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National Food Safety Standard - Determination of Galactooligosaccharides in Foods

食品安全国家标准　食品中低聚半乳糖的测定

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National Food Safety Standard - Determination of Galactooligosaccharides in Foods

1 Scope

This Standard specifies the method of liquid chromatography for the determination of galactooligosaccharides in foods.

This Standard is applicable to the determination of galactooligosaccharides in infant formula foods (excluding infant formula foods for special medical purposes), infant auxiliary foods, dairy products, beverages and baked foods.

This Standard is not applicable to the determination of galactooligosaccharides in foods that contain other oligomeric reducing substances that may cause interference.

2 Principle

The galactooligosaccharides (*n* is 2 ~ 7) in the specimen is extracted by aqueous solution and derivatized by 2-aminobenzamide, enzymatically hydrolyzed by amyloglucosidase to remove the interference of maltodextrin and starch, and then, detected by high performance liquid chromatograph - fluorescence detector. The collected chromatographic peaks are qualitatively determined based on the retention time of galactooligosaccharides with the same degree of polymerization determined by liquid chromatography - tandem mass spectrometry under the same test conditions. Adopt the external standard method of maltose glycan reference material corresponding to the degree of polymerization and relative molecular weight for quantitative determination. After deducting the lactose and maltose content, the galactooligosaccharides content is obtained.

3 Reagents and Materials

Unless it is otherwise specified, the reagents used in this Method are all analytically pure, and the water is Grade-1 water specified in GB/T 6682.

WARNING---sodium cyanoborohydride and 2-methylpyridine-*N*-borane are hazardous materials, which will release flammable gases when exposed to water and are dangerous to the environment. Please follow the instructions for use of the reagents. Please wear personal protective equipment and perform all operations under a fume hood.

3.1 Reagents

3.1.1 Dimethyl sulfoxide [$(CH_3)_2SO$]: chromatographically pure.

3.1.2 2-aminobenzamide ($C_7H_8N_2O$).

3.1.3 Sodium cyanoborohydride ($NaBH_3CN$).

3.1.4 2-methylpyridine-*N*-borane ($C_6H_7N \bullet BH_3$).

3.1.5 Acetic acid (CH_3COOH).

3.1.6 Ammonium acetate (CH_3COONH_4).

3.1.7 Ammonium formate ($HCOONH_4$).

3.1.8 Formic acid ($HCOOH$).

3.1.9 Acetonitrile (CH_3CN): chromatographically pure.

3.1.10 Amyloglucosidase (CAS: 9032-08-0): enzyme activity unit ≥ 60 U/mg.

NOTE: for the determination method of enzyme activity, see A.3 in GB 1866.174-2016.

3.2 Preparation of Reagents

3.2.1 Acetic acid-dimethyl sulfoxide solution (3 + 7, volume ratio): respectively measure-take 3 mL of acetic acid and 7 mL of dimethyl sulfoxide in a beaker, evenly mix it and reserve it for later use.

3.2.2 2-aminobenzamide derivative solution (0.35 mol/L): accurately weigh-take 476 mg of 2-amonibenzamide and 630 mg of sodium cyanoborohydride or 1,070 mg of 2-methylpyridine-*N*-borane in a beaker, use 10 mL of acetic acid-dimethyl sulfoxide solution (3 + 7) to dissolve it and evenly mix it.

3.2.3 Ammonium acetate solution (0.2 mol/L, pH 4.5 ± 0.1): weigh-take 1.54 g of ammonium acetate, use 80 mL of water to dissolve it, then, use acetic acid to adjust pH to 4.5 ± 0.1, and use water to dilute to 100 mL. Prepare it right before use.

3.2.4 Amyloglucosidase solution (about 180 U/mL): weigh-take amyloglucosidase equivalent to about 900 U of activity and dissolve it in 5 mL of ammonium acetate solution (0.2 mol/L, pH 4.5 ± 0.1), stir, until it is completely dissolved. Prepare it right before use.

