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Approved By:

William Westman

Prepared By:

Mark Petry and Bao Liting

Report Highlights:

On November 20, 2009, China notified the WTO of "National Food Safety Standard of the People's Republic of China for Determination of Aflatoxin M1 in Milk and Dairy Products" as SPS/N/CHN/176. The date for submission of final comments to the WTO is January 1, 2010. The proposed date of entry into force has not been specified.

Executive Summary:

On November 20, 2009, China notified the WTO of "National Food Safety Standard of the People's Republic of China for Determination of Aflatoxin M1 in Milk and Dairy Products" as SPS/N/CHN/176. The date for submission of final comments to the WTO is January 1, 2010. The proposed date of entry into force has not been specified.

Thanks go to the consortium of industry and 3rd country Embassies in Beijing for their assistance in

translating and reviewing this standard.

This report contains an UNOFFICIAL translation of National Standard on Determination of Aflatoxin M1 in Milk and Dairy Products.

General Information:

BEGIN TRANSLATION

ICS 67.040

C53

GB National Food Safety Standard

GB ××××—××××

**Determination of Aflatoxin M1 in Milk and Dairy Products
(Draft for Comment)**

Issued on xx-xx-xxxx

Implemented on xx-xx-xxxx

Issued by the Ministry of Health
of the People's Republic of China

Preface

This standard reference refers to AOAC 997.12; the measures are not equivalent.
Compared with AOAC 997.12, the following main changes have been made to the standard:

Appendix A, B and C of this standard are all informative.

This standard is proposed by and under the jurisdiction of the Ministry of Health of People's Republic of China.

1 Scope

This standard specifies the method for determination of aflatoxin M₁ in milk and dairy products.

The first method in this standard applies to determination of aflatoxin M₁ in milk and dairy products; the second method applies to determination of aflatoxin M₁ 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 M₁ in milk and milk powder; the fourth method applies to determination of aflatoxin M₁ in cow's milk and its products; the fifth method applies to determination of aflatoxin M₁ in raw milk, pasteurized milk, UHT sterilized milk and milk powder.

The quantization limit of the first method in this standard is (calculate on the basis of fresh milk) 0.01 µg/kg; in the second method, the lowest detectable limit of aflatoxin M1 in milk powder is 0.08 µg/ kg, and the lowest detectable limit of aflatoxin M1 in milk is 0.008 µg/ L; in the third method, the lowest detectable limit of aflatoxin M1 in milk is 0.1 µg/L, the detection limit of aflatoxin M1 in milk powder is 0.1 µg/kg; the detection limit of the fifth method is 0.5 µg/kg.

2 Normative References

The following standards contain provisions which, through reference in this text, constitute provisions of this Standard. For dated reference, subsequent amendments to, or revisions of, any of these publications do not apply (excluding the contents corrected). However, the parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards. For undated references, the latest edition of the normative document referred to applies.

GB/T 6682 Water for analytical laboratory use specification and test methods

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 immuno-affinity column; dry the eluent with N₂, 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: chromatographic pure.

4.2 Acetonitrile: chromatographic pure.

4.3 Petroleum benzine: analytical reagent.

4.4 Chloroform: analytical reagent.

4.5 Nitrogen gas.

4.6 Standard sample: M₁: purity ≥ 98%.

4.7 Test solution

4.8 20% acetonitrile water solution: add 100 mL acetonitrile into 400 mL water.

4.9 10% acetonitrile water solution: add 50 mL acetonitrile to 450 mL water.

4.10 0.1 % formic acid solution: pipette 1 mL formic acid and dilute it with water to 1000 mL.

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

4.12 0.5 mol.L^{-1} sodium hydroxide solution: weigh 2 g sodium hydroxide and dissolve with 1000 mL water.

4.13 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 $0.22 \mu\text{m}$ microporous membrane (5.7), 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°C 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^{-1} . Transfer the solution to a plastic bottle, store in a -20°C refrigerator for later use.

4.15 A series of standard solutions:

Pipette $100 \mu\text{L}$ M_1 standard stock solution (4.14) to a 100 mL volumetric flask, blow chloroform with nitrogen gas to nearly dry, and dilute to volume with acetonitrile. Dilute M_1 standard stock solution (4.14) with blank matrix solution of the test sample (4.13) to a series of standard working solutions with concentrations with 0.5, 0.8, 1.0, 2.0, 4.0, 6.0 and 8.0 ng mL^{-1} , and then dilute to 1 mL.

