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National Food Safety Standard - Determination of M Aflatoxins in Foods

食品安全国家标准

食品中黄曲霉毒素 M 族的测定

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National Food Safety Standard - Determination of M Aflatoxins in Foods

1 Scope

This Standard specifies the methods for the determination of aflatoxins M_1 and aflatoxins M_2 (hereinafter referred to as AFT M_1 and AFT M_2) in foods.

Method I is isotope dilution liquid chromatography - tandem mass spectrometry, which is applicable to the determination of AFT M_1 and AFT M_2 in milk, milk products and milk-containing special dietary food.

Method II is high-performance liquid chromatography; its scope of application is the same as Method I.

Method III is enzyme-linked immunosorbent screening assay, which is applicable to the screening determination of AFT M_1 in milk, milk products and milk-containing special dietary food.

Method I -- Isotope Dilution Liquid Chromatography - Tandem Mass Spectrometry

2 Principle

Use methanol - water solution to extract aflatoxins M_1 and aflatoxins M_2 in sample. Use water or phosphate buffer solution to dilute the supernatant, then, purify and enrich it through immunoaffinity column. After concentration, constant-volume and filtering of the purified liquid, separate it through liquid chromatography. Detect it through tandem mass spectrometry, then, quantify it through isotope internal standard method.

3 Reagents and Materials

Unless it is otherwise stipulated, all reagents adopted in this Method shall be analytical pure; water shall be Grade-1 water stipulated in GB/T 6682.

3.1 Reagents

3.1.1 Acetonitrile (CH_3CN): chromatographic purity.

calibrate the concentration (please refer to Appendix A for the calibration method).

3.4.2 Mixed standard stock solution (1.0 μg/mL): respectively and accurately absorb 1.00 mL of 10 μg/mL AFT M_1 and AFT M_2 standard stock solution, then, place them in the same 10 mL volumetric flask. Add acetonitrile to dilute to the constant volume, then, obtain 1.0 μg/mL mixed standard solution. This solution can be preserved in an airtight container in the dark at 4 °C; it shall remain valid for 3 months.

3.4.3 Mixed standard working solution (100 ng/mL): accurately absorb 1.0 mL of mixed standard stock solution (1.0 μg/mL), then, place it in a 10 mL volumetric flask. Use acetonitrile to dilute to the constant volume. This solution can be preserved in an airtight container in the dark at 4 °C; it shall remain valid for 3 months.

3.4.4 50 ng/mL isotope internal standard working solution 1($^{13}C_{17}$-AFT M_1): take 1 mL of AFT M_1 isotope internal standard (0.5 μg/mL), then, use acetonitrile to dilute to 10 mL. Preserve it at -20 °C; it shall be used for the determination of liquid sample. It shall remain valid for 3 months.

3.4.5 5 ng/mL isotope internal standard working solution 2($^{13}C_{17}$-AFT M_1): take 100 μL of AFT M_1 isotope internal standard (0.5 μg/mL), then, use acetonitrile to dilute to 10 mL. Preserve it at -20 °C; it shall be used for the determination of solid sample. It shall remain valid for 3 months.

3.4.6 Standard series of working solution: respectively and accurately absorb 5 μL, 10 μL, 50 μL, 100 μL, 200 μL and 500 μL of standard working solution, then, place them in 10 mL volumetric flask. Add 100 μL of 50 ng/mL isotope internal standard working solution. Use initial mobile phase to dilute to the constant volume. Thus, prepare AFT M_1 and AFT M_2 series of standard solution at the concentration of 0.05 ng/mL, 0.1 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL and 5.0 ng/mL.

4 Instruments and Apparatuses

4.1 Balance: division value: 0.01 g, 0.001 g and 0.00001 g.

4.2 Water bath kettle: temperature controlled at 50 °C ± 2 °C.

4.3 Vortex mixer.

4.4 Ultrasonic cleaner.

4.5 Centrifuge: ≥ 6,000 r/min.

4.6 Rotary evaporator.

4.7 Solid phase extraction device (equipped with vacuum pump).

4.8 Nitrogen blowing instrument.

vortex mixing. If milk powder cannot completely dissolve, place the centrifuge tube in 50 °C water bath; after the milk powder completely dissolves, take it out. Wait till the sample solution cools down to 20 °C, then, add 10 mL of methanol; start vortex for 3 min. Place it at 4 °C; start centrifugation at 6,000 r/min for 10 min, or, filter it through glass fiber filter paper. Transfer an appropriate amount of the supernatant or filtrate to a beaker, then, add 40 mL of water or PBS to dilute it; reserve it for later use.

5.1.3 Cream

Weigh-take 1 g (accurate to 0.001 g) of sample, then, place it in a 50 mL centrifuge tube. Add 100 μL of $^{13}C_{17}$-AFT M_1 internal standard solution (5 ng/mL); start oscillation blending, then, evenly place it for 30 min. Add 8 mL of petroleum ether; wait till cream dissolves, then, add 9 mL of water and 11 mL of methanol. Start oscillation for 30 min. Transfer all the liquid to separating funnel. Add 0.3 g of sodium chloride, then, thoroughly shake and dissolve it. Evenly place it and wait for layering, then, transfer the lower layer to a round-bottomed flask. Start rotary evaporation, till it is below 10 mL, then, use PBS to dilute it to 30 mL.

