National Standard of the People's Republic of China

GB 5413.25-2010

National food safety standard Determination of inositol in foods for infants and young children, milk and milk products

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Foreword

This standard replaces GB/T 5413.25- 1997 (Determination of Inositol in Formula Foods and Milk products for Infants and Young Children).

Compared with GB/T 5413.25- 1997, the following main changes have been made to the first method:

- modify strain storage culture medium.
- change hydrochloric acid distillation method to hydrochloric acid pressure hydrolysis for test sample treatment.
- change strain inoculation mode from mixing bacteria liquid and culture medium and then dispensing to test tubes to dripping bacteria liquid to test tubes.
- adjust the concentration of standard working solution.
- adjust the sterilization temperature from 100□to 121□.
- identify the requirements and methods for controlling the concentration of inoculation bacterial suspension.
- increase the suitability of the calculation formula.
- increase the detection limit.

The following main changes have been made to the second method:

- adopt inositol silylation derivation method.
- increase the detection limit.

Appendix A and B of this standard are informative.

The versions replaced by this standard are:

- GB 5413- 1985, GB/T 5413.25- 1997.

National food safety standard

Determination of inositol in foods for infants and young children, milk and milk products

1 Scope

This standard specifies the determination method of inositol in foods for infants and young children and milk products.

This standard applies to determination of inositol in foods for infants and young children and milk products.

2 Normative References

The normative documents referenced in the text are indispensable to the application of this standard. For dated references, only the edition bearing such date applies to this standard. For undated references, the latest edition of the normative document referred to (including all the amendments) applies.

Method 1 Microorganism method

3 Principles

Utilizing the specificity and sensitivity of *Saccharomyces uvarum* for inositol, the content of the test material in the test sample can be determined quantitatively. In culture medium containing all nutrition components except for the test material, there is a linear relationship between the growth of microorganisms and the content of the test material; through comparing transmittancy with the standard curve, the content of the test material in the test sample can be calculated.

4 Reagents and materials

Unless otherwise specified, all reagents used in this method are analytical reagents; water is secondary water specified in GB/T 6682.

4.1 Strain: *Saccharomyces uvarum (*ATCC 9080).

4.2 Standard substance of myo-Inositol: formula $C_6H_{12}O_6$, purity≥99 %.

4.3 Sodium chloride (NaCl)

4.4 Sodium hydroxide (NaOH)

4.5 Culture medium

4.5.1 Malt extract agar: refer to appendix A

4.5.2 Culture medium for inositol determination :refer to appendix A

4.6 sodium chloride solution (9 g/L): Weigh 9.0 g sodium chloride, dissolve with 1000 mL water, and dispense it to test tubes, 10 mL per tube. Sterilize at 121□ for 15 min.

4.7 hydrochloric acid solution (1 mol/L): Measure 82.0 mL concentrated hydrochloric acid; dissolve with

water, cool and dilute to 1000 mL.

4.8 hydrochloric acid solution (0.44 mol/L): Measure 36.6 mL concentrated hydrochloric acid; dissolve with water, cool and dilute to 1000 mL.

4.9 sodium hydroxide solution (600 g/L): Weigh 300 g sodium hydroxide; dissolve with water, cool and dilute to 500 mL.

4.10 sodium hydroxide solution (1 mol/L): Weigh 40 g sodium hydroxide; dissolve with water, cool and dilute to 1000 mL.

4.11 Standard solution of inositol

4.11.1 Standard stock solution of inositol (0.2 mg/mL): Place standard substance of inositol into a desiccator filled with P_2O_5 and dry for more than 24 h; weigh 50 mg standard substance of inositol (4.2) (accurate to 0.1 mg), dissolve adequately with water, dilute to 250 mL, and store in a refrigerator.

4.11.2 Intermediate standard solution of inositol (10 μg/mL): Pipette 5 mL standard stock solution of inositol (4.11.1), dilute to 100 mL with water, and store in a refrigerator.

4.11.3 Standard working solution of inositol (1 μg/mL and 2 μg/mL): Pipette 10 mL intermediate standard solution of inositol (4.11.2) for two times, and dilute to 100 mL and 50 mL with water respectively. This working solution should be prepared immediately before each determination.

4.12 Desiccant: P_2O_5.

4.13 Glass beads: with a diameter of about 5 mm.

5 Instruments & Equipment

Besides routine sterilization and culturing equipment in microbiological laboratory, other equipments and materials are as follows:

5.1 Analytical balance: with a reciprocal sensibility of 0.1mg.

5.2 pH meter: it is accurate to ≤0.02.