3.2.5 Ammonium formate solution (50 mmol/L, pH 4.4 ± 0.1): weigh-take 3.15 g of ammonium formate, use 900 mL of water to dissolve it, then, use formic acid to adjust pH to 4.4 ± 0.1, and use water to dilute it to 1,000 mL. Prepare it right before use.

3.3 Reference Materials

3.3.1 Maltose reference material ($C_{12}H_{22}O_{11}$, CAS: 69-79-4), purity ≥ 98%, or a standard substance certified by the state and awarded a standard substance certificate.

3.3.2 Maltotriose reference material ($C_{18}H_{32}O_{16}$, CAS: 1109-28-0), purity ≥ 98%, or a standard substance certified by the state and awarded a standard substance certificate.

3.3.3 Maltotetraose reference material ($C_{24}H_{42}O_{21}$, CAS: 34612-38-9), purity ≥ 95%, or a standard substance certified by the state and awarded a standard substance certificate.

3.3.4 Maltopentaose reference material ($C_{30}H_{52}O_{26}$, CAS: 34620-76-3), purity ≥ 95%, or a standard substance certified by the state and awarded a standard substance certificate.

3.3.5 Maltohexaose reference material ($C_{36}H_{62}O_{31}$, CAS: 34620-77-4), purity ≥ 95%, or a standard substance certified by the state and awarded a standard substance certificate.

3.3.6 Maltoheptaose reference material ($C_{42}H_{72}O_{36}$, CAS: 34620-78-5), purity ≥ 95%, or a standard substance certified by the state and awarded a standard substance certificate.

3.3.7 Lactose reference material ($C_{12}H_{22}O_{11}$, CAS: 63-42-3), purity ≥ 98%, or a standard substance certified by the state and awarded a standard substance certificate.

3.4 Preparation of Standard Solutions

3.4.1 Standard stock solutions (10.00 mg/mL): respectively and accurately weigh-take 100 mg (accurate to 0.1 mg) of lactose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose reference materials, use water to dissolve them, reach a constant volume of 10 mL, and evenly mix them. Transfer each standard stock solution into a liquid storage bottle and store it at 4 °C. It shall remain valid for 1 month.

3.4.2 Mixed standard intermediate solution (1.000 mg/mL): draw-take 1.00 mL of each standard stock solution (10.00 mg/mL) into a 10 mL volumetric flask, add water to reach a constant volume to the scale and evenly mix it. Prepare it right before use.

3.4.3 Mixed standard service solution (100.0 μg/mL): draw-take 1.00 mL of the mixed standard intermediate solution (1.000 mg/mL) in a 10 mL volumetric flask, add water to reach a constant volume to the scale and evenly mix it. Prepare it right before use.

3.4.4 Mixed standard series of working solutions: respectively draw-take 1.00 mL, 2.50 mL and 5.00 mL of the mixed standard intermediate solution (1.000 mg/mL) in a 10 mL volumetric flask, add water to dilute to the scale and evenly mix it. Respectively draw-take 0.10 mL, 1.00 mL and 5.00 mL of the mixed standard service solution (100.0 μg/mL) in a 10 mL volumetric flask, add water to reach a constant volume to the scale and evenly mix it. The mass concentration of the mixed standard series of working solutions is respectively 1.000 μg/mL, 10.00 μg/mL, 50.00 μg/mL, 100.0 μg/mL, 250.0 μg/mL and 500.0 μg/mL. Prepare them right before use.

4 Instruments and Equipment

4.1 High performance liquid chromatograph: equipped with fluorescence detector.

4.2 Balance: with a division value of 0.1 mg and 1 mg respectively.

following steps to process it simultaneously with the sample according to 5.1.3.

