National Standard of the People's Republic of China

GB 5009.5—2010

National Food Safety Standard

Determination of protein in foods

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Preface

This standard replaces GB/T 5009.5-2003 Determination of protein in foods, GB/T 14771-1993 Testing method of protein in foods and GB/T 5413.1-1997 Milk powder and formula foods for infant and young children--Determination of protein.

The main revised contents of this method to GB/T 5009.5-2003 are as following:

— Automatic distillation method (Kjeldahl theory) is added in “Method I”;
— Combustion method is added as “Method III”;
— Conversion factor is revised;
— Specification for significant digits of calculated results is revised;
— Determination of the titration end point via pH meter is added.

The former standards replaced by this standard are as following:

— GB/T 5009.5-1985, GB/T 5009.5-2003；

— GB/T 14771-1993.

National Food Safety Standard

Determination of protein in foods

1 Scope

This standard specifies the detection method for the determination of protein in foods.

Method I and Method II of this standard apply to the determination of protein in various foods; Method III applies to the determination of protein in solid samples foods with protein content no less than 10g/100g, such as foodstuff, beans, milk powder, rice powder, protein powder, etc, by using screening method.

This standard does not apply to those foods with inorganic nitrogen or organic non-protein content.

Method I Kjeldahl

2 Normative Documents Cited by this Standard

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

3 Principle

Food is heated and catalytically digested and protein in it is decomposed into ammonia, which reacts with sulfuric acid into ammonium sulfate. The ammonia is released by distilling the ammonium sulfate in alkali solution, absorbed in boric acid and then titrated with standard sulfuric acid solution or standard hydrochloric acid solution. The content of protein is generated by multiplying the volume of titrant by the conversion factor.

4 Reagents and material

All reagents are analytical reagent grade unless otherwise stated.

All water is 3rd grade water specified in GB/T 6682

4.1 Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$).

4.2 Potassium sulfate (K_2SO_4).

4.3 Sulfuric acid (H_2SO_4), density 1.84g/mL at 20℃.

4.4 Boric acid ($H_3BO_{3)}$.

4.5 Methyl red ($C_{15}H_{15}N_3O_2$).

4.6 Bromcresol green ($C_{21}H_{14}Br_4O_5S$).

4.7 Methylene blue ($C_{16}H_{18}ClN_3S \cdot 3H_2O$).

4.8 Sodium hydroxide (NaOH).

4.9 Ethanol (C_2H_6O), 95% (v/v).

4.10 Boric acid solution (20g/L).

Weigh 20g boric acid into a 1000mL volumetric flask, dissolve and dilute to mark with water.

4.11 Sodium hydroxide (NaOH) solution (400g/L).

Dissolve 40g sodium hydroxide in water. Allow the liquid to cool down and then dilute to 100mL with water.

4.12 Sulfuric acid standard volumetric solution [$c(H_2SO_4) = 0.0500mol/L$] or hydrochloric acid standard volumetric solution [$c(HCl) = 0.0500mol/L$]

4.13 Methyl red ethanol solution (1g/L).

Dissolve 0.1g methyl red in 95% (volume fraction) ethanol in a 100mL volumetric flask. Dilute to 100mL with ethanol and mix.

4.14 Methylene blue ethanol solution (1g/L).

Dissolve 0.1g methylene blue in 95% (volume fraction) ethanol in a 100mL volumetric flask. Dilute to 100mL with ethanol and mix.

4.15 Bromocresol green ethanol solution (1g/L).

Dissolve 0.1g bromocresol green in 95% (volume fraction) ethanol in a 100mL volumetric flask. Dilute to 100mL with ethanol and mix.

4.16 Mixed indicator

Mixed indicator A: Mix 2 parts of methyl red ethanol solution (4.13) and 1 part of methylene blue ethanol solution (4.14) prior to use.

Mixed indicator B: Mix 1 part of methyl red ethanol solution (4.13) and 5 parts of bromocresol green ethanol solution (4.15) prior to use.

