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National Food Safety Standard - Determination of Folates in Food

食品安全国家标准

食品中叶酸的测定

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State Administration for Market Regulation.

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National Food Safety Standard - Determination of Folates in Food

1 Scope

This Standard specifies the methods of determining folates in food.

This Standard is applicable to the determination of folates in food.

2 Principle

Folates is an essential nutrient for the growth of *Lactobacillus rhamnosus*. Under certain controlled conditions, inoculate the *Lactobacillus rhamnosus* bacterial solution into the medium containing the specimen solution; after culturing for a period of time, determine the light transmittance (or absorbance value). Within a certain determination range, in accordance with the standard curve of folates content and light transmittance (or absorbance value), the folates content in the specimen can be calculated.

3 Reagents and Materials

Unless it is otherwise specified, the reagents used in this Method are analytically pure; the water is Grade-2 water specified in GB/T 6682.

3.1 Reagents

3.1.1 Hydrochloric acid (HCl).

3.1.2 Sodium hydroxide (NaOH).

3.1.3 Sodium chloride (NaCl).

3.1.4 Sodium phosphate dodecahydrate ($Na_3PO_4 \bullet 12H_2O$).

3.1.5 Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \bullet 7H_2O$).

3.1.6 L-ascorbic acid ($C_6H_8O_6$).

3.1.7 Toluene (C_7H_8).

3.1.8 Anhydrous ethanol (C_2H_6O).

3.1.9 Lyophilized chicken pancreas powder: containing γ-glutamyl hydrolase.

3.1.10 Papain: enzyme activity ≥ 5 U/mg.

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

3.2 Preparation of Reagents

3.2.1 Phosphate buffer (0.05 mol/L, pH 6.8): respectively weigh-take 4.35 g of sodium phosphate dodecahydrate and 10.39 g of disodium hydrogen phosphate heptahydrate; add water to dissolve and reach a constant volume of 1 L; mix it up. Add 2 mL of toluene; preserve it at room temperature. Before use, at a ratio of approximately 5 mg/mL, add L-ascorbic acid as a protectant of the folates; adjust pH to 6.8 ± 0.1.

3.2.2 20% ethanol solution (2 + 8): measure-take 200 mL of anhydrous ethanol and 800 mL of water; mix it up.

3.2.3 Sodium hydroxide ethanol solution (0.01 mol/L): weigh-take 0.4 g of sodium hydroxide; use 20% ethanol solution to dissolve and reach a constant volume of 1 L; mix it up.

3.2.4 Sodium hydroxide solution (1 mol/L): weigh-take 40 g of sodium hydroxide; add water to dissolve and reach a constant volume of 1 L; mix it up.

3.2.5 Hydrochloric acid soaking solution: measure-take 100 mL of hydrochloric acid (concentration: 36% ~ 38%); mix it up with 50 times of water.

3.2.6 Chicken pancreas solution: weigh-take 100 mg of lyophilized chicken pancreas powder; add 20 mL of phosphate buffer; mix it up. Prepare it right before use.

3.2.7 Protease-amylase solution: respectively weigh-take 200 mg of papain and α-amylase; add 20 mL of phosphate buffer; grind to homogenate. At 3,000 r/min, centrifuge for 5 min. Prepare it right before use.

3.3 Culture Medium

3.3.1 Agar medium for strain stock: in accordance with A.1, prepare it.

3.3.2 Medium for folates determination: in accordance with A.2, prepare it.

NOTE: the above mediums may also be commercialized synthetic or finished medium, which shall be prepared in accordance with the instructions before use.

3.4 Reference Substance

Folates reference substance ($C_{19}H_{19}N_7O_6$, CAS: 59-30-3): purity ≥ 97%, or reference substances certified by the state and awarded with a reference substance certificate.

3.5 Preparation of Standard Solutions

3.5.1 Folates standard stock solution (20.0 μg/mL): accurately weigh-take 20.0 mg of folates reference substance; use sodium hydroxide ethanol solution to dissolve it; transfer it and reach

and pulverizing method may be adopted to mix them up. Before use, liquid specimens shall be shaken to mix up. The specimens prepared above can be stored in the refrigerator at 2 °C ~ 4 °C for 1 week.

6.2 Specimen Extraction

6.2.1 Direct extraction method

When determining the content of folates added in the sample, the direct extraction method can be adopted.

Accurately weigh-take 0.1 g ~ 2 g of solid specimen or 0.5 mL ~ 2 mL of liquid specimen, accurate to 0.001 g; transfer it into a conical flask. Add 80 mL of sodium hydroxide ethanol solution, with a stopper. Perform ultrasonic oscillation for 0.5 h ~ 4 h, until the specimen is completely dissolved or dispersed, then, transfer it into a 100 mL volumetric flask; use water to dilute to the scale.

6.2.2 Enzymatic extraction method

The enzymatic extraction method should be adopted for the naturally occurring folates in food specimens, such as: cereals, potatoes, meat, eggs, dairy, fruits, vegetables, bacteria, algae, beans and nuts, etc.

