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Voluntary - Public

Date: 12/3/2009

GAIN Report Number: CH9113

China - Peoples Republic of

Post: Beijing

National Dairy Standard - Folic Acid

Report Categories:

FAIRS Subject Report

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Report Highlights:

On November 20, 2009, China notified the WTO of "National Food Safety Standard of the People's Republic of China for the Determination of Folic Acid in Foods for Products for Infants and Young Children, Raw Milk and Dairy Products" as SPS/N/CHN/160. The date for submission of final comments to the WTO is January 1, 2010. The proposed date of entry into force has not been specified.

Executive Summary:

On November 20, 2009, China notified the WTO of "National Food Safety Standard of the People's Republic of China for the Determination of Folic Acid in Foods for Products for Infants and Young Children, Raw Milk and Dairy Products" as SPS/N/CHN/160. The date for submission of final comments to the WTO is January 1, 2010. The proposed date of entry into force has not been specified.

Thanks go to the consortium of industry and 3rd country Embassies in Beijing for their assistance in translating and reviewing this standard.

This report contains an UNOFFICIAL translation of National Standard on Determination of Folic Acid in Raw Milk and Dairy Products for Infants and Young Children.

General Information:

BEGIN TRANSLATION

GB National Food Safety Standard
GB ××××—××××

Determination of Folic Acid in Foods for Products for Infants and Young Children, Raw Milk and Dairy Products

Draft for Comment

Issued on xx-xx-xxxx

Implemented on xx-xx-xxxx

Issued by the Ministry of Health
of the People's Republic of China

1. Scope:

This standard is formulated a microbial method for determination of folic acid.

This standard is applicable to Determination of Folic Acid in Foods for Products for Infants and Young Children, Raw Milk and Dairy Products.

The limitation of this standard is 20µg/kg.

2. Referenced normative documents

The following standards contain provisions, which through reference in this text, constitute provisions of this Standard. For dated references, subsequent amendments (exclude correction) to or revisions of any of these publications shall not apply to this Standard. All parties are subject 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 publication referred to applies.

3. Principle

Folic acid content of infant formula is estimated from acidimetric response of *Lactobacillus casei* (ATCC 7469).

4. Reagent, strain and culture medium

For all reagents, if no special specification is stated, refers to analytic reagent; All experiment water refers to level 2 water.

- 4.1. Chick pancreas preparation—Weigh 100 mg desiccated chick pancreas, add 20mL H₂O, and stir 15 min. Centrifuge 10 min at 3000 rpm and use clear supernate. Prepare fresh daily.
- 4.2. Salt solution.—Weigh 9g NaCl and dissolved in 1000mL H₂O. Transfer 10mL portions to culture tubes, plug. with cap and sterilize 20 min at 121°C. Pre pare fresh weekly
- 4.3. Phosphate buffer:
 - 4.3.1. Phosphate bufferI: Dissolve 5.85 g KH₂PO₄ and 1.22 g K₂HPO₄ in H₂O and dilute to 1 L. Add ascorbic acid in phosphate buffer solution to make the concentration to 0.5g/100ml. Prepare just prior to use.
 - 4.3.2. Phosphate bufferII (for preparation of cereal and cereal product): Dissolve 14.2 g Na₂HPO₄ in H₂O 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 4mol/L NaOH.
 - 4.3.3. Phosphate bufferIII (for preparation of cereal and cereal product): Dissolve 14.2 g Na₂HPO₄ in H₂O 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 4mol/L NaOH.
 - 4.3.4. Phosphate bufferIV (for standard solution of cereal and cereal product): 0.1mol/L, pH7.0。
Dissolve 13.61g KH₂PO₄ in H₂O and dilute to 1 L. Adjust PH to 7.0 by 4mol/L KOH.
- 4.4. Folic acid: reference standard
- 4.5. Strong aqua ammonia
- 4.6. Toluene
- 4.7. Ascorbic acid
- 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 (25°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 (25°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 (25°C).

(note: the commercial synthetic medium is better.)

4.10 KOH solution: 4mol/L KOH solution.

4.11 Papain solution: Weight 1g papain (≥6000U/mg PH6.0,40°C), dissolve in 100ml phosphate buffer (4.3.1). Prepare just prior to use.

4.12 α-amylase: Weight 1g α-amylase(1.5U/mg), dissolve in 100 ml phosphate buffer (4.3.1). Prepare just prior to use.

4.13 Sterile filter (0.22µm)

4.14 Preparation of standard solution

4.14.1 Stock standard solution.—500 mg/mL.

Accurately weigh Folic Acid Reference Standard(4.4) equivalent to 55–56 mg folic acid,. Using 50 mL H₂O, quantitatively transfer to 100 mL volumetric flask. Add 2 mL strong aqua ammoni(4.5).

final volume of stock standard solution(mL) = $m \times 1000 \times c / (100 \times 500)$

m= weight of reference standard, mg,

c = purity of folic acid standard, g/100g

When completely dissolved, dilute to volume with H₂O, and add, by pipet, additional H₂O needed for final volume, calculated as above. Mix well. Store in red or amber bottle at 10°C. 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 H₂O, and mix thoroughly. Store in a refrigerator. Make fresh after 1 month.

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

5 Apparatus

5.2 Centrifuge.

5.3 Spectrophotometer.

5.3.1 Spectrophotometer.

5.3.2 Microplate Reader.

5.4 microplate system.

