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National food safety standard

Determination of vitamin niacin and niacinamide in foods for infants and young children，milk and milk products

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Determination of vitamin niacin and niacinamide in foods for infants and young children, milk and milk products

1. Range:

The standard specifies a method for the detection of vitamin niacin and niacinamide in foods and dairy products.

The standard applies a method for the detection of vitamin niacin and niacinamide in foods and dairy products.

2. Referenced normative documents

References application is necessary to this standard. All indication date of references for only indication date version, for undated references, the latest version (include all modification) is also applies.

Method Ⅰ HPLC Method

3 Principles

After extracted with hot water and deproteinized by acid, sample is separated by C-18 chromatogram column and determinated with UV detector.

4 Material and Regents

If no special illumination, all reagents mentioned in this method are AR grade and water is grade Ⅰ water regulated in GB/T 6682.

4.1 α-amylase, enzymatic activity ≥ 1.5U/mg

4.2 Hydrochloric acid

4.3 Sodium hydroxide

4.4 Hydrochloric acid solution (2.4mol/L):accurate pipette 10mL Hydrochloric acid(4.2) to a 50mL volumetric flask, diluted to mark with water.

4.5 Sodium hydroxide (2.5mol/L): accurate weigh 5.0g Sodium hydroxide(4.3) to a 50mL volumetric flask, diluted to mark with water.

4.6 perchloric acid 60%V/V

4.7 Anhydrous Methanol, HPLC grade

4.8 Isopropyl, HPLC grade

4.9 Heptane Sodium sulphonate

4.10 Standard Solution

4.10.1 Niacin and Niacinamide standard stock solution (1.0mg/mL):

Accurately weigh about 0.1g(accurate to 0.0001g) of Niacin and Niacinamide standard material respectively to 100mL volumetric flask, dissolved with water, and add water to the mark.

4.10.2 Niacin and Niacinamide mixed standard intermediate solution (40μg/mL):

Respectively pipette 2ml of standard stock solution (4.10.1) to 50mL volumetric flask, add

water to the mark. Fresh preparation.

4.10.3 Niacin and Niacinamide mixed standard working solution:
Respectively pipette 0.0mL, 1.0mL, 2.0mL, 5.0mL, 10.0mL standard intermediate solution (4.10.2) to 50mL volumetric flask, add water to the mark. The respective concentration of them is 0.0μg/mL, 0.80 μg/mL, 1.60 μg/mL, 4.00 μg/mL, 8.00 μg/mL. Fresh preparation.

5 Instruments and Equipments

5.1 HPLC, equipped with UV detector

5.2 PH meter, accurate to 0.01

5.3 Ultrasonator

5.4 Analytical balance, accurate to 0.1mg

5.5 Incubator, 30~80℃

6 analysis procedure

6.1 Sample Pretreatment

6.1.1 Amylum Sample: For solid sample, weigh about 5.0g(accurate to 0.0001g) and add 25mL of 45-50℃ water; for liquid sample should mix equality, weigh about 20g(accurate to 0.0001g), to a 150mL conical flask, then add about 0.5g α-amylase(4.1), and mix thoroughly. Fill nitrogen to the conical flask and seal it, placed it into 50-60□ incubator for 30min, then cool it to room temperature.

6.1.2 No amylun Sample: For solid sample, weigh about 5.0g(accurate to 0.0001g) and add 25mL of 45-50℃ water; for liquid sample should mix equality, weigh about 20g(accurate to 0.0001g), to a 150mL conical flask and mixed. Stand it for 5~10min and dissolved thoroughly. Let it stand to room temperature.

6.1.3 Extraction: place above-mentioned conical flask to ultrasonator for 10min

6.1.4 Sedimentation and Constant Volume: after the sample solution cooling to room temperature, adjust PH of it to 1.7±0.1 with hydrochloric acid solution(4.4), let it stand for 2min, then adjust pH of it to 4.5±0.1 with sodium hydroxide(4.5). Transfer the sample solution into a 50mL volumetric flask; wash the conical flask with distilled water repeatedly and combine the washing liquid to the 50mL volumetric flask then add water to the mark. Filter the solution through filter paper. Again filter the solution through 0.45μm membrane filter and collect the filtrate as injection sample.

6.2 Reference Chromatography Parameter

Column: C18, 50×4.6 mm, 5μm film, or equivalent

Mobile phase: methanol(4.7)70mL, isopropyl(4.8)20mL, heptane Sodium sulphonate(4.9)1g, dissolved with 910mL water and mixed, adjust pH of the mobile phase to 2.1±0.1 with perchloric acid and filter it through 0.45μm membrane filter.

Flow rate: 1.00mL/min

UV detector wavelength: 261nm

Oven temperature: 25□

Inject volume: 10uL

6.3 Quantitative analysis

6.3.1 Inject the Niacin and Niacinamide mixed standard working solution (4.3.10) to HPLC apparatus to get area or height (the standard chromatographic refer to chart A.1.1 in appendix A). Plot the standard curve, the area or height as y-axis and the concentration of

working standard as x-axis.