Accurately weigh-take an appropriate amount of specimen (containing 0.2 μg ~ 2 μg of folates), accurate to 0.001 g. General cereals, potatoes, meat, dairy, fresh fruits and vegetables, bacteria and algae specimens: 2 g ~ 5 g; eggs, beans, nuts, offal and dried specimens: 0.2 g ~ 2 g; liquid or semi-liquid specimens: 5 g ~ 10 g. Transfer it into a 100 mL conical flask; add 30 mL of phosphate buffer. Shake it for 5 min, with a stopper. At 121 °C (0.10 MPa ~ 0.12 MPa), hydrolyze it under high pressure for 15 min.

After the specimen is taken out, cool it down to room temperature. Add 1 mL of chicken pancreas solution, 1 mL of protease-amylase solution; mix it up. Add 3 ~ 5 drops of toluene, then, place it in a constant-temperature incubator at 36 °C ± 1 °C to perform enzymatic hydrolysis for 16 h ~ 20 h. Take it out and transfer it into a 100 mL volumetric flask; add water to reach a constant volume, then, filter it.

Take another conical flask. DO NOT add the specimen. The other steps are the same as the operation of the specimen. Regard it as the enzyme blank solution.

NOTE: For formula foods based on grains and milk powder, if the background folates content of the matrix needs to be calculated, the enzymatic extraction method may be adopted.

6.3 Dilution

In accordance with the folates content in the specimen, use water to appropriately dilute the specimen extract, so that the folates content in the specimen diluent is within the range of 0.2 ng/mL ~ 0.3 ng/mL.

6.4 Specimen Determination

6.4.1 Test tube method

6.4.1.1 Specimen and enzyme blank series tubes

Take three test tubes; respectively add 1.0 mL, 2.0 mL and 3.0 mL of specimen diluent (V_x); add water to 5.0 mL; mix it up. Take another three test tubes; adopt the same method to add enzyme blank solution. For each gradient, perform 2 parallels.

6.4.1.2 Standard series tubes

Take the test tubes and respectively add 0.00 mL, 0.25 mL, 0.50 mL, 1.00 mL, 1.50 mL, 2.00 mL, 2.50 mL, 3.00 mL, 4.00 mL and 5.00 mL of the folates standard working solution; add water to 5.00 mL, which is equivalent to the folates content in the standard series tubes: 0.00 ng, 0.05 ng, 0.10 ng, 0.20 ng, 0.30 ng, 0.40 ng, 0.50 ng, 0.60 ng, 0.80 ng and 1.00 ng; mix it up. Prepare 2 ~ 3 sets of the standard series tubes. When drawing the standard curve, calculate by the average value of each standard point.

6.4.1.3 Sterilization

Perform autoclaving on all the series tubes used for determination and the medium for folates determination at 121 °C (0.10 MPa ~ 0.12 MPa) for 15 min (or sterilize in accordance with the requirements of the medium).

6.4.1.4 Inoculation and culture

After the series tubes used for determination are cooled down to room temperature, under the conditions of aseptic operation, add 40 μL of inoculum to each 10 mL of the medium for folates determination; mix it up. Add 5 mL of the inoculated medium for folates determination to each determination tube; mix it up. Place it in a constant-temperature incubator at 36 °C ± 1 °C to culture for 20 h ~ 40 h. When the maximum turbidity is obtained, terminate the culture. Prepare another standard 0 tube (containing 0.00 ng of folates) that is not inoculated and regard it as the 0 control tube.

6.4.1.5 Determination

Use a vortex oscillator to mix the cultured standard series tubes, specimens and enzyme blank series tubes. Use a 1 cm cuvette, at 540 nm, adjust the light transmittance to 100% (or the absorbance value is 0) with the 0 control tube that is not inoculated; successively determine the light transmittance (or the absorbance value) of the standard series tubes, specimens and enzyme blank series tubes. If the 0 control tube is turbid, it suggests that it may be contaminated with bacteria, and the experiment needs to be re-performed.

NOTE: an appropriate spectral range for the determination is 540 nm ~ 610 nm.

6.4.2 Microplate method

Appendix A

Preparation of Culture Medium

A.1 Agar Medium for Strain Stock

A.1.1 Reagents

A.1.1.1 Dipotassium hydrogen phosphate (K_2HPO_4).

A.1.1.2 Potassium dihydrogen phosphate trihydrate ($KH_2PO_4 \bullet 3H_2O$).

A.1.1.3 Magnesium sulfate heptahydrate ($MgSO_4 \bullet 7H_2O$).

A.1.1.4 Ferrous sulfate heptahydrate ($FeSO_4 \bullet 7H_2O$).

A.1.1.5 Manganese sulfate monohydrate ($MnSO_4 \bullet H_2O$).

A.1.1.6 Sodium acetate trihydrate ($CH_3COONa \bullet 3H_2O$).

