biochemical Incubator: 36 ℃ ± 0.5 ℃

5.6 Centrifuge

6. Analyzing Procedures

6.1 Preparation of Bacterial Strain

6.1.1 Transfer the lyophilized powder of *lactobacillus plantarum* ATCC 8014 to the test tubes of lactobacillus broth culture medium (4.7.2), and cultivate 24 hours in the incubator under 36□±1℃. Then Transfer it to another test tube contains lactobacillus agar culture medium (4.7.1) and cultivate 24 hours in the incubator under 36 ℃±1℃. The result culture stores as the reserved strain.

6.1.2 Transfer the culture medium of lactobacillus plantarum ATCC 8014 respectively to three test tubes of lactobacillus agar culture medium (4.7.1), and cultivate 24 hours in the incubator under 36 ℃±1℃. Transfer every month, and store it in the refrigerator as the monthly inoculated tube.

6.1.3 Then inoculate another test tube of lactobacillus agar culture medium (4.7.1) with the culture tube monthly inoculated, and cultivate it for 24 hours under 36 ℃±1℃ for daily determination of daily inoculated tube.

6.1.4 Inoculate a tube of lactobacillus broth culture medium (4.7.2) from the daily inoculated tube and cultivate for 24 hours under 36℃±1℃. Centrifuge the culture solution under aseptic condition for 10 min (2000r/min), pour out the supernatant, vibrate and wash the thallus with 10 mL physiological saline (4.1), centrifuge once more for 10 min (2000r/min), and then pour out the supernatant and wash with another 10 mL physiological saline (4.1). Proceed to centrifugation as described above, pour out the supernatant and add 10 mL physiological saline (4.1). Add 1 mL bacterial suspension into 10 mL physiological saline (4.1) and mix evenly.

6.1.5 Use the spectrophotometer to measure the optical density of bacterial suspension (6.1.4) against the physiological saline (4.1) when the wavelength is 550 nm. The value should be between 60% and 80%.

6.2 Treatment of Samples

Dissolve 2 g (accurate to 0.0001g) solid sample or 5 g (accurate to 0.0001g) liquid sample (contains pantothenic acid 0.1 mg) with 10 mL tris buffer (4.8) and a little distilled water in 250 mL flask, and then be hydrolyzed under 121□ for 15 min, and cool down to room temperature. Adjust the solution to pH 4.5±0.2 by HCl (4.9), and dilute to 250 mL by distilled water. Filtrated and pipette 4 mL liquid to dilute and make the final concentration of pantothenic acid reaches 5ng/mL.

6.3 Preparation of Standard curve

Add the distilled water, standard solution and culture medium for determination of pantothenic acid into the culture tube in the order as described in Table 1, three shares each. The pantothenic acid concentration in the tubes from S2 to S10 are 0 ng, 5 ng, 10 ng, 15 ng, 20 ng, 25 ng, 30 ng, 40 ng and 50 ng.

Table 1 The preparation of standard curve tubes

Test Tube No.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Distilled water, mL	5	5	4	3	2	1	0	2	1	0
Standard solution, mL	0	0	1	2	3	4	5	3	4	5
Culture medium, mL	5	5	5	5	5	5	5	5	5	5
note 1 Add the standard solution of low concentration in the test tubes S3-S7, **note 2** add the standard solution of high concentration in the test tubes S8-S10.										

6.4 Specimen

Add the distilled water, specimen and culture medium into the test tube in the order as described in Table 2, three trials each.

Test Tube No.	1	2	3	4
Distilled water, mL	4	3	2	1
Specimen, mL	1	2	3	4
Culture medium, mL	5	5	5	5

6.5 Sterilization

Sterilize 5 min all test tubes mentioned in 6.4 and 6.5 under 121 □, and cool down to room temperature intermediately.

Note: Ensure the conditions during heating and cooling are even (if the sterilization tubes are excessive or at near distance, it may be unfavourable in the sterilizator).

6.6 Inoculation

Add 50μL appropriate bacteria solution (6.1.4) into each test tube under the aseptic condition, cover the cap, and shake adequately each tube (except for blank tube S1 for standard solution).

6.7 Culture

Cultivate the tubes in 36 □±1□ for 16-24 hours. Through the visual inspection of the reaction in the each test tube, the non-vaccinated test tube should be clear, and the standard specimen and the growth in such test tube should in gradient, and free of other bacteria. If the test tube is contaminated by other microorganism, the determination is void.

6.8 Determination

With the inoculated blank tube (Tube S2 in the table 1) as the blank, read the optical density of the highest concentration standard specimen tube S7 under the condition of 5s shaking and 550 nm wavelength. 2 hours later, measure the optical density of this tube again under the same condition. If the difference of two times of measurements of optical density equals to or less than 2%, then figure out the optical density of the determination standard solution in all testing tube and the specimen.

