National Standard of the People's Republic of China

GB 5413.37-2010

National food safety standard Determination of aflatoxin M_1 in milk and milk products

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Foreword

The first method of this standard corresponds to the ISO14501：2007 Milk and milk powder-determination of aflatoxin M_1 content -clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography; The consistency level between the first method of this standard and ISO14501：2007 is non-equivalent; The second and third method of this standard replace GB/T 18980-2003; The fourth method of this standard is from NY/T 1664-2008 Rapid determination for aflatoxin M_1 in milk-Double flow enzyme-linked immunosorbent assay.

Appendix A and B of this standard are both informative.

National food safety standard
Determination of aflatoxin M_1 in milk and milk products

1 Scope

This standard specifies the method for determination of aflatoxin M_1 in milk and milk products.

The first method in this standard applies to determination of aflatoxin M_1 in milk and milk products; the second method applies to determination of aflatoxin M_1 in milk, milk powder, low fat milk, skimmed milk, low fat milk powder and skimmed milk powder; the third method applies to determination of aflatoxin M_1 in milk and milk powder; the fourth method applies to determination of aflatoxin M_1 in liquid milk and milk powder.

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 Immunochromatography Clean-up Combined with Liquid Chromatography – Mass Spectrometry

3 Principles

Dissolve the test sample with mixed solution of water and organic solvent, extract it with ultrasound, centrifuge, take the supernatant which is then purified with an immunoaffinity column; dry the eluent with N_2, dilute to volume, filtrate with a microporous membrane, inject into a liquid chromatograph to separate, ionize it with an electrospray ion source, detect it with Multi Reaction Monitor (MRM), and then quantitate it with matrix plus external standard method.

4 Reagents and materials

Unless otherwise specified, all reagents used in this method are analytical reagents; water is the first grade water specified in GB/T6682.

4.1 Formic acid (HCOOH).

4.2 Acetonitrile (CH_3CN): chromatographic pure.

4.3 Petroleum benzene (C_nH_{2n+2}): boiling range 30℃ - 60℃.

4.4 Chloroform ($CHCl_3$).

4.5 Nitrogen gas: purity ≥99.9 %.

4.6 Standard sample: $M_{1:}$ purity ≥ 98%.

4.7 Acetonitrile water solution (1+4): add 100 mL acetonitrile into 400 mL water.

4.8 Acetonitrile water solution(1+9) add 50 mL acetonitrile to 450 mL water.

4.9 0.1 % formic acid solution: pipette 1 mL formic acid (4.1) and dilute it with water to 1000 mL.

4.10 Acetonitrile /methanol solution (50+50): add 500 mL methanol into 500 mL acetonitrile.

4.11 Sodium hydroxide solution (0.5 mol/L): weigh 2 g sodium hydroxide and dissolve with 1000 mL water.

4.12 Blank matrix solution of the test sample

Weigh 8 test samples with the same matrix as the sample to be tested and without flavacin into a 100 mL beaker. Carry out the following operations according to 6.1 Extraction of Test Solution and 6.2 Purification procedures. Combine the purification solution from the 8 test samples, filter with a single-use filter with 0.22 μm microporous membrane (5.23), discard the first 0.5 mL filtrate, and take a small amount of the filtrate and detect it with liquid chromatography - mass spectrometry.

Acquire the chromatography - mass spectrum and compare it with figure A.2 in appendix A, there shouldn't be aflatoxin M_1 at corresponding retention time. Transfer the residual filtrate to a brown bottle, and store at a - 20□ refrigerator for preparation of the series of standard solutions.

4.14 Standard stock solution of aflatoxin: weigh 0.10 mg standard substance M_1 (accurate to 0.01 mg), dissolve and dilute to 10 mL with chloroform (4.4). The concentration of this standard solution is 0.01 mg/mL. Transfer the solution to a plastic bottle, store in a - 20 □ refrigerator for later use.

4.15 A series of standard solutions: Pipette 100 μL M_1 standard stock solution (4.13) to a 100 mL volumetric flask, blow chloroform with nitrogen gas to nearly dry, and dilute to volume with acetonitrile. Dilute it with blank matrix solution of the test sample (4.12) to a series of standard working solutions with concentrations with 0.5 ng/mL, 0.8 ng/mL, 1.0 ng/mL, 2.0 ng/mL, 4.0 ng/mL, 6.0 ng/mL and 8.0 ng/mL.

5 Instruments & Equipment

5.1 Liquid chromatography - mass spectrometry: with electrospray ion source.

5.2 Chromatographic column: ACQUIT UPLC HSST3[1], with a length of 100 mm, an internal diameter of 2.1 mm; the particle diameter of the packing material is 1.8 μm, or equivalent.

5.3 Balance: with a reciprocal sensibility of 0.01 g and 0.00001 g.

5.4 Homogenizer.

5.5 Ultrasonic cleaner.

5.6 Centrifuge: ≥6000 rpm

5.7 50 mL centrifuge tube with a stopper.

[1] This information is given for ease of the user, it doesn't mean that we recognize this product, if other equivalent products have the same efficacy, they can also be used.

5.8 Water bath: the temperature is controlled at 30□±2□ and 50□±2□, with the temperature scope between 25□ and 60□.

5.9 Volumetric flask: 100 mL.

5.10 Glass beaker: 250 mL, 50 mL.

5.11 Ground glass test tube with graduation: 5 mL, 10 mL and 20 mL.

5.12 Pipette: 1.0 mL, 2.0 mL and 50.0 mL.

5.13 Glass rod.

5.14 10-mesh round-hole sieve.

5.15 250 mL separating funnel.

5.16 100 mL ground bottom flask.

5.17 Rotary evaporator

5.18 pH meter: accurate to 0.01.

5.19 250 mL conical flask with a stopper.