5 Apparatus

5.1 Weighing balance, capable of weighing to the nearest 1 mg.

5.2 Distillation apparatus, see figure 1.

5.3 Automatic Nitrogen Analyzer (Kjeldahl method)

6 Procedure

6.1 Kjeldahl

6.1.1 Preparation of sample

- Weigh a certain amount of well-mixed test sample (0.2 g ~ 2 g for solids, 2 g ~ 5 g for semi-solids and 10 g~25 g for liquid sample) (equivalent to 30 mg ~40 mg nitrogen), ca. to 0.001g.
- Transfer to a 100 mL, 250 mL or 500 mL pre-dried Kjeldahl flask;
- Add 0.2 g of copper sulfate pentahydrate(4.1), 6 g of potassium sulfate (4.2) and 20mL of sulfuric acid (4.3);
- Swirling gently to mix contents in the flask;
- Put a small funnel at the inlet of the flask;
- Hold the Kjeldahl flask in an inclined position (about 45^o) on an asbestos net with eyelet;
- Heat the flask gently in the digestion apparatus until all the contents have been carbonized and the frothing has stopped;

- Increase the heat setting until the liquid becomes clear and blue-green in colour, while liquid is under slight boiling condition;
- Continue boiling the solution for 0.5 ~ 1hr;
- Allow the flask to cool;
- Add carefully 20 mL water to the cooled flask;
- Transfer the clear digested liquid into a 100 mL volumetric flask;
- Rinse the Kjeldahl flask with water into the volumetric flask, then dilute to volume with water. Shake to mix thoroughly;
- Test sample is now ready for distillation.

Prepare a reagent blank using the same procedure and the same reagent, but omitting the test sample.

6.1.2 Determination

- Install the distillation apparatus in according to figure 1;
- Fill the steam generator with water to 2/3 of the volume;
- Add several glass beads;
- Add several drops of methyl red ethanol solution (4.13) and milliliters of sulfuric acid (4.3), ensuring water is acidic;
- Heat to boil the acidified water in the flask and keep the water boiling.

6.1.3 Titration

- Add 10.0 mL of boric acid solution (4.10) and 1~2 drops of mixed indicator solution into a conical flask.
- Immerse the outlet tip of condenser into the solution, ensuring it is below the solution's surface.
- Accurately pipette a certainty quantity (2.0~10.0 mL, in accordance with the nitrogen content of test sample) of digest solution and let it flow slowly down through the small glass cup into the reaction chamber, wash the small cup with 10 mL water and flow down to the reaction chamber. Stuff up the glass stopple at once.
- Pour 10.0 mL sodium hydroxide solution (4.11) into the small glass cup, lift up the clubbed glass stopple, let the solution flow slowly into the chamber.
- Immediately stuff up the glass stopple, then add water into the small glass cup as a seal to avoid leak.
- Clamp the clincher and begin to heat the steam generator to begin distillation.
- 10 minutes later, move conical flask to make liquid level of boracic acid leave the bottom of condenser.
- Distill continues for one minute.
- Rinse the outside of the tip of the condenser with some water.
- Remove the conical flask.
- Titrate the distillate with standard sulfuric acid volumetric solution or standard hydrochloric acid volumetric solution (4.12) until the color of boric indicator solution

changes from red purple to gray when using mixed indicator A(4.16) , pH 5.4; or from claret to green when using mixed indicator B (4.16), pH 5.1.

Carry out the reagent blank test in according to 6.1.3.

Where:

1— Electric furnace;

2— Steam generator (2 L flask) ;

3— Spiral clincher;

4— Small cup with clubbed glass stopple;

5— Reaction chamber;

6— Outer of reaction chamber;

7— Rubber hose with spiral clincher;

8— Condenser;