5.1.4 Cheese

Weigh-take 1 g (accurate to 0.001 g) of already shredded and evenly mixed sample (through aperture 1 mm ~ 2 mm round sieve), then, place it in a 50 mL centrifuge tube. Add 100 μL of $^{13}C_{17}$-AFT M_1 internal standard solution (5 ng/mL); start oscillation blending, then, evenly place it for 30 min. Add 1 mL of water and 18 mL of methanol. Start oscillation for 30 min. Place it at 4 °C; start centrifugation at 6,000 r/min for 10 min, or, filter it through glass fiber filter paper. Transfer an appropriate amount of the supernatant or filtrate to a round-bottomed flask. Start rotary evaporation, till it is below 2 mL, then, use PBS to dilute it to 30 mL.

5.2 Purification

5.2.1 Preparation of immunoaffinity column

Restore immunoaffinity column, which is preserved at low temperature, to room temperature.

5.2.2 Purification

After abandoning the liquid in the immunoaffinity column, transfer the above-mentioned sample solution to a 50 mL syringe; adjust the flow rate of dropping to 1 mL/min ~ 3 mL/min. Wait till the dropping of the sample solution finishes, add 10 mL of water to the syringe. At a stable flow rate, rinse the immunoaffinity column. Wait till water droplets stop, then, use the vacuum pump to drain the immunoaffinity column. Isolate from the vacuum system; underneath the affinity column, place a 10 mL scale test tube; take down the 50 mL syringe. Add 2 x 2 mL of acetonitrile (or methanol) to elute the affinity column. Control the flow rate of dropping at 1 mL/min ~ 3 mL/min. Use the vacuum pump to drain the affinity column, then, gather all the eluent to the scale test

When weighing-taking 1 g of milk powder, special dietary food, cream and cheese, this Method's detection limit of AFT M_1 is 0.02 μg/kg; the detection limit of AFT M_2 is 0.02 μg/kg; the quantitation limit of AFT M_1 is 0.05 μg/kg; the quantitation limit of AFT M_2 is 0.05 μg/kg.

Method II -- High-performance Liquid Chromatography

9 Principle

Use methanol - water solution to extract aflatoxins M_1 and aflatoxins M_2 in sample. After diluting the supernatant, purify and enrich through the immunoaffinity column. After concentration, constant-volume and filtering of the purified liquid, separate it through liquid chromatography. Detect it through fluorescence detector, then, quantify it through external standard method.

10 Reagents and Materials

Unless it is otherwise stipulated, all reagents adopted in this Method shall be analytical pure; water shall be Grade-1 water stipulated in GB/T 6682.

10.1 Reagents

10.1.1 Acetonitrile (CH_3CN): chromatographic purity.

10.1.2 Methanol (CH_3OH): chromatographic purity.

10.1.3 Sodium chloride (NaCl).

10.1.4 Disodium phosphate (Na_2HPO_4).

10.1.5 Potassium dihydrogen phosphate (KH_2PO_4).

10.1.6 Potassium chloride (KCl).

10.1.7 Hydrochloric acid (HCl).

10.1.8 Petroleum ether (C_nH_{2n+2}): boiling range: 30 °C ~ 60 °C.

10.2 Reagent Preparation

10.2.1 Acetonitrile - water solution (25 + 75): measure-take 250 mL of acetonitrile; add it to 750 mL of water, then, mix it up.

10.2.2 Acetonitrile - methanol solution (50 + 50): measure-take 500 mL of acetonitrile;

11.3 Vortex mixer.

11.4 Ultrasonic cleaner.

11.5 Centrifuge: rotating speed ≥ 6,000 r/min.

11.6 Rotary evaporator.

11.7 Solid phase extraction device (equipped with vacuum pump).

11.8 Nitrogen blowing instrument.

11.9 Round sieve: 1 mm ~ 2 mm aperture.

11.10 Liquid chromatography (equipped with fluorescence detector).

11.11 Glass fiber filter paper: rapid, high-load, particle retention in liquid: 1.6 μm.

11.12 Disposable microporous filter head: equipped with 0.22 μm microporous filter membrane.

11.13 Immunoaffinity column: column capacity ≥ 100 ng (please refer to Appendix B for the method of testing and verifying column capacity, recovery rate and column recovery rate).

NOTE: before use, the mass of different batches of affinity column needs to be verified.

12 Analytical Procedure

The application of immunoaffinity columns provided by different manufacturers might be slightly different in sample operation, such as sample loading, leaching and elution. Thus, operation shall comply with the requirements in the operating instructions provided by the manufacturers.

Warning: the whole analysis and operation process shall be conducted in an appointed area. This area shall be kept away from light (direct sunlight); be equipped with relatively independent operating bench and waste storage device. During the whole experiment process, operators shall adopt corresponding protective measures in accordance with the requirements of exposure to violent toxicity.

12.1 Sample Extraction

Do not add isotope internal standard solution. Other than this, the method is the same as 5.1.

