



**NATIONAL STANDARD OF THE PEOPLE'S REPUBLIC OF
CHINA**

GB 5413.16—2010

**National food safety standard
Determination of folic acid (folate activity)
in foods for infants and young children,
milk and milk products**

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Foreword

Instead of the standard GB / T 5413.16-1997 "determination of folic acid (folate activity) in foods and dairy for infants"

The major changes with GB / T 5413.16-1997 are as follows:

- Make an adjustment to the phosphate buffer;
- Add preparation method of rice;
- Add detection procedure of optical density method.

The replaced standard versions are:

- GB 5413-1985, GB / T 5413.16-1997.

Determination of folic acid (folate activity) in foods for infants and young children, milk and milk products

1. Range:

This standard is formulated a microbial method for determination of folic acid (folate activity) in foods for infants and young children, milk and milk products.

This standard is applicable to determination of folic acid (folate activity) in foods for infants and young children, milk and milk products

2. Referenced normative documents

The following standards contain provisions, which through reference in this text, are indispensable part of this Standard. For dated references, subsequent amendments (exclude correction) to or revisions of any of these publications shall not apply to this Standard. For undated references the latest edition (include correction) of the publication referred to applies.

3. Principle

Using the specificity of *Lactobacillus casei* ATCC 7469 to folic acid, determine the content of folic acid in the samples by the formation of acidity and optical density.

4. Reagent, strain and culture medium

All reagent, if no special specification, refer to analytic reagent; All experiment water, refer to level 2 water.

- 4.1. Chick pancreas preparation—Weigh 100 mg desiccated chick pancreas, add 20mL water, and stir 15 min. Centrifuge 10 min at 3000 rpm and use clear supernate. Prepare just before use.
- 4.2. salt solution.—Weigh 9g NaCl and dissolved in 1000mL water. Transfer 10mL portions to culture tubes, plug. with caps and sterilize 20 min at 121°C. Prepare fresh weekly
- 4.3. phosphate buffer:
 - 4.3.1. phosphate buffer I (0.05mol/L): Dissolve 5.85 g KH₂PO₄ and 1.22 g K₂HPO₄ in water and dilute to 1 L. Add ascorbic acid in phosphate buffer solution to make the concentration to 0.5g/100ml. Prepare just before use.
 - 4.3.2. phosphate buffer II (for preparation of cereal and cereal product): Dissolve 14.2 g Na₂HPO₄ in water and dilute to 1 L. Add ascorbic acid in phosphate buffer solution to make the concentration to 1.0g/100ml. Prepare just prior to use. Adjust PH to 7.8 by NaOH solution A (4.16).
 - 4.3.3. phosphate bufferIII(for preparation of cereal and cereal product): Dissolve 14.2 g Na₂HPO₄ in water and dilute to 1 L. Add ascorbic acid in phosphate buffer solution to make the concentration to 1.0g/100ml. Prepare just prior to use. Adjust PH to 6.8 by NaOH solution A (4.16).
 - 4.3.4. phosphate bufferIV(for standard solution of cereal and cereal product): 0.1mol/L, pH7.0。 Dissolve 13.61g KH₂PO₄ in water and dilute to 1 L. Adjust PH to 7.0 by KOH solution

(4.10).