9— Conical flask

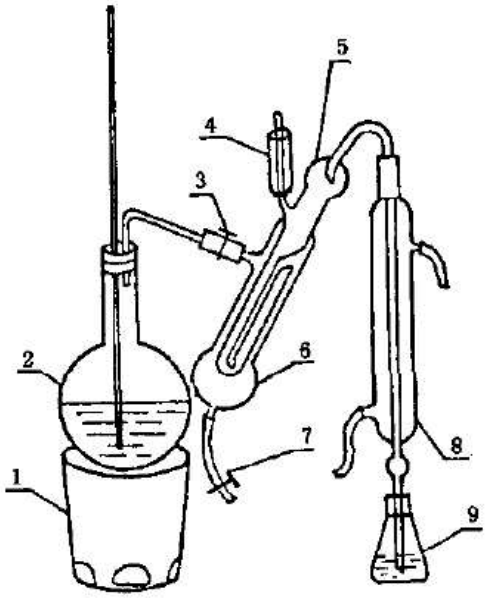

Figure 1 Distillation apparatus

6.2 Automatic distillation method (Kjeldahl theory)

Weigh a certain amount of well-mixed test sample (0.2 g ~ 2 g for solids, 2 g ~ 5 g for semi-solids and 10 g~25 g for liquid sample) (equivalent to 30 mg ~40 mg nitrogen), ca. to 0.001g. Carry out the determination following the procedure indicated in the operation manual.

7 Expression of results

Protein content of test sample is calculated according to equation (1):

$$X = \frac{(V_1-V_2) \times c \times 0.0140}{m \times V_3 / 100} \times F \times 100 \quad \text{.....(1)}$$

Where:

X –- Protein content of test sample, in g/100 g;

V_1 —- Volume of the standard acid volumetric solution (H_2SO_4 or HCl) used in the determination, in mL;

V_2 —- Volume of the standard acid volumetric solution (H_2SO_4 or HCl) used in the reagent blank test, in mL;

V_3 —- Volume of digested solution pipetted, in mL;

c —- Concentration of standard sulfuric acid volumetric solution or standard hydrochloric acid volumetric solution, in mol/L;

0.0140 –Weight of nitrogen atom that 1.0 mL standard sulfuric acid volumetric solution[c(1/2 H_2SO_4) = 1.000 mol/L] or 1.0 mL standard hydrochloric acid volumetric solution[c(HCl) = 1.000 mol/L] be equivalent to, in g;

m – Weight of test sample, in g;

F – Nitrogen-to-protein conversion factor, which is 6.25 for general foods, 6.38 for pure milk or pure dairy products, 5.70 for flour, 6.24 for corn and durra, 5.46 for peanut, 5.95 for rice, 5.71 for soybean and raw soybean products; 6.25 for soybean protein products; 6.25 for meat and meat products, 5.83 for barley, millet, oat and rye, 5.30 for sesame and sunflower, 6.25 for formulated composite food.

Express the result, i.e. With the repetitive determination, calculate the arithmetic mean value of two independent tests to three significant digits when the protein content is 1g/100g or higher, and to two significant digits when that is less than 1g/100g.

8 Precision

The absolute difference of two independent tests with the repetitive determination should agree to within 10% of the arithmetic mean value.

Method II Spectrophotometry

9 Principle

Protein in food is heated and catalytically digested to decompose and release ammonia. Ammonia reacts with sulfuric acid into ammonium sulfate, and the sulfate will react with acetyl acetone and formaldehyde in sodium acetate-acetic acid buffer solution (pH 4.8), producing a compound with yellow color, i.e. 3,5-diacetyl-2,6-dimethyl-1,4-dihydro-pyridine. Measure the absorbance at the wavelength of 400nm, quantitative analysis by comparing the value with that of standards. The content of protein is generated by multiplying the volume of titrant by the conversion factor.

10 Reagents

All reagents are analytical reagent grade unless otherwise stated.

All water is C grade water specified in GB/T 6682, i.e. distilled or demineralized water or equivalent.

10.1 Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$).

10.2 Potassium sulfate (K_2SO_4).

10.3 Sulfuric acid (H_2SO_4), density 1.84g/mL at 20℃, guaranteed reagent.

10.4 Sodium hydroxide (NaOH).

10.5 4-Nitrophenol ($C_6H_5NO_3$)

10.6 Sodium acetate ($CH_3COONa \cdot 3H_2O$).

10.7 Anhydrous sodium acetate (CH_3COONa)

10.8 Acetic acid ($C_2H_4O_2$), guaranteed reagent.

10.9 37% Formaldehyde (HCHO).

10.10 Acetyl acetone ($C_5H_8O_2$).

10.11 Sodium hydroxide (NaOH) solution (300g/L).

Dissolve 30g sodium hydroxide in water. Allow the liquid to cool down and then dilute to 100mL with water.

10.12 4-Nitrophenol indicator (1g/L).