5.3 Spectrophotometer.

5.4 Vortex mixer.

5.5 Centrifuge: with a rotary speed ≥ 2000 rpm.

5.6 Constant-temperature incubator: 30 □ ±1 □.

5.7 Shaking incubator: 30 □ ±1 □, with a shaking speed of 140 times/min to 160 times/min

5.8: Refrigerator: 2 □ to 5 □.

5.9 Sterile pipette：10ml (with 0.1ml scale) or micropipette or tip.

5.10 Bottle top dispensers: 0 ml – 10 ml.

5.11 Conical flask: 200 mL.

5.12 Volumetric flask (Type A): 100ml, 250ml, 500ml.

5.13 Transfer pipet with only one scale (Type A): with a volume of 5ml.

5.14 Funnel: with a diameter of 90mm..

5.15 Quantitative filter paper: with a diameter of 90mm.

5.16 Test tube: 18mm ×18mm

Note: Before using glassware, hard glass tube or other necessary glassware should be cleaned with activator and then should be dried at 200 □ for 2 hours.

6 Analytical procedures

6.1 Preparation of inoculation bacterial suspension

6.1.1 Resuscitation of stock strain:

Inoculate *S. cerevisiae* strain (4.1) to inclined planes of culture medium prepared with malt extract agar (4.5.1), culture at 30□ for 16 - 24 h, passage for two to three passages to prepare stock strain and then store in a refrigerator (8.5); the storage duration shouldn't exceed 2 weeks; then inoculate it to a new inclined plane of culture medium of malt extract agar.

6.1.2 Preparation of inoculation bacterial suspension:

One day before use, transfer the stock strain to a new prepared inclined plane of culture medium with malt extract agar, culture at 30□ for 16 - 24h. Scrape lawn with an inoculating loop to a sterile test tube containing sodium chloride solution (4.6). Centrifuge this bacteria liquid at 2000 rpm for 2 - 3 min, pour out the supernatant, add into 10 mL sodium chloride solution (4.6), shake and mix even, and then centrifuge for another 2 - 3 min, wash like this for 3 – 4 times. Under sterile conditions, pipette a certain amount of the bacteria liquid and transfer to a test tube filed with 10 mL sodium chloride solution (4.6), the resulted solution is inoculation bacterial suspension.

Determine the transmittance of the inoculation bacterial suspension at 550 nm wavelength with a spectrophotometer, with sodium chloride solution (4.6) as the blank; adjust the amount of bacteria liquid or add a certain amount of sodium chloride solution to make the transmittance of bacterial suspension be at 60 - 80%.

6.2 Treatment of test samples

6.2.1 Weigh a certain amount of test sample containing about 0.5 mg - 2 mg inositol (accurate to 0.1 mg) to a 250 mL triangular flask; for powder test sample, add 80 mL hydrochloric acid (4.8), for liquid test sample, add 100 mL hydrochloric acid (4.8), and then mix even to dissolve the powder test sample.

6.2.2 Cover the triangular flask with aluminium foil, incubate the solution in a sterilizer at 125□ for 1 h. Take the flask out, cool to room temperature; add about 2 mL sodium hydroxide solution (4.9) and then cool. Adjust pH to 5.2 with sodium hydroxide solution (4.10) or hydrochloric acid solution (4.7), transfer to a 250 mL volumetric flask, dilute to volume, mix even, filtrate and then collect the filtrate. Use the filtrate as the test solution. Adjust dilution, make the concentration of inositol in the test solution be within

the scope of 1μg/mL - 10 μg/mL.

6.3 Preparation of the standard curve

Add distilled water, standard working solution of inositol (4.11.3) and determination culture medium of inositol (4.5.2) to a culture tube according to the sequence in table 1, prepare in triplicate.

Table 1 Preparation of the standard curve

Test tube No	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Distilled water, mL	5	5	4	3	2	1	0	2	1	0
Standard working solution 1μg/mL, mL	0	0	1	2	3	4	5	0	0	0
Standard working solution 2μg/mL, mL	0	0	0	0	0	0	0	3	4	5
Culture medium, mL	5	5	5	5	5	5	5	5	5	5

6.4 Preparation of test solution

Add distilled water, test solution (6.2.2) and determination culture medium of inositol (4.5.2) to a culture tube according to the sequence in table 2, prepare in triplicate.