5.20 Immunoaffinity column: 3ml, syringe pump mode.

5.20 10 mL and 50 mL single-use syringe.

5.21 Solid phase extraction element.

5.22 Single-use microporous filter: with a 0.22 μm microporous membrane (aqueous phase).

6 Analytical procedures

6.1 Extraction of test solution

6.1.1 Milk: Weigh 50.0g test sample and mix it even, transfer into a 50 mL centrifuge tube with a stopper (5.7), and then heat it in a water bath (5.8) to 35□ - 37□. Centrifuge it at 6000 rpm centrifuge force for 15 min. collect all of the supernatant for purification.

6.1.2 Acidophilous milk (including solid, semisolid and milk with sarcocarp): Weigh 50.0 g test sample (accurate to 0.01g) and mix even, adjust pH to 7.4 with 0.5 mol/L sodium hydroxide solution (4.11) with pH meter indicating pH, mix at 9500 rpm (5.4) for 5 min, and carry out the following operations according to 6.1.1.

6.1.3 Milk powder and formula powder for infants: Weigh 10.0 g (accurate to 0.01g) test sample; transfer it into a 250 mL beaker. Add 50 mL water which has been preheated to 50 □ to milk powder in many times with a small amount each time, and then mix even with a glass rod. If milk powder still doesn't dissolve completely, place the beaker into a 50□ water bath (5.8) for 30 min, cool to 20 □ after the content has dissolved, and then transfer it to a 100 mL volumetric flask, wash the beaker with a small amount of water, transfer the combined eluent into the volumetric flask, dilute to volume with water, and

then transfer the solution to two 50 mL centrifuge tubes (5.7) after it is shaken even, centrifuge at 6000 rpm for 15 min, transfer 50 ml supernatant with a pipette.

6.1.4 Cheese: Weigh 5.0 g (accurate to 0.01g) test sample which has been cut into pieces and sieved through a 10-mesh round-hole sieve, mix even, transfer into a 50 mL centrifuge tube (5.7), add 2 mL water and 3 0 mL methanol, mix at 9500 rpm for 5 min, extract with ultrasound for 30 min, and then centrifuge at 6000 rpm for 15 min. Collect the supernatant, transfer the extraction solution collected to a 250 mL separating funnel, add 30 mL petroleum benzene (4.3), shake for 2min, wait until it has laminated, transfer the lower layer to a 50 mL beaker, and then discard the petroleum benzine layer. Extract with petroleum benzine ion for 2 times. Transfer the lower layer of solution to a 100 mL ground bottom flask, concentrate it at decreased pressure to about 2 mL, pour the concentrated solution to a centrifuge tube, wash the flask with acetonitrile water solution (1+4) (4.7) for 2 times, transfer the combined eluent into a 50 mL centrifuge tube, dilute to about 50 mL with water, centrifuge at 6000 rpm for 5 min, and then take the supernatant for purification.

6.1.5 Butter: Weigh 5.0g (accurate to 0.01g) test sample, transfer to a 50 mL beaker, and dissolve the butter with 20 mL petroleum benzine (4.3) and transfer to a 250ml conical flask with a stopper. Add 20 mL water and 30 mL methanol, shake it for 30 min, and then transfer all of the fluid to a separating funnel. After demixing, transfer the lower layer of solution to a 100ml round bottom flask, concentrate it at underpressure in a rotary evaporator (5.17) to 5ml, dilute with water to about 50ml and then begin purification.

6.2 Purification

6.2.1 Preparation of immunoaffinity column

Connect a 50 mL single-use syringe barrel to the top of an affinity column (5.20), and then connect the affinity column to the solid phase extraction element.

Note: the pH value of the test solution should be controlled according to the instruction of immunoaffinity column

6.2.2 Purification of the test sample

Transfer the extraction solution of the test solution in 6.1 to a 50 mL syringe barrel (5.21), adjust the vacuum system of solid phase extraction element, make the test sample flow through the column at a stable flow rate of 2 - 3 mL/ min. Take off the 50 mL syringe barrel, and put on a 10 mL syringe barrel. Fill the syringe barrel with water and wash the column with water at a stable flow rate, then suction dry the affinity column. Unconnect the vacuum system, put on a 10 mL graduated test tube to the lower part of the affinity column and another 10 mL syringe barrel to the upper part, add 4 mL acetonitrile (4.2), elute M1, collect the eluent to a graduated test tube (5.11); the elution time shouldn't be less than 60 s. Slowly evaporate the eluent with nitrogen gas at 30□ until almost dry (warning: if it is evaporated to dry, M_1 may lose). Dilute it with acetonitrile water solution (1+9) to 1ml.

6.3 Reference conditions of liquid chromatography

Mobile phase: A phase, 0.1% formic acid solution; B phase, acetonitrile / methanol (1+1).

Gradient elution: refer to A.1 in appendix A.

The flow speed of the mobile phase: 0.3 mL/min.

The temperature of the chromatographic column: 35 □.

Temperature of the test solution: 20 □.

Injection volume: 10 μL.

6.4 Reference conditions of mass spectra

Detection mode: multiple-reaction monitoring (MRM), for detailed information of parent ion, daughter ion and impact energy, see table 1; for the scanogram, refer to figure A.1 in appendix A.1

Table 1 Selection parameter of ions

Aflatoxin	Parent ion	Quantitation daughter ion	Impact energy mode	Qualitative daughter ion	Impact energy	Ionization
M1	329.0	273.5	22	259.5	22	ESI+

For control conditions of ion source, please refer to table A.2 IN appendix A。

6.5 Qualitation

The difference between the retention time of the chromatographic peak of the target compound in the test sample and that of the corresponding standard chromatographic peak should be within±2.5%.