12.2 Purification

$$X = \frac{\rho \times V \times f \times 1\,000}{m \times 1\,000} \qquad \text{.....(2)}$$

Where,

X---content of AFT M_1 or AFT M_2 in the sample, expressed in (μg/kg);

ρ---concentration of AFT M_1 or AFT M_2 in the inject solution in the chromatographic peak through the standard curve, expressed in (ng/mL);

V---final constant volume of the sample after the immunoaffinity column's purification and elution, expressed in (mL);

f---dilution factor of the sample solution;

1,000---conversion factor;

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

The calculation result shall retain 3 significant figures.

14 Precision

The absolute difference of two independent determination results obtained under repeatability conditions shall not exceed 20% of the arithmetic mean value.

15 Others

When weighing-taking 4 g of liquid milk and yogurt, this Method's detection limit of AFT M_1 is 0.005 μg/kg; the detection limit of AFT M_2 is 0.0025 μg/kg; the quantitation limit of AFT M_1 is 0.015 μg/kg; the quantitation limit of AFT M_2 is 0.0075 μg/kg.

When weighing-taking 1 g of milk powder, special dietary food, cream and cheese, this Method's detection limit of AFT M_1 is 0.02 μg/kg; the detection limit of AFT M_2 is 0.01 μg/kg; the quantitation limit of AFT M_1 is 0.05 μg/kg; the quantitation limit of AFT M_2 is 0.025 μg/kg.

Method III -- Enzyme-linked Immunosorbent Assay

16 Principle

Through certain treatment, such as homogenization, frozen centrifugation, degreasing

Add water to dissolve it, then, transfer to a 100 mL volumetric flask. Use water to dilute to the constant volume. The following step is the same as 19.1.1.

19.1.3 Cheese

Weigh-take 50 g (accurate to 0.1 g) of test sample, then, remove the inedible part on the surface. In terms of hard cheese, use a grinder to directly grind it. In terms of soft cheese, freeze it overnight at -20 °C, then, immediately, use a grinder to grind it. Weigh-take 5 g (accurate to 0.1 g) of evenly mixed test sample, then, add the extract provided by the kit. Start extraction in accordance with the instruction provided by the kit; the extract shall be considered as the test solution.

19.2 Quantitative Detection

In accordance with the operational procedure described by the enzyme-linked immunosorbent assay kit, conduct quantitative detection of the test sample (solution).

20 Expression of Analytical Result

20.1 Draw a Standard Working Curve of Enzyme-linked Immunosorbent Assay Kit

In accordance with the standard substance's concentration and absorbance variation relation, draw a standard working curve.

20.2 Concentration Calculation of Test Solution

Substitute the absorbance of the test solution into the formula obtained in 20.1, then, calculate the concentration ρ of the test solution.

20.3 Result Calculation

The content of AFT M_1 in foods shall be calculated in accordance with Formula (3):

$$X = \frac{\rho \times V \times f}{m} \qquad \cdots\cdots (3)$$

Where,

X---content of AFT M_1 in foods, expressed in (μg/kg);

ρ---concentration of AFT M_1 in the test solution, expressed in (μg /L);

V---constant volume (in terms of milk powder, special dietary food and liquid sample) or extract volume (in terms of cheese) expressed in (L);

f---dilution factor;

Appendix B

Column Capacity Verification Method for Immunoaffinity Column

B.1 Column Capacity Verification

In 30 mL of PBS, add 300 ng of AFT M_1 standard stock solution; thoroughly mix it up. Respectively take 3 immunoaffinity columns from the same batch; the amount of sample loading shall be 10 mL per column. Through sample loading, leaching, elution and eluent collection, use nitrogen to blow it to 1 mL. Use initial mobile phase to reach the constant volume of 10 mL; use liquid chromatograph to separate and determine the content of AFT M_1.

Result determination: the result is AFT $M_1 \geq 80$ ng, which signifies that it is a usable commodity.

B.2 Column Recovery Rate Verification Method

In 30 mL of PBS, add 300 ng of AFT M_1 standard stock solution; thoroughly mix it up. Respectively take 3 immunoaffinity columns from the same batch; the amount of sample loading shall be 10 mL per column. Through sample loading, leaching, elution and eluent collection, use nitrogen to blow it to 1 mL. Use initial mobile phase to reach the constant volume of 10 mL; use liquid chromatograph to separate and determine the content of AFT M_1.

Result determination: the result is AFT $M_1 \geq 80$ ng, which signifies that it is a usable commodity.

B.3 Cross Reactivity Verification

In 30 mL of PBS, add 300 ng of AFT M_2 standard stock solution; thoroughly mix it up. Respectively take 3 immunoaffinity columns from the same batch; the amount of sample loading shall be 10 mL per column. Through sample loading, leaching, elution and eluent collection, use nitrogen to blow it to 1 mL. Use initial mobile phase to reach the constant volume of 10 mL; use liquid chromatograph to separate and determine the content of AFT M_2.

Result determination: the result is AFT $M_2 \geq 80$ ng, which signifies that it is a commodity used when AFT M_1 and AFT M_2 needs to be simultaneously determined.

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