Table 2 Preparation of test solution

Test tube No	1	2	3	4
Distilled water, mL	4	3	2	1
Test solution, mL	1	2	3	4
Culture medium, mL	5	5	5	5

6.5 Sterilization

Add a glass bead to each test tube (4.13), put on the cap to the test tube and then place it into a sterilizer, sterilize at 121□ for 5 min (commercial culture medium should be sterilized according to the specification in the label).

6.6 Inoculation

Take out the test tube from the sterilizer, rapidly cool to it to below 30□. Add a drop of inoculation bacterial suspension (6.1.2) with a pipette or transferpettor to the above-mentioned test tubes, respectively (about 50 μL) (except for the S1 tube for preparation of the standard curve).

6.7 Culture

Fix a test tube in a shaking incubator, shake and culture for 22 - 24 h at about 140 – 160 times/min and under 30℃ ± 1□.

6.8 Determination

Make visual inspection of each test tube, the culture medium in S1 tube should be clear, if it is turbid, the results will be invalid.

6.8.1 Take the test tube out of the desk top-incubator and place it into a sterilizer, and then heat at 100□ for 5 min to stop the growth of microorganisms.

6.8.2 Use the blank test tube S1(6.3) as blank, adjust the transmittance of the spectrophotometer to 100% (or absorbance A is 0), read the value of the blank test tube S2(6.3). Then use the blank test tube S2 as blank, adjust the transmittance to 100% (or A is 0), read the transmittance (or absorbance A) of other test tubes one by one.

6.8.3 Thoroughly mix the content of each test tube with a vortex mixer (or through adding a drop of antifoaming agent), and then immediately transfer the culture medium to a cuvette to carry out determination at the wavelength of 540 - 660 nm; after the reading is stable for 30 s, read the transmittance, the stabilization time of each test tube should be the same. Plot a standard curve with the content of standard substance of inositol as X-axis and transmittance as Y-axis.

6.8.4 According to the transmittance of the test solution, find the concentration of inositol in the test solution from the standard curve, and then calculate the content of inositol in the test sample according to dilution factor and the amount weighed. The test tubes with transmittance outside the range of S3 –S10 should be deleted.

6.8.5 For the test tubes with a certain concentration of test solution, calculate the concentration of inositol in the test solution according to the transmittance of each test tube, and the average value of the concentration of inositol in the test solution; the concentration determined for each test tube shouldn't exceed ±15% of the average value. If the number of tubes satisfying the requirements is less than 2/3 of the total number of tubes with four levels of test solution, the data is not sufficient for calculation of the content of the test sample. If the number of tubes satisfying the requirements is 2/3 or more of the total number, calculate the average content of inositol in each millimeter of test solution in the numbered test tubes, acquire the overalll average C_x, and then calculate the content of inositol in the test sample.

Note: 1. When plotting the standard curve, the transmittance (T%) or the absorbance (A) can be read.

7 Calculation and expression of results

The content of inositol in the test sample is calculated according to formula (1):

$$X = \frac{Cx}{m} \times \frac{F}{1000} \times 100 \quad \text{.....(1)}$$

Where,

X - the content of inositol in the test sample, mg/100 g;

Cx - the average value of the content of inositol in each milliliter of test solution, μg;

m - mass of the test sample, g;

f - dilution factor. For foods for infants and children and milk products, f is 250.

The results of two independent determinations acquired under repeatability conditions are expressed with arithmetic mean. The results should have three significant numbers.

8 Precision

The absolute error of the results of two independent determinations acquired under repeatability conditions shouldn't exceed 10% of the arithmetic mean.

Method 2 Gas chromatography

9 Principles

Extract the inositol in the test sample with water and ethanol, derivative with silylating reagent, extract with N-hexane ion, and then quantitate it with gas chromatographic separation external standard method.

10 Reagents and materials

Unless otherwise specified, all reagents used in this method are analytical reagents, and water is first grade water specified in GB/T 6682.

10.1 Absolute alcohol (C_2H_6O).

10.2 N-hexane (C_6H_{14}).

10.3 ethanol (95%).

10.4 ethanol (70%).

10.5 Trimethyl chloro silicane (C_3H_9ClSi).

10.6 Hexamethyl disilylamine ($C_6H_{19}NSi_2$).

10.7 N, N- dimethylformamide (C_3H_7NO).

10.8 Silylating reagent: Pipette trimethyl chloro silicane, hexamethyl disilylamine and N, N dimethylformamide with the volume ratio of 1: 2: 8, treat with ultrasound; it should be prepared before use.