A.1.1.7 Glucose ($C_6H_{12}O_6$).

A.1.1.8 Peptone: nitrogen content ≥ 10%.

A.1.1.9 Yeast extract (dry powder): nitrogen content ≥ 10%.

A.1.1.10 Agar.

A.1.1.11 Hydrochloric acid solution (1 mol/L): measure-take 83.3 mL of hydrochloric acid (concentration: 36% ~38%); use water to reach a constant volume of 1,000 mL; mix it up.

A.1.2 Preparation of reagents

A.1.2.1 Formate salt solution: respectively weigh-take 25 g of dipotassium hydrogen phosphate and 25 g of potassium dihydrogen phosphate trihydrate; add water to dissolve it and reach a constant volume of 500 mL; mix it up. Add 1 mL of toluene; mix it up. This solution can be stored in a refrigerator at 2 °C ~ 4 °C for 1 year.

A.1.2.2 Ethyl salt solution: respectively weigh-take 10 g of magnesium sulfate heptahydrate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate heptahydrate and 0.5 g of manganese sulfate monohydrate; add water to dissolve it and reach a constant volume of 500 mL. Add 5 drops of 1 mol/L hydrochloric acid solution; mix it up. This solution can be stored in a refrigerator at 2 °C ~ 4 °C for 1 year.

A.1.2.3 Agar medium for strain stock: in accordance with Table A.1, weigh-take or absorb-take each reagent; add water to 100 mL; mix it up. Heat it up in a boiling water bath, until the agar is completely dissolved. While it is still hot, use 1 mol/L hydrochloric acid solution or 1 mol/L

A.2.2.4 Adenine-guanine-uracil solution: respectively weigh-take 0.1 g of adenine sulfate, guanine hydrochloride and uracil into a 250 mL beaker; add 75 mL of water and 2 mL of hydrochloric acid; heat up to completely dissolve it, then, cool it down. If precipitation is generated, add a few drops of hydrochloric acid, then, heat it up. Repeat, until no precipitation is generated after cooling, then, add water to 100 mL. Add 3 ~ 5 drops of toluene and store in a brown reagent bottle. It can be stored in a refrigerator at 2 °C ~ 4 °C for 1 year.

A.2.2.5 Xanthine ($C_5H_4N_4O_2$) solution: weigh-take 0.4 g of xanthine, add 10 mL of ammonia; heat it up to dissolve it; add 100 mL of water. Add 3 ~ 5 drops of toluene; store it in a brown reagent bottle. It can be stored in a refrigerator at 2 °C ~ 4 °C for 1 year.

A.2.2.6 Acetic acid buffer (1.6 mol/L, pH 4.5): weigh-take 63 g of sodium acetate trihydrate; use 200 mL of water to dissolve it. Add about 20 mL of glacial acetic acid to adjust pH to 4.5 ± 0.1. Mix it up, then, use water to dilute to 500 mL.

A.2.2.7 Vitamin solution: weigh-take 100 mg of riboflavin and use 400 mL of acetic acid buffer to dissolve it. Take 25 mg of sodium bicarbonate and dissolve in 500 mL of water. Add 2 mg of biotin, 200 mg of para-aminobenzoic acid, 400 mg of pyridoxine hydrochloride, 40 mg of thiamine hydrochloride, 80 mg of calcium pantothenate and 80 mg of niacin to dissolve it. Mix the above-mentioned two solutions, then, add water to 1,000 mL. Add 3 ~ 5 drops of toluene; store it in a brown reagent bottle. It can be stored in a refrigerator at 2 °C ~ 4 °C for 1 year.

A.2.2.8 Polysorbate-80 solution (tween-80): dissolve 10 g of polysorbate-80 in absolute ethanol and dilute it to 100 mL. Store it in a refrigerator at 2 °C ~ 4 °C.

A.2.2.9 Reduced glutathione ($C_{10}H_{17}N_3O_6S$) solution: weigh-take 0.1 g of reduced glutathione; add 100 mL of water to dissolve it; store it in a brown bottle. Prepare right before use.

A.2.2.10 Phosphate buffer (0.05 mol/L, pH 6.8): in accordance with 3.2.1, prepare it.

A.2.2.11 Hydrochloric acid solution (1 mol/L): in accordance with A.1.11, prepare it.

A.2.2.12 Ethyl salt solution: in accordance with A.1.2.2, prepare it.

A.2.3 Medium for folates determination

Prepare 1,000 mL of the culture medium for folates determination. In accordance with Table A.2, absorb-take the liquid reagent. After mixing it up, add 300 mL of water; successively add the solid reagents; boil and stir it for 2 min. Use 1 mol/L sodium hydroxide solution and 1 mol/L hydrochloric acid solution to adjust pH to 6.8 ± 0.1. Add 20 mL of ethyl salt solution; use phosphate buffer to supplement to 1,000 mL. When preparing, it can be proportionally increased or decreased in accordance with the dosage. Prepare it right before use.

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