The signal to noise ratio of the remodeling ion chromatographic peak of the qualitative ions in the test compound should be no less than 3 (S/N≥3), and the noise ratio of the remodeling ion chromatographic peak of the quantitative ions should be no less than 10 (S/N≥10).

In the mass spectra of each compound, there must be qualitative ions, and it should at least include a parent ion and two daughter ions; in a same detection batch, for a same compound, compared with standard solution with equivalent concentration, the deviation of the relative abundance of the two daughter ions of the target shouldn't exceed the scope specified in table 2.

Table 2 The largest permitted deviation of the relative ion abundancy when qualitation

Relative ion abundancy	＞50%	＞20% to 50%	＞10% to 20%	≤10%
Permitted relative deviation	±20%	±25%	±30%	±50%

The target compounds are qualitated with their retention time and the relative abundance of the corresponding LC - MS/MS chromatographic peak area of the two pairs of ions (characteristic ion pair /quantitative ion pair). It is required that the retention time of the target compound in the test sample is consistent with that in the standard solution (the deviation should be less than 20%), and it is also required that for the two pairs of ions in of the target compound in the test sample, the ratio of LC - MS/MS chromatographic peak areas should be consistent with the area ratio of the target compound in

the standard solution.

6.6 Determination of test sample

According to the conditions established in 6.3 and 6.4, determine the ion intensity of M_1 in the test solution (6.2) and the series of standard solutions (4.14), and quantitate M_1.in the test sample with external standard method. For chromatogram, please see figure A.2 in appendix A.

The retention time of chromatographic reference: M_1 3.23 min.

6.7 Blank test

No test sample is weighed; carry out blank experiments according to procedures in 6.5. It should be confirmed that there are no materials interfering the component to be tested.

6.8 Preparation of the standard curve

Inject the series of stand solution (4.14) from low to high concentration, plot with peak area-concentration to acquire a regression equation of calibration curve.

6.9 Quantitative determination

The response value of the test solution should be within the linear range of the standard curve, if it is outside of the linear range, dilutee the blank solution with test solution and then inject and analyze it, or decrease the sampling amount, treat it according to 6.1 and then inject and analyze it.

7 Expression of results

Quantitate the content with the external standard method, and calculate the residual volume of aflatoxin M_1 according to formula (1).

$$X = \frac{A \times V \times f \times 1}{m} \quad \cdots\cdots (1)$$

Where,

X - the content of aflatoxin M_1 in the test sample, μg/kg,;

A – the concentration of aflatoxin M_1 in the test sample, ng/mL;

V – constant volume, mL;

f – dilution factor of the test solution;

m – the mass of the test sample, with a unit of g;

Calculate the results which should be calculate to truncate to three significant figures.

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 Immunochromatography Clean-up Combined with High Performance Liquid Chromatography

9 Principles

When the test sample passes through the immunoaffinity column, aflatoxin M_1 will be extracted. The specific monoclonal antibodies of aflatoxin M_1 contained in the affinity column crosslink to solid support, when the sample passes through the affinity column, the antibodies selectively combine with aflatoxin M_1 (antigen) to form antibody - antigen complex. Wash the column with water to remove impurities, then elute the aflatoxin M_1 absorbed onto the column with eluent and collect the eluent. Determine the content of aflatoxin M_1 in the eluent with high performance liquid chromatograph with a fluorescence detector.

10 Reagents and materials

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

10.1 Immunoaffinity column: it should contain antibodies to aflatoxin M_1. The maximum volume of the affinity column shouldn't be less than 100 ng aflatoxin M_1 (corresponding to 50 mL test sample with a concentration of 2 μg/ L); when the standard solution contains 4 ng aflatoxin M_1 (corresponding to 50 mL test sample e with a concentration of 80 ng/ L), the recovery rate shouldn't be lower than 80%. Regularly inspect the column efficiency and recovery rate of the affinity column; the affinity column used for each batch should be at least inspected once (see 10.1.1 and 10.1.2).

10.1.1 Inspection of column efficiency

Transfer 1.0 mL aflatoxin M_1stock solution (10.5.2) to a 20 mL tapered test tube (11.9) with a pipette (11.4). Slowly blow the fluid dry with continuous flow nitrogen gas (10.3), then dissolve the residues with 10 mL l0% acetonitrile (10.2.2) and shake heavily.

Transfer the solution to 40 mL water, mix it even, and then inject all of them to the immunoaffinity column (10.1). Use the immunoaffinity column according to the requirements in the directions. Elute aflatoxin M_1 from the immunoaffinity column after it is rinsed. Appropriately dilute the eluent, determine the content of aflatoxin M_1 combined onto the immunoaffinity column with high performance liquid chromatograph.

Calculate the recovery rate of aflatoxin M_1, and compare the results with the indices required in 10.1.

10.1.2 Inspection of recovery rate

Transfer 0.8 mL 0.005 μg/ mL standard working solution of aflatoxin M_1 (10.5.3) to 10mL water with a pipette (11.4), mix it even and inject all of them into the immunoaffinity column. Use the immunoaffinity column according to the directions. Elute aflatoxin M_1 from the immunoaffinity column after it is rinsed. Appropriately dilute the eluent, determine the content of aflatoxin M_1 combined onto the immunoaffinity column with high performance liquid chromatograph determination. Calculate the recovery rate of aflatoxin M_1, and compare the results with the indices required in 10.1.

10.2 Acetonitrile (CH_3CN): chromatographic grade.

10.2.1 25% acetonitrile - water solution: Dissolve 250 mL acetonitrile (10.2) with 750 mL water (needing degassing before being used).

10.2.2 10% acetonitrile - water solution: Dissolve 100 mL acetonitrile (10.2) with 900 mL water (needing degassing before being used).