6.3.2 Inject equal volume of sample solution to HPLC and get area or height. Based on the standard curve, calculated the concentration c_i of Niacin and Niacinamide in the sample solution.

7 Calculations and Expression

7.1 calculate the content of Niacin and Niacinamide in sample
Calculate the contents of Niacin and Niacinamide as formula (1):

$$X_{1或2} = \frac{c_i \times V \times 100}{m} \quad \cdots\cdots (1)$$

In this formula,
$X_{1\ or\ 2}$——the content of Niacin or Niacinamide in sample, the unit is milligram per hundred grams (μg/100g)
m——Sample weight, the unit is gram（g）;
ci——the concentration of Niacin or Niacinamide in sample solution, the unit is microgram per milliliter (μg/mL);
V——the volume of sample solution, the unit is milliliter (mL).

7.2 calculate the total content of Vitamin PP
Calculate the content of total vitamin PP as formula (2):

$$X = X_1 + X_2 \quad \cdots\cdots (2)$$

In this formula,
X – The content of Vitamin PP in sample, the unit is μg/100g
X_1 –The content of Niacin in sample, the unit is μg/100g
X_2 –The content of Niacinamide in sample, the unit is μg/100g

The result is the arithmetical mean of two independent tests, and reserved two decimal digits.

8 Precision

Absolute difference of two independent test results should not exceed 10% of arithmetical mean under the repeated test condition.

Method Ⅱ Microbiological method

9. **Principle**

Vitamin niacin and niacinamide content of infant formula is estimated from acidimetric or densitometer response of *Lactobacillus plantarum*(ATCC 8014).

10. Reagent and culture medium

All reagent, if no special specification, refers to analytic reagent; All experiment water, refers

to level 2 water.

10.1. H_2SO_4 solution A: 10mol/L.
Pour the 95%-98%(v/v)concentrated sulfuric acid 560ml slowly in 600 water, with stirring. Then cool-off and dilute to 1000mL.

10.2. H_2SO_4 solution B: 1mol/L.
Dilute 100ml H_2SO_4 solution A with water to 1000mL.

10.3. NaOH solution A: 3.75mol/L
Weigh 150g NaOH in 1000ml beaker, dissolved by 400ml water. Cool-off to room temperature and dilute to 1000ml.

10.4. NaOH solution B: 0.375mol/L
Dilute 100ml NaOH solution A with water to 1000ml.

10.5. 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

10.5.1 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 CO_2, 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).

10.5.2 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.

10.6. HCL solution: 0.1mol/L.
Dilute 8.3mL hydrochloric acid to 1000ml with water.

10.7. Ethanol solution: 25%(v/v)
Mix 250ml absolute ethanol and 750ml water.

10.8. Strain: *Lactobacillus plantarum*(ATCC 8014)

10.9. culture medium:

10.9.1. 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℃). Sterilize 15min at 121 ℃

10.9.2. 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℃). Sterilize 15min at 121 ℃

10.9.3. Vitamin niacin and niacinamide determine medium: Casamino Acid for witamin determination 12g, glucose 40g, Sodium 20g, L-Cystine 0.4g, DL-Tryptophan 0.2g, adenine hydrochloride 20mg, guanine hydrochloride 20mg, uracil 20mg, thiamine

hydrochloride 200μg, Calcium Pantothenate 200μg, pyridoxine hydrochloride 400μg, Riboflavin 400μg, β-amino acid 100μg, Biotin 0.8μg, dipotassium hydrogen phosphate 1g, potassium dihydrogen phosphate 1g, Magnesium sulfate 0.4g, sodium 20mg, Ammonium Sulfate Iron 20mg, manganese sulfate 20mg, add distilled water to total 1000ml, adjust PH to 6.7±0.2(20-25℃).

(note: the commercial synthetic medium is better.)

10.10. Standard solution

10.10.1. Stock standard solution.—100 μg/mL.

Weigh dried niacin Reference Standard to 50.0mg (accurate to 0.1mg) from phosphorus pentoxide desiccator. Using 25%(v/v) Ethanol solution(4.7) volume to 500ml, store at refrigeratory for 4 months.

10.10.2. Inter mediate standard solution.—10 μg/mL.

Accurately pipet 10 mL stock standard solution(10.10.1), into 100 mL volumetric flask, dilute to volume with 25%(v/v) Ethanol solution(10.7), store at refrigeratory for 1month.

10.11. Normal saline: Weigh 0.9g NaCL into 100ml volumetric flask, dilute to volume, mix thoroughly. Dispense each tube 10ml, and plug the caps, sterilize 15min at 121℃. Prepare fresh weekly.

10.12. Bromothymol blue indicator: weigh 0.1 g bromothymol blue in a mortar, add 1.6 mL NaOH solution B (10.17) and grinding, add a little water until completely dissolved, then transfer to 250 mL volumetric flas volume by water.