- 4.4. Folic acid: reference standard
- 4.5. Aqua ammonia (10.8%)
- 4.6. Toluene (C₇H₈)
- 4.7. ascorbic acid (C₆H₈O₆)
- 4.8. Strain: *Lactobacillus casei* (ATCC 7469)
- 4.9. culture medium
 - 4.9.1. *Lactobacillus* agar culture medium: peptonized milk 15g, yeast extract 5g, glucose 10g, tomato juice 100ml, monopotassium phosphate 2g, Poly-sorbose Monooleate 1g, agar 10g, add distilled water to total 1000ml, adjust PH to 6.8±0.2(20-25°C). Sterilize 15min at 121 °C
 - 4.9.2. *Lactobacillus* broth culture medium: peptonized milk 15g, yeast extract 5g, glucose 10g, tomato juice 100ml, monopotassium phosphate 2g, Poly-sorbose Monooleate 1g, add distilled water to total 1000ml, adjust PH to 6.8±0.2(20-25°C). Sterilize 15min at 121 °C.
 - 4.9.3. Folic acid determine medium: Acid hydrolysis casein 10g, glucose 40g, Sodium acetate 40g, dipotassium hydrogen phosphate 1g, potassium dihydrogen phosphate 1g, DL-Tryptophan 0.2g, L-aspartic acid 0.6g, L-cysteine hydrochloride 0.5g, adenine sulphate 10mg, guanine hydrochloride 10mg, uracil 10mg, xanthine 20mg, polyethylene sorbitol 0.1g, Glutathione 5mg, Magnesium sulfate 0.4g, sodium 20mg, ferrous sulfate 20mg, manganese sulfate 15mg, riboflavin 1mg, p-amino benzoic acid 2mg, vitamin B6 4mg, thiamine hydrochloride 400µg, Calcium Pantothenate 800µg, nicotinic acid 800µg, Biotin 20µg, add distilled water to total 1000ml, adjust PH to 6.7±0.1(20-25°C).
(note: the commercial synthetic medium is better.)
- 4.10 KOH solution: Weigh 224 g potassium hydroxide in 1000 mL beaker, dissolved by 400 mL water, cool-off to room temperature, then transfer to 1000 mL volumetric flask and constant volume by water.
- 4.11 Papain solution: Weigh 1g papain(≥6000U/mg PH6.0, 40 °C), dissolve in 100ml phosphate buffer(4.3.1). Prepare just before use.
- 4.12 α-amylase: Weigh 1g α-amylase(1.5U/mg), dissolve in 100 ml phosphate buffer(4.3.1). Prepare just before use.
- 4.13 Sterile filter (0.22 µ m)
- 4.14 Preparation of standard solution
 - 4.14.1 Stock standard solution.—500 mg/mL.
Weigh Folic Acid Reference Standard (4.4) equivalent to 55–56 mg folic acid (accurate to 0.1mg). Dissolved by 50 mL water and transferred into 100 mL volumetric flask. Add 2 mL aqua ammoni(4.5).
Final volume of stock standard solution (mL) = $m \times 1000 \times c / (100 \times 500)$
Or: stock standard solution (mL) = $m \times c / 50$
 m = weight of reference standard, mg,
 c = purity of folic acid standard, g/100g
When completely dissolved, dilute to volume with water, and add, by pipet, additional water needed for final volume, calculated as above. Mix well. Store in red or amber bottle in refrigeratory. Prepare fresh after 4 months.
 - 4.14.2 Inter mediate standard solution.—50 mg/mL. Accurately pipet 10 mL stock standard

solution(4.14.1), into 100 mL amber or red volumetric flask, dilute to volume with water, and mix thoroughly. Store in refrigerator. Make fresh after 1 month.

4.14.3 Working standard solution: low concentration solution:0.05ng/ml; high concentration solution: 0.1ng/ml.

Pipet 1mL intermediate standard solution(4.14.2), into amber or red 100mL volumetric flask, dilute to volume with water and mix. Pipet 1mL this first solution into another 100mL amber or red volumetric flask and dilute to volume, mixing well. Pipet 5mL this second solution separately into 250 mL and 500mL amber volumetric flask, dilute to volume with phosphate buffer solution, and mix. Label this as high working standard solution (0.0001g/mL or 0.1ng/mL) and low working standard solution (0.00005g/mL or 0.05ng/mL). Prepare fresh for each assay.

4.15 HCl solution: 1mol/L. Dilute 83.0ml Concentrated hydrochloric acid (37%, v/v) to 1000ml by water.

4.16 NaOH solution A: 4mol/L

Weigh 160g NaOH in 1000ml beaker, dissolved by 400ml water. Cool-off to room temperature and dilute to 1000ml.

4.17 NaOH solution B: 0.1mol/L

Dilute 2.5ml NaOH solution A with water to 100ml.

4.18 NaOH Standard Solution : 0.1mol/L±0.0002mol/L

Weigh 4g NaOH(accurate 0.1mg) in 1000ml beaker, demarcate by potassium acid phthalate. This solution container should be sealed to prevent penetration of carbon dioxide

4.18.1 Calibration of NaOH standard solution:

Weigh 0.18g (accurate to 0.1mg) potassium hydrogen phthalate drying to constant weight at 105 ° ~ 110 °. Dissolved in conical flask by 50mL water than CO₂, add two drops of 5 g/L phenolphthalein indicator, and titration with NaOH standard solution to pink. Do blank test at the same time, than calculate the concentration of the NaOH standard solution according to the formula:

$$c=m/ \{ (V_1-V_2) \times 0.2042 \}$$

c - the concentration of NaOH, (mol / L);

m - mass of potassium hydrogen phthalate, (g);

V1 - the amount of NaOH solution, (mL);

V2 - the amount of NaOH solution of blank test, (mL).

