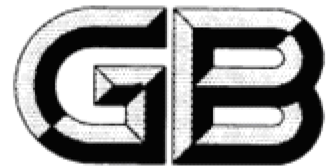

National Food Safety Standard of the People's Republic of China

GB5009.33-2010

National Food Safety Standard
Determination of Nitrite and Nitrate in Foods

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Preface

The Standard substitutes the GB/T 5009.33-2008 “Determination of Nitrite and Nitrate in Foods”.
Compared with the GB 5009.33-2008, main changes are following:

- In the Method I, the eluting condition is added for powder infant formula foods.
- The Method III: Oscillopolarography is deleted, and add determination of nitrite and nitrate in milk and dairy products as the Method III.

The replaced former editions are:

- GB 5009.33-85, GB/T 5009.33-1996, GB/T 5009.33-2003, GB/T 5009.33-2008.

Determination of Nitrite and Nitrate in Foods

1. Scope
   The Standard defines a method of determining nitrite and nitrate in foods.
   The Standard is applicable to the determination of nitrite and nitrate in foods.

Method I Ion chromatography

2. Principle
   Sample is extracted and purified using relevant method after protein precipitated and fat skimmed before separated by anion exchange column with KOH solution as an eluate and detected with a conductivity detector. It is then determined with an external standard method by taking retention time as for quantitative analysis.
3. Reagents and materials

3.1 Ultrapure water: with its conductivity of 18.2MΩ.cm.
3.2 CH3COOH: analytically pure
3.3 KOH: analytically pure
3.4 CH3COOH solution (3%): 3ml CH3COOH (3.2) into 100ml volumetric flask, diluted to a mark with water and fully homogenized.
3.5 Nitrite ion (NO_2^-) stock solution (100mg/L, aqueous solution).
3.6 Nitrate ion (NO_3^-) stock solution (1000mg/L, aqueous solution).
3.7 Mixed standard solution of nitrate (counted on NO_3^- ion, the same herein below) and nitrite (counted on NO_2^- ion, the same herein below): accurately pipette 1.0mL of nitrite ion (NO_2^-) stock solution and nitrate ion (NO_3^-) stock solution to 100mL volumetric flask, diluted to a mark with water, which 1mL of this solution contains 1.0μg of nitrite ion and 10.0μg of nitrate ion.

4 Instruments and equipments
4.1 Ion chromatograph: including a conductivity detector, suppressor, high capacity anion exchange column, measuring ring in 25μl.
4.2 Food disintegrator.
4.3 Supersonic cleaner.
4.4 Analytical balance: readability 0.1mg and 1mg.
4.5 Centrifuge: rotational speed no less than 10000rpm with 5ml or 10ml centrifugal tubes.
4.6 0.22μm syringe filters with hydrophilic filterable membrane.
4.7 Decontaminating column: including C_{18} column, Ag column and Na column or its equivalent.
4.8 Syringe: 1.0ml and 2.5ml.
All glassware should be soaked in 2mol/L of NaOH solution and water for 4h, respectively, followed by rinsing with water for 3-5 times before ready for use later.

5 Analytical Procedures
5.1 Sample pre-treatment

5.1.1 Fresh vegetable and fruit: the whole piece of vegetable and fruit is washed with deionized water, the edible portion of these vegetable and fruit is then disintegrated to uniformity after air dried. The adequate amount of disintegrated sample is then taken by quartering, and prepared into slurry with a stamp mill for use later. Water addition should be recorded if water is required to add.
5.1.2 Meat, egg, aquatic products and their processed products: an adequate amount or full of materials is taken with quartering, and then prepared into slurry with a stamp mill for use later.
5.1.3 Solid dairy products such as milk powder, soybean milk powder, and infant formula powder (excluding cheese): sample is put in a container with lid in capacity of two folds of sample; the sample is finally homogenized by repeatedly shaking and reversing the container.
5.1.4 Fermented milk, milk, condensed milk and other liquid dairy products: the sample isc or repeatedly shaking and reversing the container.
5.1.5 Cheese: an adequate amount of sample is uniformly ground to a muddy form. In order to avoid the loss of water content the excessive heat should be avoidable during the process of grinding.