10.3 Nitrogen gas (N_2).

10.4 Chloroform: add ethanol with a mass ratio of 0.5% - 1.0% (the ratio with chloroform) to stabilize it.

10.5 Standard solution of aflatoxin M_1, purity度≥98%.

10.5.1 Calibration solution

The concentration of the chloroform standard solution of aflatoxin M_1 is 10 μg/mL. According to the following method, determine the absorbance of the solution at the maximum absorption wave band, so as to determine the actual concentration of aflatoxin M_1.

Determine the absorbance at 340 nm - 370 nm with a spectrophotometer (11.11), subtract the blank background of chloroform, and read the absorbance value of the standard solution. Near the 360 nm maximum absorption wave band λ_{max}, acquire the absorbance value A, and then calculate the concentration according to formula (2).

$$c_i = \frac{A \times M \times 100}{\varepsilon} \quad \cdots\cdots (2)$$

Where,

c_i – the actual concentration of aflatoxin M_1, μg/mL

A - the absorbance value determined at λ_{max};

M - 328 g/mol, molar mass of aflatoxin M_1, with a unit of g/mol

ε - 1995, the absorbance coefficient of aflatoxin M_1 dissolved in chloroform, with a unit of m^2/mol.

10.5.2 Standard stock solution

Determine the actual concentration value of the standard solution of aflatoxin M_1 (10.5.1), dilute it to stock solution with a concentration of 0.1 μg/mL with chloroform. Seal up the stock solution, put it into a refrigerator below 5□ and store it protecting from light. Under such conditions, the stock solution can be

stable for two months.

10.5.3 Standard working solution of aflatoxin M_1

Take the stock solution (10.5.2) from the refrigerator and place at room temperature, transfer a certain amount of the stock solution, dilute and prepare it to working solution. The working solution should be prepared before usage.

Preparation of the standard working solution of aflatoxin M_1: accurately transfer 1.0 mL stock solution (10.5.2) to a 20mL tapered test tube (11.9) with a pipette (11.4), slowly blow the solution dry with nitrogen gas (10.3), then dissolve the residues with 20.0 mL 10% acetonitrile (10.2.2), shake for 30 min and mix it even, prepare it to the standard working solution of aflatoxin M_1 with a concentration of 0.005 μg/mL. During the process of blowing with nitrogen gas, it must be handled with care; the temperature shouldn't be decreased too much so as to prevent dew forming.

When plotting the standard curve, the injection volumes of aflatoxin $M_1$are respectively 0.05 ng, 0.1 ng, 0.2 ng and 0.4 ng. According to the volume of the injection ring of the high performance liquid chromatograph, prepare a series of standard solutions of aflatoxin M_1 with appropriate concentrations with the working solution. The dilution solution used is 10% acetonitrile (10.2.2)

11 Instruments & Equipment

11.1 Single-use syringe: 10 mL and 50 mL.

11.2 Vacuum system.

11.3 Centrifuge: with a speed ≥ 7000 rpm.

11.4 Pipette: 1.0 mL, 2.0 mL and 50.0 mL.

11.5 Glass beaker: 250 mL.

11.6 Volumetric flask: 100 mL.

11.7 Water bath: the temperature is controlled at 30□±2□ and 50□±2□; with the temperature scope 36□ ± 1□.

11.8 Filter paper: moderate-speed qualitative filter paper.

11.9 Graduated ground tapered glass test tube: 5 mL, 10 mL and 20 mL.

11.10 High performance liquid chromatograph

11.10.1 Pulse-free pump: a pump which is suitable for constant flow of about 1 mL/min.

11.10.2 Injection system: an injection ring with constant or variable volume of 50 μL - 500 μL.

11.10.3 Reversed phase chromatographic column: guard column packed with 3μm or 5μm octadecyl silica gel together with reversed phase materials.

11.10.4 Fluorescence detector: with excitation wavelength of 365 nm and emission wavelength of 435 nm, which can determine 0.02 ng aflatoxin M_1 (corresponding to 5 times of noise) under appropriate chromatographic conditions.

11.11 Spectrophotometer: with a wavelength scope of 200 nm - 400 nm.

11.12 Balance: accurate to 0.0l g.

12 Analytical procedures

All operational analysis should be done protecting from light as far as possible.

12.1 Preparation of test sample

12.1.1 Milk: Heat the milk sample in a water bath (11.7) to 35□ - 37□. Filter it with a filter paper (11.8) (it may needs several pieces of filter paper according to circumstances), or centrifuge at 7000 rpm centrifuge force for 15 min. Collect at least 50 mL milk test sample, and then continue analysis according to 12.4.

12.1.2 Milk powder: Weigh 10g sample (accurate to 0.1 g) and place to a 250 mL beaker (11.5). Add 50 mL water which has been preheated to 50□ in many times to the milk powder, and mix even with a stirrer rod. If the milk powder can't completely dissolve, place the beaker into a 50□ water bath (11.7) for at least 30 min, carefully mix even. Cool the dissolved milk powder to 20□, transfer to a 100 mL volumetric flask (11.6), rinse the beaker with a small amount of water in several times, transfer the combined eluting solution to the volumetric flask and then dilute to volume with water. Filer the milk with a piece of filter paper (11.8) or centrifuge under 7000 rpm centrifuge force for 15 min. Collect at least 50 mL milk test sample, and then continue to analyze it according to 11.4.

12.1.3 Fermented milk: it is treated according to the treatment method in "first method" in 6.1.2.

12.1.4 Cheese: it is treated according to the treatment method in "first method" in 6.1.4.

12.1.5 Butter: it is treated according to the treatment method in "first method" in 6.1.5.

12.2 Preparation of immunoaffinity column

Connect a 50 mL single-use syringe barrel (11.1) to the top of an affinity column (10.0), and then connect the affinity column to vacuum system (11.2).