11. Apparatus

Common lab equipment and

11.1. Spectrophotometer.

11.2. pH meter

11.3 vortex mixer

11.4 Analytical Balance: resolution 0.1mg.

11.5 Biochemical Incubator: 36℃±1℃

11.6 centrifuge: ≥2000r/min

11.7 burette: Sub-scale value of 0.1mL

12. Determination

12.1 preparation of strain

12.1.1 Transfer Lactobacillus (Lactobacillus plantarum) ATCC 8014 freeze-dried powder into Lactobacillus broth culture medium (10.9.2) tube, incubate 24 h at 36 □ ± 1 □. Transfer to Lactobacillus agar culture medium (10.9. 1) tube, incubate 24 h at 36 □ ± 1 □. The cultured strain is reserve strain.

12.1.2 Make transfer of a pure (Lactobacillus plantarum) ATCC 8014 strain from reserve strain to 3 Lactobacillus agar culture medium tube(10.9.1). Incubate 24h at 36 □ ± 1 □. Subculture monthly, and store at refrigeratory as monthly tube. Then subculture a Lactobacillus agar culture medium tube from monthly tube as daily tube, incubate 24h at 36 □ ± 1 □. Subculture 3 new tubes from monthly tube every month.

12.1.3 Inoculate a tube of Lactobacillus broth culture medium (10.9.2) from daily tube, and incubate 24h at 36 □ ± 1 □. Centrifuge culture for 10minutes at 2000r/min under sterilized

condition, and then decant supernate. Resuspend cells by 10ml normal saline (10.11) and centrifuge it again. Repeat above steps again. Then resuspend cells by 10 normal saline (10.11), and transfer 1ml suspension into 10ml normal saline (10.11), mix thoroughly.

12.1.4 Using normal saline (10.10) as blank reference, the transmittance of the suspension (12.1.3) at 550nm spectrophotometer should between 60%-80%..

12.2 preparation of sample:

Weigh 2g (accurate 0.1mg) solid sample or 5g (accurate 0.1mg) liquid sample (equivalently contain 0.1mg niacin) into 250 mL conical beaker. Dissolved with 20 mL 1mol/L H_2SO_4 solution B (10.2), and sterilize 30min at 121℃.Adjust PH to 6.0-6.5 with NaOH solution A(10.3). And adjust PH to 4.5 with HCL solution (10.6). Dilute volume to 100ml with water. Filter the solution, pipet 25ml filtrate into 100ml beaker. Adjust PH to 6.8± 0.1 with NaOH Standard Solution (10.5). Transfer into 250ml volumetric flask, and dilute to volume.

12.3 preparation of standard curve

Add distilled water, standard solution and medium in tubes according to the table1, making triplet.

Table1:

Tube No:	S1	S2	S3	S4	S5	S6	S7
Distilled water:(ml)	5	5	4	3	2	1	0
Standard solution:(ml)	0	0	1	2	3	4	5
Medium:(ml)	5	5	5	5	5	5	5

12.4 assay solution:

Add distilled water, sample solution and 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

12.5 sterilize

Sterilize all tubes 10min at 121℃ and cool-off rapidly to room 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).

12.6 Inoculation

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

12.7 Incubation

12.7.1 Titrimetric method

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

12.7.2 Densitometer method

Incubate 16h-24h at 36 ℃ ± 1 ℃.. Follow other step from 6.8.1.

12.8 assay

12.8.1 Titrimetric method

12.8.1.1 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 (10.5), using bromthymol blue indicator, 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 1.5mL greater than titer of uninoculated blank.

12.8.1.2 Transfer standard tubes and sample tubes to flasks with 10 mL water. Titrate contents of each tube with NaOH standard solution (10.5), using bromthymol blue indicator, 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 8–12mL.

12.8.2 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 ≤2%, that mean you can take out all the tubes and assay them.

12.9 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 niacin in standard solution as X-axis, the titer of NaOH standard solution or value of optical density as Y-axis, draw standard curve.

12.10 Calculation

Quantitative determine the content vitamin niacin and niacinamide from the standard curve in accordance with test value of 12.8. Calculate the average value of the 3 same serial number tubes, compare each value with the average value and abandon the one exceeding ±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 100ng or high than 500ng of vitamin niacin and niacinamide in sample.

13. Indication

Content of niacin in sample according to this formula:

$$X(\mu g/100g) = Cx/m \times F/1000 \times 100$$

X=the content of vitamin niacin and niacinamide in sample, μg/100 g

Cx = average value of niacin check from standard curve, μg;

F = dilution factor based on preparation of sample

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 two decimal.

14. Allowable error

The difference between the values of the twice tests to the same sample should ≤10%.

15. Limitation

The limitation of the method Ⅰ is 30 μg/100 g for niacin，40 μg/100 g for niacinamide。

Appendix A

(Informative Annex)

Liquid chromatogram of niacin and niacinamide standard solution

A.1 Liquid chromatogram of niacin and niacinamide standard solution