5.2 Extraction
5.2.1 Fruit, vegetable, fish, meat, egg and their processed products: 5g (accurately weighed to 0.001g) of sample in a homogeneous slurry form are taken and washed into an 100mL volumetric flask with 80mL water, extracted for 30min with an ultrasonic generator, shaken once every 5min to make sure that the solid phase is fully distributed. Leave it on a water bath at 75℃ for 5min before making volume with water. A portion of solution after filtered is then subjected to centrifuge in 10000rpm for 15min; the supernatant is ready for use later.
5.2.2 Salted fish, salted meat, and other processed products: 2g (accurately weighed to 0.001g) of sample in a homogeneous slurry form are taken and washed into an 100mL volumetric flask with 80mL water, extracted for 30min with an ultrasonic generator, shaken once every 5min to make sure that the solid phase is fully distributed. Leave it on a water bath at 75℃ for 5min before making volume with water. A portion of solution after filtered is then subjected to centrifuge in 10000rpm for 15min; the supernatant is ready for use later.
5.2.3 Milk: 10g (accurately weighed to 0.001g) of sample are put into an 100mL volumetric flask with 80mL water added, shaken uniformly, ultrasonic treated for 30min, 2mL of 3% of glacial acetic acid is then added before leaving it at 4℃for 20min and resting it to ambient temperature, making volume with water. The supernatant after filtered is ready for use later.
5.2.4 Milk powder: 2.5g (accurately weighed to 0.001g) of sample are put into an 100mL volumetric flask with 80mL water added, shaken uniformly, ultrasonic treated for 30min, 2mL of 3% of glacial acetic acid is then added before leaving it for 20min at 4℃ and resting it at ambient temperature, making volume with water. The supernatant after filtered is ready for use later.
5.2.5 15mL of supernatant are taken to run through a 0.22μm syringe filters with

hydrophilic filterable membrane and C_{18} column, the front segment in 3mL is discarded (if Cl^- ion is over 100mg/L, the supernatant should be successively run through syringe filters, C_{18} column, Ag column and Na column, the front segment in 7mL shall be discarded), the eluate collected is then determined.
The solid phase extraction column should be activated before applied. The activation is carried out as follows: if OnGuard II RP column (1.0mL), OnGuard II Ag column (1.0mL) and OnGuard II Na column (1.0mL) are engaged in the application: OnGuard II RP column is run through with 10mL of methanol, 15mL of water before use, and then activated by resting for 30min. OnGuard II Ag column (1.0mL) and OnGuard II Na column (1.0mL) are run through with water before activated with resting for 30min.

5.3 Chromatographic conditions for reference
5.3.1 Chromatographic column: selectivity of hydroxide, high capacity anionic exchange column compatible to gradient elution, such as Dionex IonPac AS11-HC 4mm×250mm (with a protection column in IonPac AG11-HC type 4mm×50mm)[1)], or equivalent ion chromatographic column.
5.3.2 Elution solution
5.3.2.1 General samples: KOH solution with its concentration of 6 mmol/l-70mmol/l, elution gradient is 6mmol/l for 30min, 70mmol/l for 5min and 6mmol/l for 5min. Flow rate is 1.0ml/min.
5.3.2.2 Powder infant formula foods: KOH solution with its concentration of 5 mmol/l-50mmol/l, elution gradient is 5mmol/l for 33min, 50mmol/l for 5min and 5mmol/l for 5min. Flow rate is 1.3ml/min.
5.3.3 Inhibitor: anion inhibitor with regenerated membrane in automatic and continuous mode, or its equivalent.
5.3.4 Detector: conductivity detector with its temperature of detector cell at 35℃.
5.3.5 Sample volume: 25μL (enabled to be modified according to the content of ion to be measured).