12.3 Extraction and purification of the sample

Transfer 50mL test sample (12.2.1 or 12.2.2) to a 50 mL syringe (11.1) with a pipette, adjust the vacuum system (11.2), inject the test sample and make it flow through the column at a stable flow rate of 2 mL / min - 3 mL / min.

Take off the 50 mL syringe barrel, and put on a 10 mL syringe barrel. Fill the syringe barrel with 10 mL water and wash the column with water at a stable flow rate, then suction dry the affinity column. Unconnect the vacuum system, put on another 10 mL syringe barrel, add 4 mL acetonitrile (10.2), slowly push the plunger to control the flow rate to elute M1, collect the eluent to a tapered tube (11.9); the elution time shouldn't be less than 60 s. Slowly evaporate the eluent with nitrogen gas (10.3) at 30℃ until the volume is within 50 μL - 500 μL (warning: if it is evaporated to dry, aflatoxin M_1 may lose). Dilute it 10 times with 10%water to the final volume V_f (500 μL - 5 000 μL).

Note: if a sample containing aflatoxin M_1 with acetonitrile content more than 10% is injected into the high

performance liquid chromatograph, the chromatographic peak will broaden. If water content exceeds 90%, it has no influence on the shape of the chromatographic peak.

12.4 High performance liquid chromatograph

12.4.1 Chromatographic conditions

Chromatographic column: c18, with a length of 25cm, internal diameter 4.6mm.

Mobile phase: 25 % acetonitrile water solution (10.2.1)

Flow speed: 1ml/min.

12.4.2 Standard curve of aflatoxin M_1

According to the volume of HPLC injection ring, select appropriate volume V_e, and inject standard solutions containing 0.05 ng, 0.1 ng, 0.2 ng and 0.4 ng aflatoxin M_1 respectively. Plot a standard curve with peak area or peak height versus the mass of aflatoxin M_1.

12.4.3 Chromatographic analysis

According to the peak height or peak area value of aflatoxin M_1 in the chromatogram of the sample eluent, determine the mass of aflatoxin M_1 (ng) in the sample eluent according to the standard curve.

If the peak area or peak height of aflatoxin $M_1$of the sample eluent is higher than that of the standard solution, dilute the diluted sample eluent to volume with water, and then inject and analyze.

13 Expression of results

13.1 Milk

Calculate the content ω_m of aflatoxin M_1 in the test sample according to formula (3).

$$\omega_m = \frac{m_A \times V_f \times 1}{V_i \times V} \quad \cdots\cdots (3)$$

Where,

ω_m – the content of aflatoxin M_1 μg/ L;

m_A - the mass of aflatoxin M_1 acquired from the standard curve according to the peak or peak height of aflatoxin M_1 in the sample eluent, ng;

V_i - the volume of the sample eluent, μL;

V_f – the final volume of the sample eluent, μL;

V - the volume of the test sample passing through the immunoaffinity column, mL;

Formula (3) is suitable for undiluted sample, otherwise a diluted factor should be considered.

The calculated result should be have three significant figures.

13.2 Milk powder

Calculate the content ω_p of aflatoxin M_1 in the test sample according to formula (4).

$$\omega_p = \frac{m_A \times V_f \times 1}{V_i \times m} \quad \cdots\cdots (4)$$

Where,

ω_p - the content of aflatoxin M_1 in the sample, μg/kg;

m - the mass of milk powder in 50mL test solution (12.4), g;

The meaning of m_A, V_f and V_i are the same as that of 14.1.

The results are expressed with two independent arithmetic mean determined at reproducible conditions and have three significant figures.

14 Precision

The precision of laboratory test results is summarized in appendix B; these data don't apply to other concentration scope and materials.

Method 3 Immunochromatography Clean-up Combined with Fluorescence Spectrophotometry

15 Principles

After the test sample is centrifuged, defatted and filtered, the filtrate is then purified when passing through the immunoaffinity column containing specific monoclonal antibodies of aflatoxin M1; during the process, aflatoxin M_1 cross-links to antibodies on the chromatographic media. This antibody has specificity for aflatoxin M1; when the sample passes through the affinity column, the antibodies selectively combined to aflatoxin M_1 (antigen). Remove impurities on the immunoaffinity column with methanol - water (1+9), elute with methanol - water (8+2) passing through the immunoaffinity column, and add the eluent derivative with bromine solution to the fluorophotometer to determine the content of aflatoxin M1.

16 Reagents and materials

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

16.1 Methanol (CH_3OH): chromatographic pure.

16.2 Sodium chloride (NaCl).

16.3 Methanol - water (1+9): it is prepared by adding 10 mL methanol to 90 ml water.

16.4 Methanol - water (8+2): it is prepared by adding 80 mL methanol to 20 mL water.

16.5 Stock solution of bromine solution (0.01%): weigh sufficient amount of bromine, dissolve it to water to prepare 0.01% stock solution, and then store the solution protecting from light.

16.6 Working solution of bromine solution (0.002%): add 10 mL 0.01 % bromine solution to 40 mL water and mix even, store in a brown bottle for later use. It should be prepared immediately before use.

16.7 Quinine sulfate dihydrate ($C_2OH_{24}N_2O_2 \cdot H_2SO_4 \cdot 2H_2O$).

16.8 Sulphuric acid solution (0.05 mol/L): take 2.8 ml concentrated sulphuric acid, slowly add to sufficient water, cool and dilute to 1000 ml.

16.9 Calibration solution for fluorophotometer: weigh 0.340 g sulphuric acid quinine, dissolve and dilute to 100 mL with 0.05 mol/L sulphuric acid solution. The fluorophotometer reading of this solution is corresponding to 2.0 μg/L standard solution of aflatoxin M1. The fluorophotometer reading of 0.05 mol/L sulphuric acid solution (16.8) is corresponding to 0.0 μg/L aflatoxin M1.