Dissolve 0.1g 4-nitrophenol with 20mL ethanol (95% v/v) in a 100mL volumetric flask. Dilute to 100mL with water and mix.

10.13 Acetic acid solution (1mol/L).

Pipette 5.8mL glacial acetic acid (10.8) in a volumetric flask. Dilute to 100mL with water.

10.14 Sodium acetate solution (1mol/L).

Dissolve 41g anhydrous sodium acetate (10.7) or 68g sodium acetate (10.6) with water in a 500mL volumetric flask, then dilute to mark with water.

10.15 Sodium acetate- acetic acid buffer solution.

Weigh 60mL sodium acetate solution (10.14) and 40mL acetate acid solution (10.13) respectively. Mix them together and the pH of the mixture is 4.8.

10.16 Chromogenic agent.

Mix 15mL formaldehyde (10.9) with 7.8mL acetyl acetone (10.10), dilute the mixture to 100mL with water. Shake violently to mix well. (Allow it to stabilize by store it at room temperature for 3 days).

10.17 Ammonia nitrogen standard stock solution (1.0g/L, as nitrogen).

Accurately weigh 0.4720g of ammonium sulfate (dried at 105 □ for 2 hrs) and dissolve with water, move the solution into a 100mL volumetric flask, and then dilute to scale. Mix well. The nitrogen content in 1mL solution is equivalent to 1.0mg.

10.18 Ammonia nitrogen standard working solution (0.1g/L, as nitrogen)

Accurately pipette 10.00 mL ammonia nitrogen standard stock solution (10.17) into a 100mL volumetric flask, dilute to the scale with water. Mix well. The nitrogen content in 1mL solution is equivalent to 0.1mg.

11 Apparatus

11.1 Spectrophotometer.

11.2 Electric-heated thermostatic water bath (100℃ ± 0.5℃).

11.3 Glass colorimetric tubes with stopple, 10mL

11.4 Balance, weighing to 1 mg

12 Procedure

12.1 Digestion of sample

- Weigh a certain amount of test sample (0.1 g ~ 0.5 g (ca. to o.001g) for milled and well-mixed solids which has pass through 40 mesh screen, 0.2 g ~ 1 g (ca. to o.001g) for semi-solids and 1 g~5 g (ca. to o.001g) for liquid sample);
- Transfer to a 100 mL or 250 mL pre-dried Kjeldahl flask;
- Add 0.1 g of copper sulfate pentahydrate, 1 g of potassium sulfate and 5 mL of sulfuric acid (10.3);
- Swirling to mix contents in the flask;
- Put a small funnel at the inlet of the flask;
- Hold the Kjeldahl flask in an inclined position (about 45^{o}) on an asbestos net with eyelet;
- Heat the flask gently in the digestion apparatus until all the contents have been carbonized and the frothing has stopped;
- Increase the heat setting until the liquid becomes clear and blue-green in colour, while liquid is under slight boiling condition;
- Continue boiling the solution for half hour;
- Allow the flask to cool;
- Slowly add 20 mL water to the cooled flask;
- Transfer the clear digested liquid into a 50 mL or 100 mL volumetric flask;
- Rinse the Kjeldahl flask with some water into the volumetric flask, then dilute to volume with water. Mix thoroughly.

Prepare a reagent blank using the same procedure and the same reagent, but omitting the test sample.

12.2 Test solution preparation

- Pipette 2.00 mL ~ 5.00 mL sample digested solution or reagent blank digest solution into a 50mL or 100mL volumetric flask.
- Add 1~2 drops 4-Nitrophenol indicator (10.12), shake to mix.
- Add droplets of sodium hydroxide solution (10.11) to neutralize until color of solution changes to yellow.
- Add droplets of acetate acid (10.13) until the solution is colorless.
- Dilute the solution to scale with water and mix well.

12.3 Standard curve drawing

- Pipette 0.00, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.00 mL ammonia nitrogen standard working solution to 10mL glass colorimetric tubes respectively. (The nitrogen content in these tubes is equivalent to 0.0, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100.0 μg).
- Add 4.0 mL sodium acetate- acetic acid buffer solution (10.15) and 4.0 mL chromogenic agent (10.16), dilute the mixture to scale with water, mix well.
- Place the tubes in water bath of 100℃, heating for 15min.
- Take out and cool the tubes to room temperature by using water.
- Transfer the cooled solution into 1mL cuvette.
- Take the tube with ammonia nitrogen standard working solution as reference, measure the absorbance at the wavelength of 400nm.
- Draw standard curve by using the values obtained to get the linear regression equation.