6.9 Drawing of Standard Curve

Draw the curve with the standard nicotinic acid content as the horizontal coordinate and the optical density as the longitudinal coordinate

6.10 Calculation the content of Pantothenic acid

Calculate the content of pantothenic acid per mL of the test solution at each concentration. Figure out the average value from the three repeat tubes; the measured value of each concentration should not exceed ±15% average value. And the invalid record should be discarded. If the final number of test tubes that can be used for calculation is more than 2/3 of total number of tubes. The final number of test tubes that can be used for calculation should be equals to or more than 2/3 of the total number. To recalculate the valid tubes' average Pantothenic acid content, and then calculate the total average value C_x base on the recalculated average value.

7. Calculation and Expression of Results

The content X of pantothenic acid in the specimen is calculated according to formula (1):

$$X = \frac{C_x}{m} \times \frac{f}{1000} \times 100 \quad \cdots\cdots (1)$$

Where,

X –Pantothenic acid content in the specimen,μg/100g;

Cx – total average content of pantothenic acid get from 6.10, μg;

F - Dilution factor;

m - Mass of the specimen, g.

The calculation result is the arithmetic mean of the two times of independent determination result, retained to the three significant figures.

8. Precision

The absolute difference of the two independent determination results obtained under the repetitive condition shall not exceed 10% arithmetic mean.

The 2nd Method High Performance Liquid Chromatography

9. Principle

After the pre-treatment including hot water extraction, separate it with the C18 chromatographic column, detect with the UV detector and determine the content of the pantothenic acid by the external standard method.

10. Reagents and Materials

Unless otherwise specified, the reagents used in the method are the ones of analytical pure, and the water used is the Level 1 Water regulated in GB/T 6682.

10.1 Taka-diastase: activity unit≥1.5 U/mg

10.2 Acetonitrile: chromatographically pure

10.3 Hydrochloride acid

10.4 $ZnSO_4$

10.5 Hydrochloride acid solution: c(HCl) is 0.1 mol/L.

Pipette 8.3 mL Hydrochloride acid (10.3), and dilute to 1000 mL with distilled water.

10.6 ZnSO4 solution (15 g/100 mL)

Dissolve 15 g ZnSO4 (10.4) with distilled water to 100 mL.

10.7 Potassium dihydrogen phosphate solution：0.05 mol/L

weigh 6.8g potassium dihydrogen phosphate, dissolve it in 800 mL water, adjust the pH value with phosphoric acid to 3, make the constant volume to 1000 mL, and filter with 0.45μm filter membrane.

10.8 Standard solution of pantothenic acid

10.8.1 Standard stock solution of pantothenic acid: concentration 1mg/mL.

Weigh accurately 1.087 g calcium pantothenate, add water to dissolve it and adjust to the constant volume 1000 mL.

Concentration of pantothenic acid=concentration of calcium pantothenate ×0.920.

10.8.2 Mediate standard solution of pantothenic acid (0.1mg/mL)

Pipette 10 mL standard stock solution (10.8.1) in the volumetric flask and add water to the constant volume. Prepare it before use.

11. Instruments and Equipment

11.1 Analytical balance: sense quantity 0.1 mg.

11.2 High performance liquid chromatographm with UV detector

11.3 Ultrasonic

11.4 pH meter: accurate to 0.01

11.5 Incubator: 55 □ ± 2 □

12. Analyzing Procedures

12.1 Specimen Treatment

12.1.1 Treatment of no-starch specimen

Weigh about 5g evenly mixed solid specimen or about 20 g liquid specimen (to the accuracy of 0.1 mg) and put into a 150 mL triangular flask. For the solid specimen, add 30 mL 40 □-50 □ lukewarm water, shake and dissolve it well and then proceed with ultrasonic extraction for 20min.

12.1.2 Treatment of Starch-Containing Specimen

If the specimen contains starch, weigh about 5 g evenly mixed solid specimen or about 20g liquid specimen (to the accuracy of 0.0001 g), add about 0.2 g Taka-diastase (10.1), and add about 30 mL 40□～50□ lukewarm water for the solid specimen and shake to dissolve it adequately. Then cover the cap and do the enzymolysis for 30 min under 50□-60□.

12.2 Preparation of Determination Solution

After the specimen solution has been cooled to room temperature, adjust the pH value to 4.50 with hydrochloride acid solution (10.5), add 5 mL zinc sulfate solution (10.6), and mix adequately. Make transfer to the 50 mL volumetric flask, add water to the specified scale, mix evenly and filter with the filter paper. After subjecting to filtration by 0.45 μm filter membrane, the filtrate becomes the specimen solution to be measured.