17 Instruments & Equipment

17.1 Fluorophotometer

17.2 Centrifuge: the centrifuge force is no less than 7000 r/min

17.3 Glass fiber filter paper: with a diameter of 11 cm, and pore size of 1.5 μm.

17.4 Aflatoxin M_1 immunoaffinity column.

17.5 Pressure air pump.

17.6 Glass test tube: with a diameter of 12 mm, length of 75 mm and no characteristics of fluorescence.

17.7 Glass syringe.

18 Analytical procedures

18.1 Sample extraction

18.1.1 Milk: Take 50 mL milk sample, add 1.0 g sodium chloride (16.2), centrifuge at 7000 r/min for 10 min, carefully transfer the defatted milk to be analyzed from the underlayer without agitating the top fat layer, filter the defatted milk with a piece of glass fiber filter paper (17.3) and then store the filtrate for later use.

18.1.2 Milk powder: Weigh 5.0 g milk powder (accurate to 0.01g), slowly dissolve and dilute to 50 mL with 30□ - 60□ water, add 1.0 g sodium chloride, and then operate according to the procedures in 18.2.

18.2 Purification

Connect an immunoaffinity column (17.4) to a 10 mL glass syringe (17.7). Accurately transfer 10.0 mL of the above-mentioned filtrate (18.1) to a glass syringe, connect the pressure air pump (17.5) with the syringe and adjust the pressure to make the solution slowly flow through the immunoaffinity column (17.4) at a flow rate of about 6 mL /min until 2 mL - 3 mL air has passed through the column. Wash the column with 10 mL methanol - water (1+9) for two times, discard all of the effluent and make 2 mL - 3 mL air pass through the column. Add accurately 1.0 mL (V_1) methanol - water (8+2) to elute the column at a flow rate of 1 mL /min - 2 mL /min, collect all methanol - water eluent to a glass test tube (17.6) and store it for later use.

18.3 Determination

18.3.1 Calibration of the fluorophotometer

With the excitation wavelength of 360 nm and the emission wavelength of 3.250 nm, adjust the reading of fluorophotometer to be 0.0μg/L with 0.05 mol/L sulphuric acid solution as blank; adjust the reading of the fluorophotometer to be 2.0 μg/L with fluorophotometer calibration solution (16.9).

18.3.2 Determination of the test solution

Take the above-mentioned eluent and add to 1.0 mL (V) 0.002% bromine solution, and then immediately determine the content c of aflatoxin M1 with a fluorophotometer within 1 min.

18.3.3 Blank test

Replace the test sample with water, and then carry out the blank test according to procedures in 18.1 – 18.3.

19 Expression of results

19. 1 Milk

Calculate the results of milk detection according to formula (5):

$$X_1 = \frac{(C_1 - C_0) \times V_1 \times 10}{V} \quad \cdots\cdots (5)$$

Where,

X_1 - the content of aflatoxin M_1 in the test sample, with a unit of μg/ L;

c_1 - the concentration of aflatoxin M in the test solution read from the fluorophotometer, with a unit of μg/ L;

c_0 - the concentration of aflatoxin M read from the fluorophotometer in blank test, with a unit of μg/ L;

V_1 - the eluent volume of the final purified methanol –water (8+2), with a unit of mL;

V - the volume of the test sample passing through the affinity column, with a unit of mL;

10 - coefficient of the instrument reading

The calculated results should be accurate to the first decimal place.

19.2 Milk powder

Calculate the detection result of the milk powder according to formula (6):

$$X_2 = \frac{(C_2 - C_0) \times V_1 \times 10 \times V}{m} \quad \cdots\cdots (6)$$

Where,

X_2 - the content of aflatoxin M_1 in the test sample, with a unit of μg/kg;

C_2 - the concentration of aflatoxin M_1 in the test solution read from the fluorophotometer, with a unit of μg/ L;

C_0 - the concentration of aflatoxin M_1 read from the fluorophotometer in blank test, with a unit of μg/ L;

V_l - - the eluent volume of the final purified methanol –water, with a unit of mL;

V - the volume of the test sample passing through the affinity column, with a unit of mL;

m - the mass of milk powder contained in 50 mL test sample, with a unit of g/mL;

10 - coefficient of the instrument reading.

The results are expressed with two independent arithmetic mean determined at reproducible conditions and are accurate to the first decimal place.

Method 4 Bidirectional Enzyme-linked Immunosorbent Assay Method

20 Principles

Utilizing the principle of enzyme-linked immunosorbent assay competition, the residual aflatoxin M_1 in the sample reacts with quantitative specific enzyme labeling antibodies, and the remaining free enzyme labeling antibodies will combine with the coating antigen on the enzyme labeling plate; after washing, add chromogenic substrate of the enzyme to develop color, and then compare with the standard spot for qualitation .

21 Reagents and materials

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

21.1 Bidirectional enzyme-linked immunosorbent assay kit of aflatoxin M_1, stored at 2 □ - 7 □.

21.1.1 A series of standard solutions of flavacin M_1.

21.1.2 Particles of enzyme-linked immunosorbent assay reagent (containing specific enzyme labeling antibody).

21.1.2.1 Antibody for flavacin M_1.

Warning – the kit shouldn't be damaged, if so, it should be immediately destroyed.