5.4 Determination
5.4.1 Standard curve
The mixed standard solution of nitrite and nitrate pipetted is diluted with water to prepare a series of standard solutions with nitrite ion concentration of 0.00mg/L, 0.02mg/L, 0.04mg/L, 0.06mg/L, 0.08mg/L, 0.10mg/L, 0.15mg/L, 0.20mg/L, and with nitrate ion concentration of 0.0mg/L, 0.2mg/L, 0.4mg/L, 0.6mg/L, 0.8mg/L, 1.0mg/L, 1.5mg/L, 2.0mg/L. The chromatographic diagram of standard solution above with each concentration is obtained by successive injection of samples one by one from the lowest concentration. The calibration curve is plotted using concentration (mg/L) of nitrite and nitrate ions as abscissa and peak height (μS) and peak area as ordinate to calculate the linear regression equation.

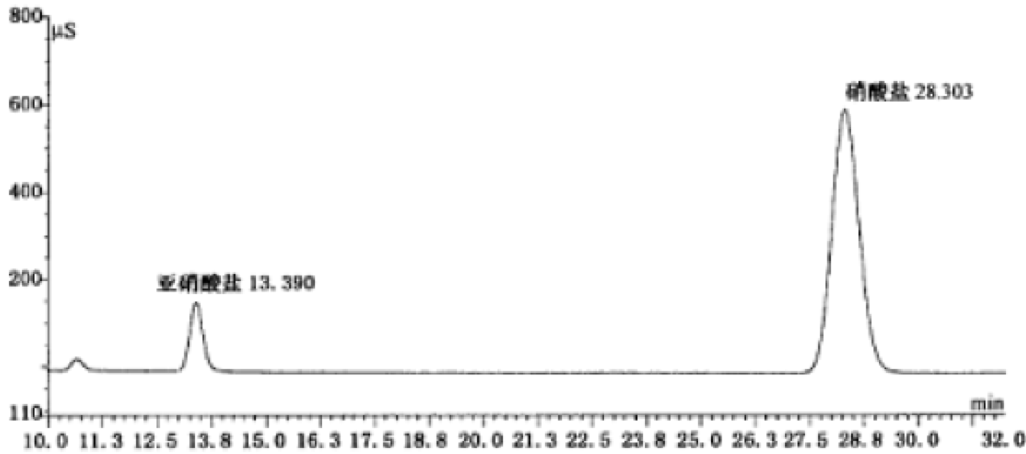


Fig.1 Chromatographic diagram of mixed standard solution of nitrite and nitrate

5.4.2 Determination of samples

50ul blank solution and 50ul sample solution are injected into ion chromatograph one by one at the same working condition, respectively, chromatographic diagrams are then recorded. The peak height (μS) and peak area are individually measured using retention time for qualitative analysis.

6 Formulation of analytical results

The contents of nitrite (counted on NO_2^- ion) and nitrate (counted on NO_3^- ion) in samples are calculated in accordance with formula (1):

$$X = \frac{(c - c_0) \times V \times f \times 1\,000}{m \times 1\,000} \qquad \cdots\cdots (1)$$

where,

X—the content of nitrite or nitrate in samples, mg/kg;

C—the content of nitrite or nitrate in samples for measurement, mg/L;

C_0—the content of nitrite or nitrate in blank solution, mg/L;

V—the volume of sample solution, mL;

f—dilution factor of sample solution;

m—sample taken, g.

Note: The content of $NO2^-$ in the sample multiplies by 1.5, to represent the nitrite content (calculation per sodium nitrite). The content of $NO3^-$ in the sample multiplies by 1.37, to represent the nitrate content (calculation per sodium nitrate).

The result is represented by the mean arithmetical value from two independent determination results under the same condition, and keeps two digits.

7. Precision

The absolute difference of two independently measured results under the same condition will not be over 10% of arithmetic mean.

Method II: Spectrophotometry

8. Principle

Nitrite determination applies to the method of naphthyl ethylenediamine hydrochloride.

Nitrate determination applies to the method of Cd column deocidization.