12.3 Reference Chromatographic Condition

Chromatographic column: ODS-C_{18}, 250×4.6 mm, 5 μm; or the one with the same performance

Preparation of moving phase: Mix the potassium dihydrogen phosphate solution (10.7) 900 mL with methanol (10.2) 100 mL, and then filtrate by 0.45 μm microfiltrator.

Flow rate: 1.0 mL/min

Determination wavelength: 200 nm

Column temperature: 30 □±1□

Injection volume: 10 ul

12.4 Determination

12.4.1 Preparation of Standard Curve

Respectively inject （10.8.2）1.0 mL,2.0 mL,4.0 mL,8.0 mL and 12.0 mL mediate standard solution of pantothenic acid into 100 mL volumetric flask, add water to the specified scale to get the standard working solution of pantothenic acid at the concentration of 1.0 ug/mL,2.0 ug/mL,4.0 ug/mL,8.0 ug/mL and 12.0 μg/mL respectively. Prepare it before use.

Respectively inject 10 μL standard working solution into the high performance liquid chromatograph and get the corresponding peak height (or peak area). Draw the standard curve with the peak height (or peak area) as the longitudinal coordinate and the concentration of the standard working solution as the horizontal coordinate.

12.4.2 Determination of specimen solution

Inject the 10 μL specimen solution to be determined (12.2) into the high performance liquid chromatograph and get the peak height (or peak area). Then get the concentration of the pantothenic acid from the standard curve.

13. Calculation and Expression of Results

The content of pantothenic acid in the specimen is calculated according to formula (2):

$$X = \frac{V \times C \times K}{m} \times 100 \quad \cdots\cdots (2)$$

Where,

X- The content of pantothenic acid in the specimen, μg/100 g

C- Mass concentration of pantothenic acid in the specimen solution, μg/mL;

m - Mass of the specimen, g.

V- Total volume of the measured specimen

K- Dilution factor of specimen solution.

Note: The calculation result is corrected to the 1 decimal place.

The calculation result is the arithmetic mean of the two independent determination results. It is correct to the 3 decimal place.

14. Precision

The absolute difference of the two independent determination results obtained under the repetitive condition shall not exceed 10% arithmetic mean.

15. Others

The detection limit of the2nd method in this standard is 100μg/100g.

Appendix A

(Informative Appendix)

Chromatogram of Pantothenic Acid Standard Solution

A.1 Chromatogram of Pantothenic Acid Standard Solution

Chromatogram of Pantothenic Acid Standard Solution refers to Figure A.1

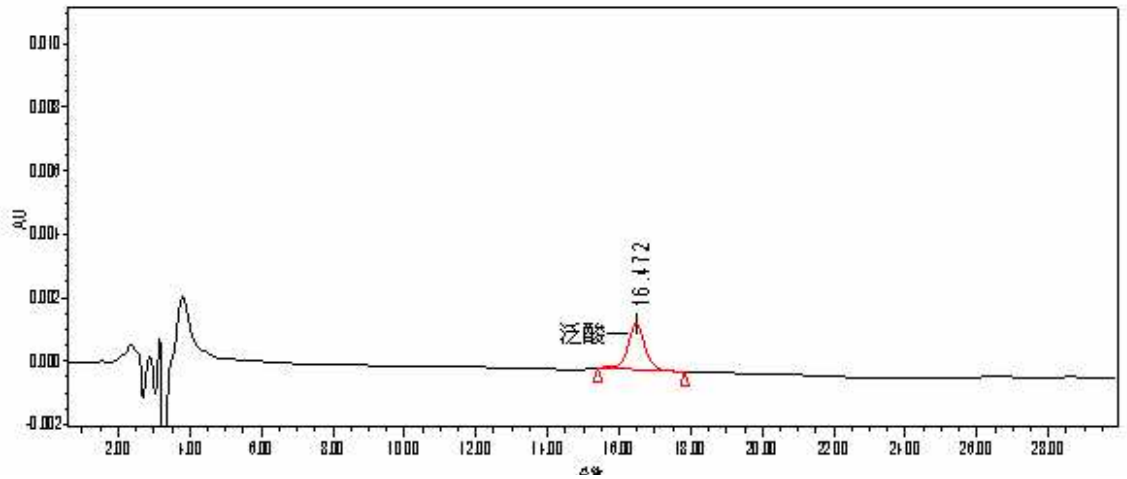

Figure A.1 Standard Chromatogram of Pantothenic Acid