21.1.2.2 Enzyme - enzyme conjugates

21.1.3 Chromogenic substrate of the enzyme

22 Instruments & Equipment

22.1 Sample test tube, with a sealing cap and particles of enzyme-linked immunosorbent assay reagents inside (21.1.2).

22.2 Transferpettor (tube), 450 μL±50 μL.

22.3 Enzyme-linked immunosorbent assay detection heater, 40℃±5℃.

22.4 Enzyme-linked immunosorbent assay detection reader.

23 Analytical procedures

23.1 Preheat the heater (22.3) to 45 □±5 □, and maintain at least for 15 min.

23.2 Shake the fluid test sample or milk powder test sample after it is recovered and mix it even, transfer 450 μL to a sample test tube (22.1), shake well to dissolve the particles of enzyme-linked immunosorbent assay reagents (21.1.2) completely.

23.3 Put the sample test tube (21.1) and the enzyme-linked immunosorbent assay detection kit (21.1) together into a preheated heater and maintain for 5 min - 6 min.

23.4 Pour all of the content in the sample test tube into the sample pool of the kit (21.1); the sample will flow through the “result display window” and to the green activation ring.

23.5 When the green color of the activated ring begin turning to white, press the ring activation button immediately.

23.6 Maintain the kit (21.1) in the heater (22.3) for 4 min to complete color reaction.

23.7 Take out the kit and insert the reader horizontally, and carry out the assessment program of the detection results immediately. The assessment program should be completed in 1 min.

24 Expression of results

24.1 visual observation and reading of the results

If the color of the test sample spot is deeper than that of the quality control spot, or they are equivalent, the detection result is negative.

If the color of the test sample spot is lighter than that of the quality control spot, the detection result is positive.

24.2 Assessment of the result of the enzyme-linked immunosorbent assay detection reader

If the numerical value is smaller than 1.05 and it displays Negative, the detection result is negative.

If the numerical value is greater than 1.05 and it displays Positive, the detection result is positive.

Note: positive samples should be further validated with the first method - quantitation detection method.

25 Other issues

The limit of quantitation of the first method in this standard is 0.01 μg/kg(based on milk); for milk powder, the lower detection limit of aflatoxin M1 of the second method is 0.08μg/kg, for milk, the lower limit is 0.008μg/L; for milk, the detection limit of aflatoxin M1 of the third method is 0.1μg/L, for milk powder, the detection limit is 0.l μg/kg; the detection limit of the fourth method is 0.5 μg/kg.

Appendix A

(Informative)

The chromatogram and reference conditions of immunochromatography clean-up combined with liquid chromatography – mass spectrometry of aflatoxin M1

A.1 The chromatogram and reference conditions of immunochromatography clean-up combined with liquid chromatography – mass spectrometry of aflatoxin M1

For scanogram of immunochromatography clean-up combined with liquid chromatography – mass spectrometry of aflatoxin M1, see figure A.1

For chromatogram-mass spectrum of immunochromatography clean-up combined with liquid chromatography – mass spectrometry of aflatoxin M1, see figure A.2

For gradient elution conditions of immunochromatography clean-up combined with liquid chromatography – mass spectrometry, see table A.1.

For control conditions of ion source of immunochromatography clean-up combined with liquid chromatography – mass spectrometry, see table A.2.