The sample after its protein precipitated, fat removed and nitrite diazotized with p-animobenzenesulfonic acid at weak acidic condition is coupled with naphthyl ethylenediamine hydrochloride (NEAH) to form a dye in purple red, the nitrite content is then determined by comparing with a standard. The nitrate content is determined after nitrate reduced to nitrite with the help of Cd column to give a total nitrite content to be determined, followed by the nitrite content deducted from the total nitrite content.

9. Reagents

9.1 $K_4Fe(CN)_6 \cdot 3H_2O$

9.2 $Zn(CH_3COO)_2 \cdot 2H_2O$

9.3 CH_3COOH

9.4 $Na_2B_4O_7 \cdot 10H_2O$

9.5 HCl (ρ=1.19g/ml)

9.6 Ammonia (25%)

9.7 $C_6H_7NO_3S$

9.8 $C_{12}H_{14}N_2 \cdot 2HCl$

9.9 $NaNO_2$

9.10 $NaNO_3$

9.11 Rolled tin or zinc bar

9.12 Cadmium sulfate

9.13 Potassium ferrocyanide (106g/L): 106.0g of potassium ferrocyanide (9.1) weighed is dissolved with water before diluted to 1000mL.

9.14 Zinc acetate solution (220g/L): 220.0g of zinc acetate (9.2) weighed is dissolved by adding 30ml glacial acetic acid and then diluted to 1000ml.

9.15 Saturated borax solution (50g/L): 5.0g of sodium borate (9.4) is dissolved in hot water, and after cooled it is ready for use later.

9.16 Ammonia buffer solution (pH 9.6-9.7): 30mL of HCl (9.5) is taken, then added with 100mL water and 65mL ammonia water (9.6) after mixed, and finally diluted with water to 1000mL, mixed to adjust it to pH 9.6-9.7.

9.17 Diluted ammonia buffer solution: 50mL of ammonia buffer solution(9.16) is taken and diluted with water to 500mL, and mixed.

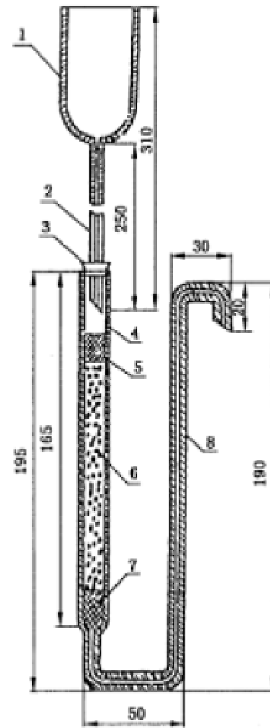
9.18 HCl solution (0.1mol/L): 5mL of HCl is taken and diluted with water to 600mL.

9.19 p-animobenzenesulfonic acid solution (4g/L): 0.4g of p-animobenzenesulfonic acid(9.7) is dissolved with 100mL of 20% HCl in a brown flask, mixed and stored, and kept away from the direct sunlight.

9.20 Naphthyl ethylenediamine hydrochloride (2g/L): 0.2g of naphthyl ethylenediamine hydrochloride(9.8) is dissolved in 100mL water, mixed and stored, and kept away from the direct sunlight.

9.21 Sodium nitrite stock solution: 0.1000g of sodium nitrite which is dried to constant weight at 110-120□ is put into a 500mL volumetric flask, diluted with water to a mark, and mixed. This solution contains 200μg/mL of sodium nitrite.
9.22 Sodium nitrite standard solution: 5.00mL of sodium nitrite stock solution is taken before use to a 200mL volumetric flask, diluted with water to a mark. This solution contains 5.0μg/mL of sodium nitrite.
9.23 Sodium nitrate stock solution: 0.1232g of sodium nitrate which is dried to constant weight at 110-120□ is put into a 500mL volumetric flask, diluted with water to a mark, and mixed. This solution contains 200μg/mL of sodium nitrate
9.24 Sodium nitrate standard solution: 2.50mL of sodium nitrate stock solution is taken before use to a 100mL volumetric flask, diluted with water to a mark. This solution contains 5μg/mL of sodium nitrate.