10.9 Standard substance of inositol: putiry≥99 %

10.10 Standard solution of inositol (0.010mg/ml): Weigh 100mg (accurate to 0.1 mg) standard substance of inositol (10.9) which has been dried at 105□ ± 1□for 2 hours to a 100mL volumetric flask, and dissolve it completely with 25mL water. Dilute to volume with ethanol (10.3),mix even. Transfer 1mL of this solution to a 100mL volumetric flask and dilute to volume with ethanol (10.4),mix even.

11 Instruments & Equipment

11.1 Analytical balance: with a reciprocal sensibility of 0.1mg.

11.2 Gas chromatograph: with FID detector.

11.3 Centrifuge: over 5000r/min.

11.4 Rotary evaporator.

11.5 Ultrasonoscope.

11.6 Hot water bath of constant temperature

11.7 25mL test tube with a screw cap.

12 Analytical procedures

12.1 Treatment and derivation of the test sample

12.1.1 Treatment of the test sample: weigh 1g solid state test sample which has been mixed even and 12g liquid test sample (accurate to 0.0001g) to a 50mL volumetric flask, add to it 12mL warm water at about 40□ to dissolve the test sample. Extract it with ultrasound for 5 min, dilute to volume with ethanol (10.3), mix even and allow to stand for 20 min. Transfer 10mL of the solution to a 15mL centrifuge tube, centrifuge at 4000r/min for 5 min. Transfer 5mL supernatant to a stock bottle on the rotary evaporator.

12.1.2 Drying and derivation: add 10mL absolute alcohol to the stock bottle, rotate and concentrate it at 80□±2 ℃until nearly dry, add 5mL absolute alcohol and continue to concentrate it until completely dry (if there is water remaining, the next silylation won't be thorough). Add 10mL silylating reagent (10.8), dissolve with ultrasound for 5 min and transfer to a 25mL centrifuge tube with a screw cap, place the tube into a 80□±2 ℃ water bath so that the content can react for 75 min. Take out the tube and shake every 20 min during the reaction process, than take out the tube and cool to room temperature. Add 5mL N-hexane (10.2), shake to mix the content, then allow to stand and laminate, take 3mL supernatant into a centrifuge tube with a screw cap with a little absolute sulphuric acid sodium, shake and then centrifuge at 4000r/min, the resulted solution is the test sample solution.

12.2 Preparation of standard solution

Pipette 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0mL standard solution of inositol (10.10) to a stock bottle respectively, according to procedures in (12.1.2).

12.3 Determination

12.3.1 Reference chromatographic condition:

Chromatographic column: a capillary column with 50% cyanopropyl - methylpolysiloxane as the packing material (with a length of 60m, an internal diameter of 0.25mm, film thickness of 0.25μm); or an equivalent chromatographic column.

Temperature of the injection port: 280 □

Temperature of the detector: 300□

Split ratio: 10: 1

Injection volume: 1.0μL

For temperature programming, see table 3:

Table 3 Temperature programming

Heating rate (/□ min)	Target temperature (□)	Maintaining time (min)
	120	0
10	190	50
10	220	3

12.3.2 Preparation of the standard curve

Inject the standard solutions (12.2) into the gas chromatograph respectively (for chromatogram, refer to appendix B), and then plot the standard curve with the determined peak area (or peak height) as Y-axis, with the content of inositol (mg) in the standard solution of inositol as X-axis.

12.3.3 Determination of the test sample solution

Inject the test sample solutions (12.1.2) into the gas chromatograph and get the peak area (or peak height); find the content of inositol (mg) in the test sample solution from the standard curve.

13 Calculation and expression of results

The content of inositol in the test sample is calculated according to formula (2):

$$X = \frac{C_s \times f_i}{m_i} \times 100 \quad \cdots\cdots (2)$$

Where,

X - the content of inositol in the test sample, mg/100 g;

Cs - find the content of inositol in the test sample solution from the standard curve, mg.

mi - mass of the test sample, g.

fi – the coefficient of the content of inositol in test sample solution converting to the content of inositol in test sample is 10.

The results of two independent determinations acquired under repeatability conditions are expressed with arithmetic mean. The results should have three significant numbers.

14 Precision

The absolute error of the results of two independent determinations acquired under repeatability conditions shouldn’t exceed 10% of the arithmetic mean.

15 Other

For the first and second method of this standard, the detection limits are both 2.0 mg/100 g.