4.18.2 Phenolphthalein solution:

Weigh 0.5 g phenolphthalein dissolve in 75 mL 95% ethanol(v/v), add 20 mL of water, then add NaOH standard solution (4.18) until formation of pink by a drop, then volume to 100 mL.

4.19 Bromothymol blue indicator: weigh 0.1 g bromothymol blue in a mortar, add 1.6 mL NaOH solution B (4.17) and grinding, add a little water until completely dissolved, then transfer to 250 mL volumetric flask volume by water.

5 Apparatus

5.1 PH meter

5.2 Centrifuge: ≥ 2000 r/min

5.3 Spectrophotometer.

5.4 Analytical Balance: resolution 0.1mg.

- 5.5 Biochemical Incubator: $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$
- 5.6 burette: Sub-scale value of 0.1mL
- 5.7 vortex mixer

6 analytical procedure

6.1 preparation of strain

6.1.1 Transfer Lactobacillus (*Lactobacillus casei*) ATCC 7469 freeze-dried powder into Lactobacillus broth culture medium (4.9.2) tube, incubate 24 h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Transfer to Lactobacillus agar culture medium (4.9.1) tube, incubate 24 h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The cultured strain is reserve strain.

6.1.2 Make transfer of a pure strain from reserve strain to 3 Lactobacillus agar culture medium tube(4.9.1). Incubate 24h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Subculture monthly, and store at refrigeratory as monthly tube. Subculture 3 new tubes from monthly tube every month.

6.1.3 Subculture a Lactobacillus agar culture medium tube from monthly tube as daily tube, incubate 24h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

6.1.4 Inoculate a tube of Lactobacillus broth culture medium(4.9.2) from daily tube, and incubate 24h at 37°C . Centrifuge culture for 10minutes at 2000r/min under sterilized condition, then decant supernate. Resuspend cells by 10ml salt solution(4.2) and centrifuge it again. Repeat above steps again. Then resuspend cells by 10ml salt solution, transfer 1ml suspension into 10ml salt solution, mix thoroughly.

6.1.5 Using salt solution(4.2) as blank reference, test the optical density of this suspension by spectrophotometer at 550nm, the value should between 60%-80%.

6.2 preparation of sample:

6.2.1 dairy product

Weigh 2g (accurate 0.1mg) sample(equivalently contain $5\mu\text{g}$ folic acid) into 100 mL beaker. Reconstitute in 25–30 mL water and quantitatively transfer to 100 mL volumetric flask. Dilute to volume with water. Its concentration of folic acid is about 50ng/ml. Pipet 1 mL diluted test solutions and 1 mL chick pancreas preparation(4.1) into 180×15 mm screw top culture tube, and mix well. Add 18 mL 0.05mol/L phosphate buffer–ascorbic acid solution□(4.3.1), and 1 mL toluene(4.6). Mix. For blank, pipet 1 mL water and 1 mL chick pancreas preparation into a empty tube and add 18 mL 0.05mol/L phosphate buffer I (4.3.1) and 1 mL toluene. Incubate test solution and blank tubes 16 h at 37°C . Sterilize 5 min in water bath at 100°C . Dilute with phosphate buffer I (4.3.1) to the concentration of folate about 0.1ng/ml.

If the strengthen folic acid account large proportion in the sample compare with the native folic acid, directly add 1ml sample solution with 19ml 0.05mol/L phosphate buffer □(4.3.1), Sterilize 5 min in water bath at 100°C . Dilute with 0.05mol/L phosphate buffer □ (4.3.1) to the concentration of folate about 0.1ng/ml.

6.2.2 Cereal and cereal product

Weigh equivalently contain $1\mu\text{g}$ folic acid sample into 150 mL beaker. Mixed with 20ml phosphate buffer□(4.3.2) and add 50ml water, 1.0ml Toluene(4.6). Plug the caps and sterilize 15min at 121°C , then cool-off the tubes rapidly. Add 1ml Papain solution (4.11), incubate 3h at 37°C , then sterilize 3min at 100°C , cool-off. Add 1mlα-amylase(4.12) incubate 2h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$, add 4ml Chick pcreas preparation(4.1), plug the caps and incubate 16h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Then sterilize 3min at 100°C , cool-off and adjust PH to 4.5 by 1mol/L HCl solution (4.15). Dilute to

100ml by water. Filter the diluted solution to get the filtrate. Pipet 1 mL filtrate solutions to 100 mL volumetric flask, dilute to volume with phosphate buffer (4.3.3). The concentration of folate about 0.1ng/ml

If the strengthen folic acid account large proportion in the sample compare with the native folic acid, directly add 20ml 0.05mol/L phosphate buffer–ascorbic acid solution (4.3.2)and 50ml water with sample. Sterilize 15 min at 121°C. Filter and dilute 1ml the filtrate with 0.05mol/L phosphate buffer (4.3.3) to the concentration of folate about 0.1ng/ml.