10. Instruments and equipments
10.1 Analytical balance: readability 0.1mg and 1mg
10.2 Stamp mill.
10.3 Ultrasonic cleaner.
10.4 Constant temperature oven.
10.5 Spectrophotometer.
10.6 Cd column
10.6.1 Preparation of sponge Cd: sufficient zinc sheet or zinc rod is put in 500mL of $CdSO_4$ solution (200g/L) for 3-4h, after cadmium therein completely replaced by zinc the cadmium on glass rod is softly scratched off to be sunk at the bottom of container, the remnants of zinc rod is taken out and the supernatant is then removed, the remained material is washed several times with water before transferred to a stamp mill, added with 500mL water, smashed to 2s, washed with water onto a standard sieve, 20-40 mesh of portion of powder is finally taken.
10.6.2 Packing of Cd column as shown in Fig. 2: filled with water in the Cd column and packed at bottom of the column with 2cm in height of glass cotton as a pad, removing all air entrapped out of the column, packed with 8-10cm sponge cadmium in height while softly knocked, finally covered with 1cm in height of glass cotton. A funnel is equipped at upper position and inserted through a rubber stopper and connected with a glass tube of the cadmium column.
25mL of acid burette can be used to replace the Cd column glass tube if it is not available, but the attention should be paid that the liquid level should be always above the Cd layer.
After packed, the Cd column will be washed with 25mL of HCl (0.1mol/L) and 25mL of water twice. The Cd column should be sealed with water if it is not in use, water level should be always kept above the Cd layer, and bubble is not allowed to be entrapped inside the Cd layer.



Unit: mm

1—funnel, i.d. 35mm, o.d. 37mm;
2—inlet capillary, i.d. 0.4mm, o.d. 6mm;
3—rubber stopper;
4—Cd column glass tube, i.d. 12mm, o.d. 16mm;
5,7—glass cotton;
6—sponge Cd;
8—outlet capillary, i.d. 2mm, o.d. 8mm.

Fig. 2 Schematic diagram of Cd column

10.6.3 After completed with use the Cd column should be washed with 25mL of HCl (0.1mol/L) and 25mL of water twice. The Cd column should be finally covered with water.

10.6.4 Determination of reduction efficiency of Cd column: 20mL of sodium nitrate standard solution is taken, added with 5mL diluted ammonia buffer solution, poured in the funnel after mixed to reduce Cd column by running through, and collected the effluent to the previous beaker, after all solution in funnel being run off, added with 5mL water to replace all inside remained sample solution. 10.0mL of reduced solution is taken (equivalent to 10μg sodium nitrite) in a 50mL cuvette, followed by a procedure as described in section of 12.4 as “taking 0.00mg/L, 0.20mg/L, 0.40mg/L, 0. 60mg/L, 0. 80mg/L, 1.00mg/L……” to calculate the results according to a calibration curve, keeping the addition in consistency, reduction efficiency should exceed 98% as required.

10.6.5 Calculation of reduction efficiency

reduction efficiency should be calculated based on formula (2):

$$X = \frac{A}{10} \times 100\% \qquad \cdots\cdots (2)$$

where,
X—Reduction efficiency, %;
A—the determined content of nitrite, μg;
10—Solution for measurement equivalent to the content of nitrite, μg.

11. Analytical Procedures

11.1 Pretreatment of samples

The same to 5.1

11.2 Extraction

5g (accurately weighed to 0.001g) of sample in a homogeneous slurry form are taken into a 50mL beaker (amount of water added during processing should be corrected accordingly), added with 12.5mL of saturated borax solution(9.15), mixed homogeneously, washed to a 500mL volumetric flask with 300mL water at about 70□, heated on a hot water bath for 15min, removed to a cooling bath for cooling, and finally placed at ambient temperature.

11.3 Purification of extracting solution

5mL of potassium ferrocyanide solution(9.13) are added into the extracting solution while rotating it, added with 5mL of zinc acetate solution(9.14) to precipitate protein. It is then added with water to a mark, mixed homogeneously, resting for 0.5h to remove fat at upper layer, the supernatant is filtered, the first 30mL of filtrate is discarded, and the filtrate is ready for use later.