5.1.5 Derivatization of standard series working solution

Transfer 20 μL of standard series working solution into a 2 mL centrifuge tube with a screw cap, add 200 μL of 2-aminobenzamide derivative solution; vortex and mix for 30 s; incubate in a 60°C water bath for 120 min; take it out and place it to reach room temperature; add 1 mL of ammonium acetate solution (0.2 mol/L, pH 4.5 ± 0.1) and vortex for 30 seconds. Then transfer 0.50mL of the mixed solution into a 2mL centrifuge tube; add 200μL of ammonium acetate solution (0.2mol/L, pH4.5±0.1); incubate in a 50℃ water bath for 30min; take it out and place it to reach room temperature; add 0.70mL of acetonitrile; mix well; centrifuge at 6000r/min for 5min; the supernatant is filtered with a 0.22μm organic microporous membrane for high-performance liquid chromatography measurement.

5.2 Reference Conditions of Instrument

5.2.1 Chromatographic column: amide bonded surface porous silica gel column [150 mm × 4.6 mm (inner diameter), particle size 2.7 μm], or equivalent column.

5.2.2 Mobile phase: mobile phase A is ammonium formate solution (50 mmol/L, pH 4.4 ± 0.1); mobile phase B is acetonitrile.

5.2.3 Mobile phase flow rate: 1.0 mL/min. For the mobile phase gradient elution procedure, see B.1 in Appendix B.

5.2.4 Detector: fluorescence detector, with an excitation wavelength of 355 nm and an emission wavelength of 430 nm.

5.2.5 Column temperature: 30 °C.

5.2.6 Injection volume: 20 μL.

5.3 Drawing of Standard Curve

Respectively inject the mixed standard series of working solution derivative solutions into the high performance liquid chromatograph to determine the peak areas of the chromatograms of maltose reference materials and lactose derivatives with different degrees of polymerization. Take the concentration of maltose reference materials and lactose reference material in the mixed standard series of working solutions as the x-coordinate, and the peak area of the maltose reference material derivatives with different degrees of polymerization as the y-coordinate to draw a standard curve of maltose reference materials and lactose derivatives with different degrees of polymerization. The chromatogram of maltose reference materials and lactose derivative solutions with different degrees of polymerization is shown in Figure C.1 in Appendix C.

5.4 Determination of Specimen Solution

Inject the specimen solution into the high performance liquid chromatograph. Refer to

Appendix D to determine the retention time periods of sugars with different degrees of polymerization, respectively record the sum of the peak areas of sugar derivatives with each degree of polymerization, and the peak areas of lactose and maltose derivatives. In accordance with the standard curve of the reference material derivatives with the corresponding polymerization degree, respectively calculate the concentration of sugars with different degrees of polymerization in the specimen solution. In accordance with the standard curve of lactose and maltose reference material derivatives, respectively calculate the concentration of lactose and maltose in the specimen solution. For the chromatogram of the specimen after derivatization, see Figure C.2 in Appendix C.

6 Expression of Analysis Results

The content X of galactooligosaccharides in the specimen is calculated in accordance with Formula (1).

$$X=\frac{(\sum\rho_{\mathrm{DPi}}-\rho_0-\overset{\text{lactose}}{\rho_{乳糖}}-\overset{\text{maltose}}{\rho_{麦芽糖}})\times V\times 1\ 000}{m\times 1\ 000\times 1\ 000} \quad \cdots\cdots(1)$$

Where,

X---the content of galactooligosaccharides in the specimen, expressed in (g/kg);

ρ_{DPi}---the concentration of sugar derivatives with different degrees of polymerization obtained through the standard curve, expressed in (μg/mL) (i = 2 ~ 7);

ρ_0---the concentration of derivatives in the blank specimen solution, expressed in (μg/mL);

ρ_{lactose}---the concentration of lactose derivatives in the specimen solution, expressed in (μg/mL);

ρ_{maltose}---the concentration of maltose derivatives in the specimen solution, expressed in (μg/mL);

V---the constant volume of the specimen, expressed in (mL);

1,000---conversion factor;

m---the mass of the specimen, expressed in (g).

The calculation results, which are expressed as the arithmetic mean of the results of two independent determinations obtained under repeatability conditions, shall retain 3 significant figures.

7 Precision

The absolute difference between the results of two independent determinations obtained under repeatability conditions shall not exceed 15% of the arithmetic mean.