Appendix A

(Normative)

Culture medium and Reagents

A.1 Malt Extract Agar

A.1.1 Components

12.*75* g maltose, 2.75 g dextrin, 2.35 g glycerol, 0.78 g peptone, 15.0 g agar, 1000 mL distilled water, pH 4.7±0.2 (25□±5□).

A.1.2 Preparation method:

Dissolve all the other components except for agar with water, adjust pH, add agar, heat to boil to lyze agar. Dispense the solution to test tubes after mixing even, 10 mL per tube. Autoclave at 121□ for 15 min, arrange to an inclined plane and store for later use.

A.2. Culture medium for inositol determination

A.2.1 Components

100 g glucose, 10 g potassium citrate, 2 g citric acid, 1.1 g potassium dihydrogen phosphate, 0.85 g potassium chloride, 0.25 g magnesium sulfate, 0.25 g calcium chloride, 50 mg manganese sulfate, 50 mg ferrous chloride, 80 mg DL - tryptophane, 0.1 g L - cystine, 0.5 g L - isoleucine, 0.5 g L - leucine, 0.5 g L - lysine, 0.2 g L - methionine, 0.2 g DL - phenylalanine, 0.2 g L - tyrosine, 0.8 g L - aspartic acid, 0.2 g DL - aspartic acid, 0.1 g DL - serine, 0.2 g glycine, 0.4 g DL - threonine, 0.5 g L - valine, 0.124 g L - histidine, 0.2 g L - proline, 0.4 g DL - alanine, 0.6 g L - glutamic acid, 0.48 g L - arginine, 500 μg thiamine hydrochloride, 16 μg biotin, 5 mg calcium pantothenate, 1 mg pyridoxine hydrochloride, 1000 mL distilled water, pH 5.2±0.2 (25□±5□).

A.2.2 Preparation method

Dissolve the above-mentioned components with water, adjust pH and store for later use.

Note: some commercial synthetic culture mediums have good effects; prepare commercial synthetic culture medium according to specifications in the label.

Appendix B
(Informative)

The gas chromatograms of the derivative of inositol in the standard substance

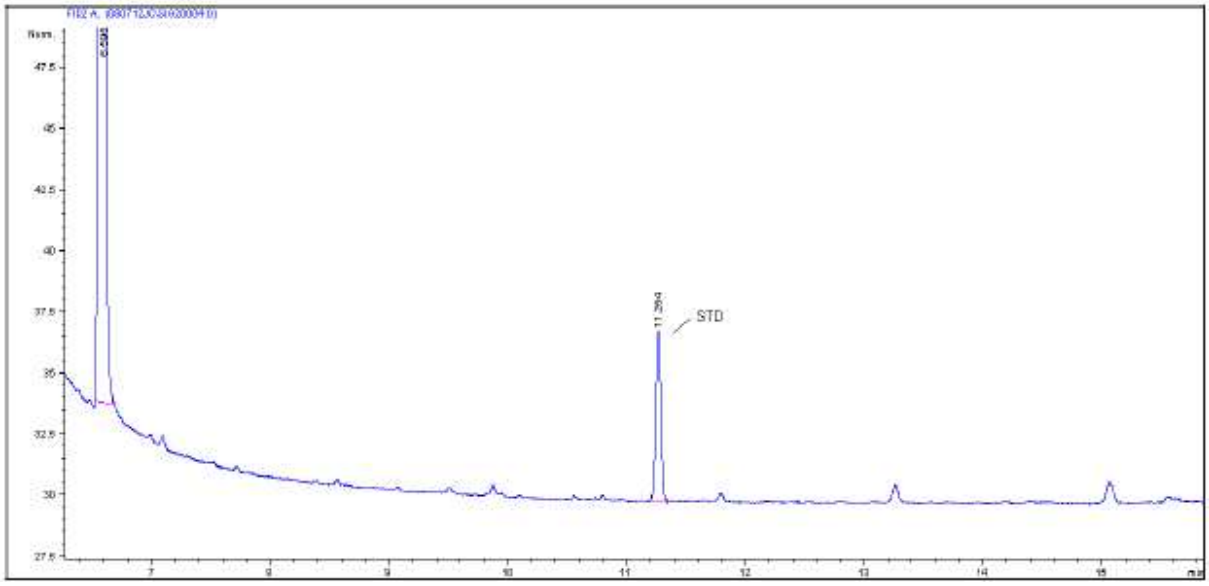

Figure B.1 The gas chromatogram of the derivative of inositol in the standard substance