11.4 Determination of nitrite

Pipette 40.0ml of the filtrate mentioned above into a 50mL capped cuvette, and pipette another 0.00mL, 0.20mL, 0.40mL, 0.60mL, 0.80mL, 1.00mL, 1.50mL, 2.00mL and 2.50mL of nitrite standard solution (equivalent to 0.0μg, 1.0μg, 2.0μg, 3.0μg, 4.0μg, 5.0μg, 7.5μg, 10.0μg and 12.5μg of sodium nitrite) into a 50mL capped cuvette, respectively. 2mL of p-animobenzenesulfonic acid (9.19) are respectively added into a cuvette for standard and a cuvette for sample before added with 1mL of naphthyl ethylenediamine hydrochloride (2g/L) after rested for 3-5min, then added with water to a mark, mixed thoroughly, rested for 15min before poured into a 2cm cuvette, zeroing with a cuvette of blank solution and absorbance measured at 538nm to plot a calibration curve, the same procedure is carried out for blank.

11.5 Determination of nitrate

11.5.1 Reduction of Cd column

11.5.1.1 Rinse Cd column with 25mL of diluted ammonia buffer solution(9.17), the flow rate is controlled at 3-5mL/min (2-3mL/min for burette replaced).

11.5.1.2 20mL of the filtrate is pipetted in a 50mL beaker, added with 5mL of ammonia buffer solution(9.18) and then poured into the funnel after mixed to reduce Cd column by running through, and collected the effluent to the previous beaker, after all solution in funnel is run off, added with 5mL water to replace all inside remained sample solution.

11.5.1.3 All collected effluents are reduced again by running through the Cd column, the secondary effluent is collected in the 100mL volumetric flask, the Cd column is

then washed three times with 20mL of water each, washing solution is collected in the same flask before added with water to a mark, and mixed thoroughly.

11.5.2 Determination of total sodium nitrite

10-20mL of reduced sample solution is pipetted into a 50mL cuvette, processed with the procedure in 12.4 as described as “taking 0.00mL, 0.20mL, 0.40mL, 0.60mL, 0.80mL, 1.00mL……”

12. Formulation of analytical results

12.1 Calculation of nitrite content

The content of nitrite (calculation per sodium nitrite) should be calculated based on formula (3):

$$X_1 = \frac{A_1 \times 1\,000}{m \times \frac{V_1}{V_0} \times 1\,000} \quad \cdots\cdots (3)$$

where,

X_1—the nitrite content in sample, mg/kg;

A_1—the mass of nitrite in sample in measurement, μg;

m—the mass of sample, g;

V_1—the volume of sample in measurement, mL;

V_2—the total volume of effluent, mL.

The result is represented by the mean arithmetical value from two independent determination results under the same condition, and keeps two digits.

12.2 Determination of nitrate

The content of nitrate (calculation per sodium nitrate) should be calculated based on formula (4):

$$X_2 = \left(\frac{A_2 \times 1\,000}{m \times \frac{V_2}{V_0} \times \frac{V_4}{V_3} \times 1\,000} - X_1\right) \times 1.232 \quad \cdots\cdots (4)$$

where,

X_2—the sodium nitrate content in sample, mg/kg;

A_2—the measured mass of total Cd-reduced sodium nitrite, μg;

m—the mass of sample, g;

V_2—the volume of sample solution in measurement of total sodium nitrite, mL;

V_0—the total volume of effluent, mL;

V_4—the volume of the Cd column reduced sample solution, mL;

V_3—the total volume of the Cd-reduced sample solution, mL;

X_3—the sodium nitrite content in sample calculated based on Eq. (3), mg/kg;

1.232—factor of sodium nitrite converted to sodium nitrate.

The result is represented by the mean arithmetical value from two independent determination results under the same condition, and keeps two digits.

13. Precision

The absolute difference of two independently measured results under the same condition shall not exceed10% of the arithmetic mean.