Appendix A

Method for the Identification of Other Oligomeric Reducing Substances

A.1 Principle

In the specimen solution, amyloglucosidase is used to remove maltodextrin and starch interference, the lactose and galactooligosaccharides in the sample are hydrolyzed by β-galactosidase, then, derivatized by 2-aminobenzamide. The high performance liquid chromatograph - fluorescence detector is used for detection. The collected signals are segmented in accordance with the retention time of the polymerization degree, then, the peaks are summed. The external standard method of the maltose glycan reference material with the corresponding degree of polymerization is adopted to respectively perform the quantitative determination. Then, the total derivative content is calculated, and after deducting the blank solution and maltose derivative content, the total content of other oligomeric reducing substances is calculated. If the total content of other oligomeric reducing substances in the specimen is greater than the quantitation limit, then, the standard method is not applicable to the determination of galactooligosaccharides in the specimen.

A.2 Reagents

A.2.1 β-galactosidase solution: enzyme activity ≥ 4,000 U/mL.

NOTE: the determination method for enzyme activity refers to GB/T 33409-2016. For different batches of β-galactosidase solutions, before use, they need to be subject to quality verification.

A.2.2 Acetonitrile solution (75%, volume fraction): measure-take 75 mL of acetonitrile and add water to dilute to 100 mL.

The others are the same as Chapter 3.

A.3 Instruments and equipment

Same as Chapter 4.

A.4 Analytical procedures

A.4.1 Verification of β-galactosidase solution

A.4.1.1 Treatment of β-galactosidase solution

Draw-take 700 μL of water into a 2 mL centrifuge tube, add 50 μL of β-galactosidase solution (4,000 U/mL), and conduct vortex mixing for 30 seconds. In 60 °C water bath, perform thermal insulation for 120 minutes. Then, raise the water bath temperature to 100 °C and perform thermal insulation for 5 minutes, take it out and cool it at 4 °C for 10 minutes. Draw-take 20 μL of the mixed solution into a 2 mL centrifuge tube with a screw cap, add 200 μL of 2-aminobenzamide derivative solution, conduct vortex mixing for 30 seconds. In 60 °C water bath, conduct thermal insulation for 120 minutes, take it out and bring it to room temperature. Add 1 mL of acetonitrile solution (75%), evenly mix it, then, at 6,000 r/min, centrifuge for 5 minutes. Filter the supernatant through a 0.22 μm organic microporous filter membrane and determine it through high performance liquid chromatography.

A.4.1.2 Treatment of standard series of working solutions

Transfer-take 500 μL of standard series of working solutions, add 250 μL of water, and conduct vortex mixing for 30 seconds. In 60 °C water bath, perform thermal insulation for 120 minutes. Then, raise the water bath temperature to 100 °C and perform thermal insulation for 5 minutes. Take it out and cool it at 4 °C for 10 minutes. Draw-take 20 μL of mixed solution in a 2 mL centrifuge tube with a screw cap, add 200 μL of 2-aminobenzamide derivative solution, and conduct vortex mixing for 30 seconds. In 60 °C water bath, perform thermal insulation for 120 minutes, take it out and bring it to room temperature. Add 1 mL of acetonitrile solution (75%), evenly mix it, then, at 6,000 r/min, centrifuge it for 5 minutes. Filter the supernatant through a 0.22 μm organic microporous filter membrane and determine it through high performance liquid chromatography.

A.4.1.3 Validation rules

In accordance with A.4.3, A.4.4 and A.4.5, determine the treated β-galactosidase derivative solution, and calculate the content of other oligomeric reducing substances in the solution. If the content is less than the detection limit of 1.0 g/kg, then, the enzyme solution passes the validation, and the specimen pre-treatment steps can be carried out.