12.4 Determination

- Pipette 0.50 mL~2.00 mL test sample (nitrogen content less than 100μg) into a 10mL glass colorimetric tube.
- Pipette same volume of reagent blank test solution into a 10mL glass colorimetric tube.
- Proceed as indicated in 12.3, from “Add 4 mL sodium acetate- acetic acid buffer solution (10.15) “ to the end.
- The content of protein can be quantified by comparing the absorbance with standard curve or be calculated using the linear regression equation.

Quantitatively analysis

13 Expression of results

Protein content of test sample is calculated according to equation (2):

$$X = \frac{(c - c_0)}{m \times \frac{V_2}{V_1} \times \frac{V_4}{V_3} \times 1000 \times 1000} \times 100 \times F \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (2)$$

Where:

X –- Protein content of test sample, in g/100 g;

c — Nitrogen content in test solution, in μg;

c_0 — Nitrogen content in reagent blank test solution, in μg;

V_1 —Constant volume of sample digest sample, in mL;

V_2— Volume of digestion solution used in test solution preparation, in mL;

V_3— Total volume of test sample solution, in mL;

V_4— Volume of sample solution used in determination, in mL;

m — Weight of sample, in g;

F – Nitrogen-to-protein conversion factor, which is 6.25 for general foods, 6.38 for pure milk or pure dairy products, 5.70 for flour, 6.24 for corn and durra, 5.46 for peanut,

5.95 for rice, 5.71 for soybean and raw soybean products; 6.25 for soybean protein products; 6.25 for meat and meat products, 5.83 for barley, millet, oat and rye, 5.30 for sesame and sunflower, 6.25 for formulated composite food.

Express the result, i.e. With the repetitive determination, calculate the arithmetic mean value of two independent tests to three significant digits when the protein content is 1g/100g or higher, and to two significant digits when that is less than 1g/100g.

14 Precision

The absolute difference of two independent tests with the repetitive determination should agree to within 10% of the arithmetic mean value.

Method III Combustion Method

15 Principle

Test sample burns at 900℃～1200℃, producing gas mixture, among which carbon, sulfur and other interfering gases and minerals are absorbed by absorption tube, and nitrogen oxides are completely reduced to nitrogen gas. The nitrogen gas flow will pass through a thermal conductivity detector (TCD). Commence the detection.

16 Apparatus

16.1 Nitrogen / protein analyzer;
16.2 Weighing balance, capable of weighing to the nearest 0.1mg.

17 Procedure

- Accurately weigh 0.1g~1.0g well-mixed test sample (ca. to 0.001g) following the instructions indicated in operational manual of analyzer.
- Wrap the sample with tin foil and put it on the sample plate.
- Combust the sample fully in combustion furnace (900 □ ~ 1200 □) using high pure oxygen (purity ≥ 99.99%).
- Outcome from the furnace (NO_x) is carried to a reduction furnace (800 □) with carrier gas CO_2 to be reduced to nitrogen gas.
- Commence the detection.

18 Expression of results

Protein content of test sample is calculated according to equation (3):

$$X = C \times F \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (3)$$

Where:

X – Protein content of test sample, in g/100g;

C – Nitrogen content in test sample, in g/100g;

F – Nitrogen-to-protein conversion factor, which is 6.25 for general foods, 6.38 for pure milk or pure dairy products, 5.70 for flour, 6.24 for corn and durra, 5.46 for peanut, 5.95 for rice, 5.71 for soybean and raw soybean products; 6.25 for soybean protein products; 6.25 for meat and meat products, 5.83 for barley, millet, oat and rye, 5.30 for sesame and sunflower, 6.25 for formulated composite food.

19 Precision

The absolute difference of two independent tests with the repetitive determination should agree to within 10% of the arithmetic mean value

20 Others

The limit of detection (LOD) of method I is 8 mg/100g for sample volume of 5.0g.

The limit of detection (LOD) of method II is 0.1 mg/100g for sample volume of 5.0g.