5.5 Analytical Balance: resolution 0.1mg.

6 Determination

6.1 Preparation of strain

6.1.1 Transfer a pure Lactobacillus casei strain from strain culture medium to Lactobacillus agar culture medium (4.9.1). Incubate 24h at 37°C.

6.1.2 Inoculate a tube of Lactobacillus broth culture medium(4.9.2) from strain culture medium, and

incubate 24h at 37°C centrifuge culture for 10 minutes at 2000r/min under sterilized conditions, then decant supernate. Re-suspend cells by 10ml salt solution (4.2) and centrifuge it again. Repeat above steps again. Then re-suspend cells by 10 salt solution, transfer 1 ml suspension into 10 ml salt solution, mix thoroughly. Adjust the transmittance of this suspension in spectrophotometer with water as blank reference between 60%-80% by salt solution (4.2) before using.

6.2 Preparation of sample:

6.2.1 Dairy product

Weigh 2g (accurate 0.1mg) sample (equivalently contain 5 μ g folic acid) into 100 mL beaker. Reconstitute in 25–30 mL H₂O and quantitatively transfer to 100 mL volumetric flask. Dilute to volume with H₂O. The concentration of the folic acid is about 50ng/ml. Pipet 1 mL diluted test solution and 1 mL chicken 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 H₂O and 1 mL chicken pancreas preparation into a empty tube and add 18 mL 0.05mol/L phosphate buffer–ascorbic acid solution and 1 mL toluene. Mix. Incubate test solution and blank tubes 16 h at 37 C. Sterilize 5 min in water bath at 100°C. Dilute with 0.05mol/L phosphate buffer–ascorbic acid solution to the folate concentration of about 0.1ng/ml.

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

6.2.2 Cereal and cereal products

Weigh equivalent container of 1 μ g folic acid sample into 150 mL beaker. Mixed with 20ml phosphate buffer (4.3.2) and add 50ml H₂O, 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 1ml α -amylase (4.12) incubate 2h at 37, add 4ml Chicken pancreas preparation (4.1), plug the caps and incubate 16h at 37°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 H₂O. 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 should be about 0.1ng/ml.

If the strengthen of the folic acid accounts for a large proportion in the sample compared with the natural folic acid, directly add 20ml 0.05mol/L phosphate buffer–ascorbic acid solution (4.3.2) and 50ml H₂O to the sample. Sterilize 15 min at 121°C. Filter and dilute 1ml the filtrate with 0.05mol/L phosphate buffer–ascorbic acid solution (4.3.3) to get a concentration of folate of about 0.1ng/ml.

6.2.3 If using a microplate kit, follow the step from 6.2.1-6.2.2 to get the concentration of folate to about 0.1ng/ml. Sterilize the solution by 0.22 μ m Sterile filter (4.13) and store the filtrate in dark place until assay.

6.2.4 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 H₂O and mix. Pipet 1mL this first solution into an other 100mL amber or red volumetric flask, dilute to volume, and mix. 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.

6.3 Preparation of standard curve

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

Table1:

Tube No:	1	2	3	4	5	6	7	8	9	10
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, and make 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 the formation of the lightest color. Ensure the heating and cooling under regular conditions (incorrect results may occur if the tubes are too congested in the autoclave).

6.6 Inoculation

Add sterile inoculate 50µl suspension to each tubes except standard No1. Plug the caps, mix well all tubes.

6.7 Incubation

6.7.1 Titrimetric method

Choose a selected temperature ($\pm 0.5^{\circ}\text{C}$) between 30-40°C, incubate 72h. Predict the growth situation through visually inspecting each tube: incubation tube should clear, the sample tubes and standard tubes should have gradual growth and free of other bacteria. If the tube is contaminated by other microorganisms, the result is invalid.

6.7.2 Densitometer method

Choose a selected temperature ($\pm 0.5^{\circ}\text{C}$) between 30-40°C, incubate 16-24h. Follow other step from 6.7.1.

6.8 Assay

6.8.1 Densitometer method

Choose a suit parameter, assay the maximum concentration tube with using a blank inoculate tube as reference at 550nm. And assay this tube again after 2h. If the difference between this two result is $\leq 2\%$, that mean you can take out all the tubes and assay them.

6.8.2 Titrimetric method

6.8.2.1 Titrate

Titrate contents of each tube with 0.1mol/L NaOH, using bromthymol blue indicator, or using pH meter to pH 6.8. Disregard results of assay if titer of inoculated blank is more than 1.5mL greater than titer of un-inoculated blank. Titer at 5.0mL level of standard solution should be 8–12mL.

6.8.2.2 assay PH

When determining pH, read pH values to nearest 0.01 pH unit after incubation.

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 value of densitometer(PH) as Y-axis, draw standard curve. As far as possible line through the middle of two discrete points and smooth the standard curve.

Quantitative determine vitamin of each concentration of assay solution. Abandon the absorption value less than 0.5ml standard solution or high than 4.5ml standard solution.

For each concentration of assay solution, calculate the concentration of folic acid per ml. Calculate the average value, and each concentration assay solution value should not exceed the average $\pm 15\%$. 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, you can calculate content of samples according to the average of the value.

7 Calculations and indication

Content of folic acid in per 100g/ml sample according to this formula:

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

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 one decimal.

8 Allowable error

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