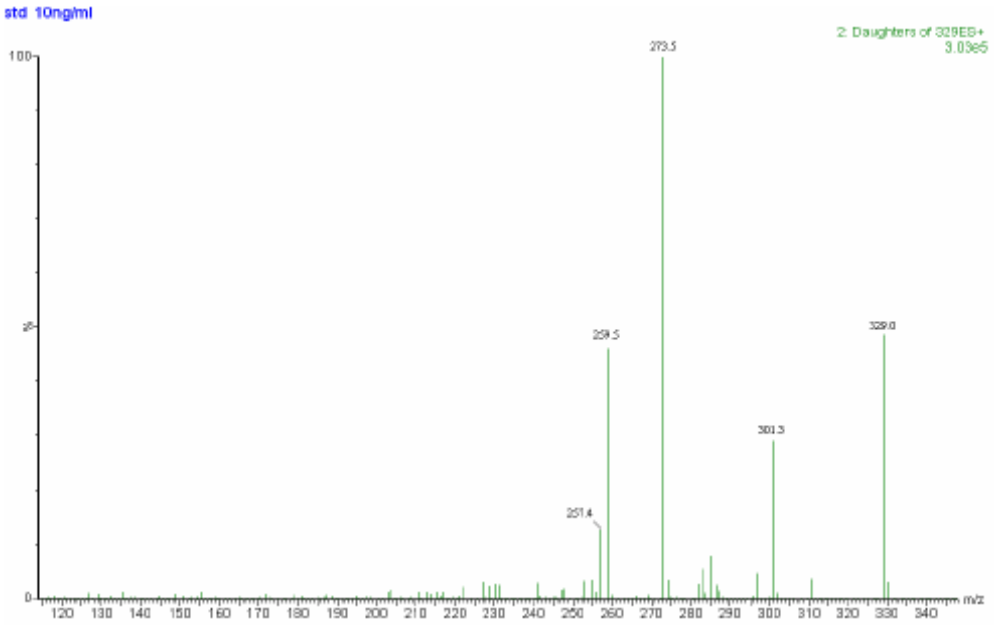

Figure A.1 The scanogram of M_1 daughter ion

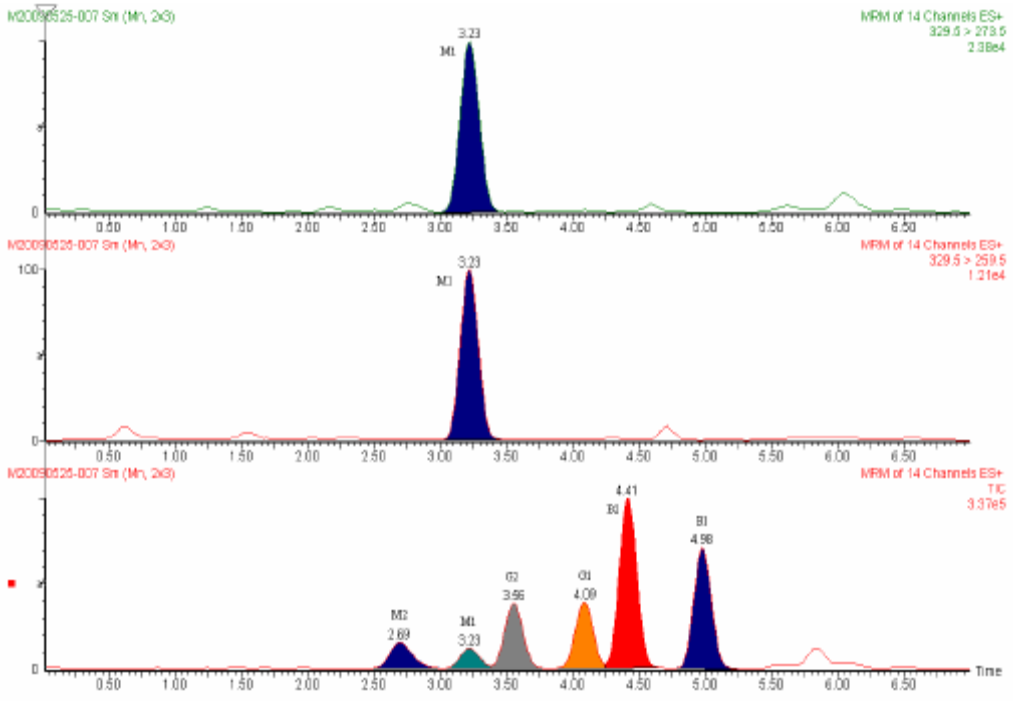

Figure A.2 The chromatogram-mass spectrum of M_1 daughter ion

A.1 Gradient elution conditions of liquid chromatography

Time (min)	Mobile phase A%	Mobile phase B%	Gradient change curve
0	68.0	32.0	-
4.20	55.0	45.0	6
5.00	0.0	100.0	6
5.70	0.0	100.0	1
6.00	68.0	32.0	6
Note: 1 represents immediate change, 6 represents linear change			

A.2 Control conditions of ion source

Ionization mode	Electric spray ionization, negative ion
Voltage of capillary tube (kV)	3.5
Cone voltage (V)	45
Voltage of radio-frequency lens 1 (V)	12.5
Voltage of radio-frequency lens 2 (V)	12.5
Ion source temperature (□)	120
Cone gas flow (L/h)	50
Desolvation temperature (□)	350
Desolvation gas glow (L/h)	500
Electric multiplying voltage (V)	650

Appendix B

(Informative)

Test results from many different laboratories

16 laboratories from all over the world have taken part in the coordination test of low fat (1%) and high fat (28%) milk powder samples. The high-fat samples are residues used to prepare reference materials [4], therefore, the content of aflatoxin M_1 is known.

For milk powder, its contamination level is 0.08 μg/ kg - 0.6 μg/ kg, i.e. for milk, the contamination level is 8ng/ L - 60ng/ L.

The test results are acquired with the statistical method specified in ISO 5725 – l and ISO 5725 – 2 [2; 3], the data of precision is listed in table B.1

(Note: test data is from reference literature [1] and [2]).

Table B. 1 Precision data

Number of the sample	1	2	3	4	5
Number of laboratories [a]	12	4	13	11	14
Average value / (ng/ kg)	81	150	80	202	580
repeatability value r/ (ng/ kg)	23	60.1	15	27	203
Reproducibility value R/ (ng/ kg)	52	98	41	61	310
Repeatability variation coefficient / (%)	9.9	14.0	6.8	4.7	12.5
Reproducibility variation coefficient / (%)	23	22.7	18.3	10.8	19.1
[a] The laboratories decreased is the data which should be rejected from the sample according to Cochran and Grubbs statistical method					

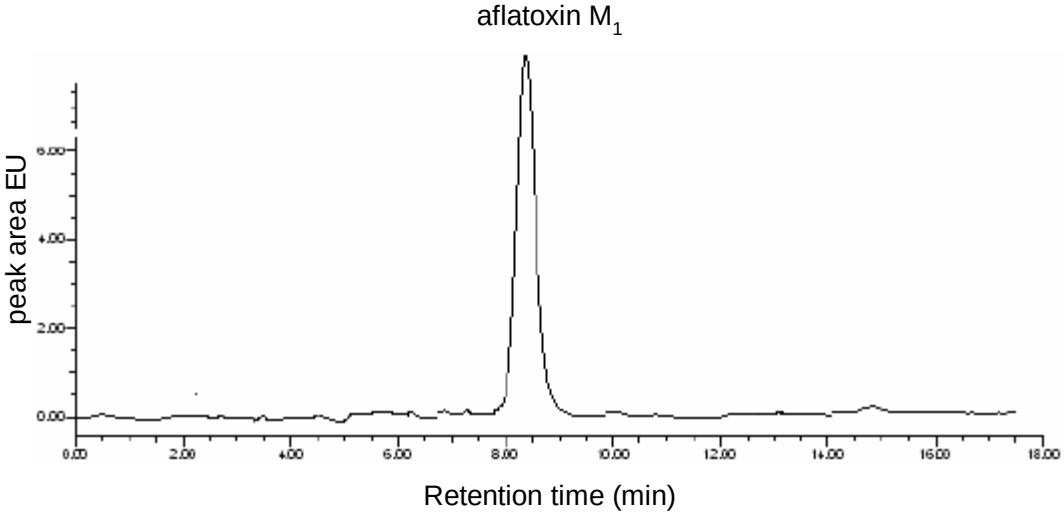

Figure B.1The standard chromatogram of high performance liquid chromatography,