A.4.2 Sample pre-treatment

A.4.2.1 Specimen treatment

Weigh-take 1.000 g of specimen in a 50 mL beaker, use water to dissolve it, then, transfer it to a 50 mL volumetric flask, add water to reach a constant volume to the scale and evenly mix it, and take it as the specimen solution for later use. Draw-take 500 μL of the specimen solution into a 2 mL centrifuge tube with a screw cap, add 200 μL of amyloglucosidase solution (about 180 U/mL) and 50 μL of β-galactosidase solution (4,000 U/mL); conduct vortex mixing for 30 seconds. In 60 °C water bath, conduct thermal insulation for 120 minutes. Then, raise the water bath temperature to 100 °C and conduct thermal insulation for 5 minutes; take it out and cool it at 4 °C for 10 minutes. Draw-take 20 μL of mixed solution into a 2 mL centrifuge tube, add 200 μL of 2-aminobenzamide derivative solution, conduct vortex mixing for 30 seconds. In 60 °C water bath, conduct thermal insulation for 120 minutes; take it out and bring it to room temperature. Add 1 mL of acetonitrile solution (75%), evenly mix it, then, at 6,000 r/min, centrifuge for 5 minutes. Filter the supernatant through a 0.22 μm organic microporous filter membrane and determine it through high performance liquid chromatography.

A.4.2.2 Blank test

Appendix D

Method of Determining Retention Time Periods of Sugars with Different Degrees of Polymerization through Liquid Chromatography - Mass Spectrometry

D.1 Principle

The galactooligosaccharides (n is 2 ~ 7) in the specimen is extracted by aqueous solution, derivatized by 2-aminobenzamide, enzymatically hydrolyzed by amyloglucosidase to remove maltodextrin and starch interference, and then, determined by liquid chromatography - tandem mass spectrometry. Extract the total ion currents of the derivatives of sugars with different degrees of polymerization to determine the retention time periods of sugars with different degrees of polymerization.

D.2 Reagents and solutions

Same as Chapter 3.

D.3 Instruments

D.3.1 Liquid chromatograph - mass spectrometer.

D.3.2 Balance: with a division value of 0.1 mg and 1 mg.

D.3.3 pH meter: with an accuracy of 0.01.

D.3.4 Vortex mixer.

D.3.5 Constant-temperature water bath.

D.3.6 Centrifuge.

D.4 Analytical procedures

D.4.1 Pre-treatment of samples and standard solutions

Same as Chapter 5.

D.4.2 Reference conditions of instrument determination

D.4.2.1 Chromatography conditions: maintain consistent with the conditions for liquid chromatographic determination in 5.2. A post-column 2 : 1 spit is recommended.

D.4.2.2 Mass spectrometry conditions: high-resolution mass spectrometer is recommended.

The reference conditions are as follows:

a) ESI source;

b) Negative ion mode detection;

c) Full scan mode scanning;

d) Scanning range: 100 *m*/*z* ~ 1,500 *m*/*z*.

The recommended mass spectrometry parameters:

a) Nozzle voltage: 1,000 V;

b) Capillary voltage: 3,500 V;

c) Collision voltage: 130 V;

d) Cone voltage: 65 V;

e) Drying gas temperature: 350 °C;

f) Drying gas flow rate: 5 L/min;

g) Atomizer pressure: 45 psi (1 psi ≈ 6.89 kPa);

h) Sheath gas temperature: 350 °C;

i) Sheath gas flow rate: 11 L/min.

D.4.3 Solution determination

Inject the specimen solution into the liquid chromatogram - mass spectrometer to test the corresponding signal responses of the derivatives of sugars with different degrees of polymerization.

Meanwhile, inject the standard solution derivative solution into the liquid chromatogram - mass spectrometer to test the signal responses of the derivatives of reference material with different degrees of polymerization.

D.5 Time period determination method

Analyze the total ion chromatograms of the derivatives of sugars with different degrees of polymerization and extract the number of molecular ions of derivatives with different degrees of polymerization in the negative ion mode. The values are shown in Table D.1. In accordance with the rules of recording the starting time ($t_{DPistart}$) to the end time (t_{DPiend}) of each peak of the degree of polymerization, determine the time period ($t_{Dpi\text{-}MS}$) of different degrees of polymerization. The reference chromatogram is shown in Figure D.1.

------ The End ------