6.3 preparation of standard curve

Add distilled water, working standard solution(using phosphate buffer(4.3.4) instead of phosphate buffer(4.3.1) when detect cereal and cereal products)and folic acid determine medium (4.9.3) in tubes according to the table1, making triplet.

Table1:

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
Medium:(ml)	5	5	5	5	5	5	5	5	5	5
#:add low concentration standard solution in No.3-7; add high concentration standard solution in No.8-10										

6.4 assay solution:

Add distilled water, sample solution and folic acid determine medium into according to the table2, making triplet.

Table2:

Tube No:	1	2	3	4
Distilled water :(ml)	4	3	2	1
Sample solution: (ml)	1	2	3	4
Medium:(ml)	5	5	5	5

6.5 sterilize

Sterilize all tubes 10min at 121°C and cool-off rapidly to culture temperature, to formation of lightest color. Ensure the heating and cooling condition regular(bad impact may occur if too congest or too much tubes in the autoclave).

6.6 Inoculation

Sterile inoculate 50 μl suspension to each tubes except standard uninoculation S1. Plug the caps, mix well all tubes.

6.7 Incubation

6.7.1 Titrimetric method

Incubate 72h at 36 °C ± 1 °C. Predict the growth situation through visually inspect each tubes: uninoculation tube should clear, the sample tubes and standard tubes should have gradual growth and free of other bacteria. If the uninoculation tube is turbid, the result is invalid.

6.7.2 Densitometer method

Incubate 16h-24h at 36 °C ± 1 °C.. Follow other step from 6.7.1.

6.8 assay

6.8.1 Titrimetric method

6.8.1.1 Transfer uninoculated blank tubes S1 and inoculate blank tubes S2 to flasks with 10 mL water. Titrate contents of each tube with NaOH Standard solution (4.18), using bromthymol blue indicator (4.19), or using pH meter to pH 6.8. Record the consumption of NaOH standard solution volume.

Note: Disregard assay results if titer of inoculated blank is 1.5 mL greater than titer of uninoculated blank.

6.8.1.2 Transfer standard tubes and sample tubes to flasks with 10 mL water. Titrate contents of each tube with NaOH standard solution (4.18.1), using bromthymol blue indicator (4.19), or using pH meter end to pH 6.8. Record the consumption of NaOH standard solution volume.

Note: The titer of S7 tubes usually between 8–12 mL.

6.8.2 Densitometer method

Read the optical density of S7 tubes after 5s oscillation with using S2 as reference at 550nm. And read the optical density of this tube again after 2h. If the difference between this two values are $\leq 2\%$, that mean you can take out all the tubes and assay them.

6.9 draw standard curve

According to the microorganism growth characteristic of logarithmic phase and plateau phase, draw 2 sect of logarithmic curve. With the value of folic acid in standard solution as X-axis, the titer of NaOH standard solution or value of optical density as Y-axis, draw standard curve.

6.10 calculation

Quantitative determine the content vitamin niacin and niacinamide from the standard curve in accordance with test value of 6.8. Calculate the average value of the 3 same serial number tubes, compare each value with the average value and abandon the one exceeding $\pm 15\%$. If calculable value you received is less than 2 / 3 of total tubes, must be redone; If calculable value is more than 2 / 3 of total tubes, recalculate the average value with calculable value. This average value can calculate the total average value of all serial number tubes Cx .

Note: Abandon the content value less than 0.05ng or high than 0.5ng of folic acid in sample.

7 Indication

Content of folic acid in sample according to this formula:

$$X = [(Cx \times D) - EB] \times 100 / 1000 \text{ m}$$

X = Content of folic acid in sample, $\mu\text{g}/100 \text{ g}$

Cx = average value of folic acid check from standard curve, ng;

D = dilution factor based on preparation of sample

EB = folic acid content in Chick pancreas blank tube, ng/mL

m = test portion weight or volume, g or mL

100 = conversion to per 100g

1000 = conversion from ng to μg .

The result indicated with average of two separate calculation, and keep to three decimal.

8 Allowable error

The difference between the values of the twice tests to the same sample should $\leq 10\%$.

10 **Limitation**

The limitation of this standard is 2 μ g/100g.