5 Apparatus

5.1 Liquid chromatography - mass spectrometry: with electrospray ion source; mass scope: 1 mass charge ratio (m/z) - 1500 mass charge ratio (m/z); resolution: 0.1 atomic mass unit (AMU).

5.2 Chromatographic column: ACQUIT UPLC HSST3, with a length of 100 mm, an internal diameter of 2.1 mm; the particle diameter of the packing material is $1.8 \mu\text{m}$, 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: 4000 g centrifugal force (centrifugal force $g=1.12 \times 10^{-5} \times \text{rpm} \times \text{turning radius}$).

5.7 Centrifuge tube with a stopper: 50 mL.

5.8 Water bath: the temperature is controlled at $30^\circ\text{C} \pm 2^\circ\text{C}$ and $50^\circ\text{C} \pm 2^\circ\text{C}$, with the temperature scope between 25°C and 60°C .

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 Oscillator.
- 5.18 250 mL conical flask with a stopper.
- 5.19 Immunoaffinity column.
- 5.20 Single-use syringe: 10 mL and 50 mL.
- 5.21 Vacuum system.
- 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°C - 37°C. Centrifuge it at 4 000 g 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 and mix even, adjust pH to 7.4 with 0.5 mol.L⁻¹ sodium hydroxide solution (4.3.5), 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 test sample; transfer it into a 250 mL a beaker (5.10). Add 50 mL water which has been preheated to 50 °C to milk powder in many times with a small amount each time, and then mix even with a glass rod (5.14). If milk powder still doesn't dissolve completely, place the beaker (5.10) into a 50°C water bath (5.7) for 30 min, cool to 20 °C after the content has dissolved, and then transfer it to a 100 mL volumetric flask (5.9), 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 4000 g centrifuge force for 15 min, transfer 50 ml supernatant with a pipette (5.12).

6.1.4 Cheese

Weigh 5.0 g test sample which has been cut into pieces and sieved through a 10-mesh round-hole sieve (5.14), 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 4 000 g

centrifuge force for 15 min. Collect the supernatant, transfer the extraction solution collected to a 250 mL separating funnel (5.16), add 30 mL petroleum benzene, shake for 2min, wait until it has laminated, transfer the lower layer to a 50 mL beaker (5.10), and then discard the petroleum benzene layer. Extract with petroleum benzene ion for 2 times. Transfer the lower layer of solution to a 100 mL ground bottom flask (5.17), concentrate it at decreased pressure to about 2 mL, pour the concentrated solution to a centrifuge tube, wash the flask with 5 mL 20% acetonitrile water solution (4.3.1) for 2 times, transfer the combined eluent into a 50 mL centrifuge tube, dilute to about 50 mL with water, centrifuge at 4 000 g for 5 min, and then take the supernatant for purification.

6.1.5 Butter

Weigh 5.0g test sample, transfer to a 50 mL a beaker (5.10), dissolve the butter with 20 mL petroleum benzene (4.2.3) and transfer to a conical flask with a stopper (5.18). Add 20 mL water and 30 mL methanol, shake it for 30 min, and then transfer all of the fluid to a separating funnel. The following operations are carried out according to (6.1.4).

6.2 Purification

6.2.1 Preparation of immunoaffinity column

Connect a 50 mL single-use syringe barrel (5.20) to the top of an affinity column (5.19), and then connect the affinity column to vacuum system (5.21).

6.1.2 Purification of the test sample

Transfer the extraction solution of the test solution in 6.1 to a 50 mL syringe barrel (5.20), adjust the vacuum system (5.21), make the test sample flow through the column at a stable flow rate of 2 - 3 mL min^{-1} . 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. Disconnect 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 (4.2.2), 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 (4.2.5) at 30°C until the volume V_e is within 50 μL - 500 μL (warning: if it is evaporated to dry, M₁ may lose). Dilute V_e 10 times with 10% acetonitrile water solution to the final volume V_f (500 μL - 5 000 μL).

6.2 Reference conditions of liquid chromatography

Mobile phase: A phase, 0.1% formic acid solution (4.3.3); B phase, 1 : 1acetonitrile / methanol (volume ratio).

Gradient elution: refer to A.3 in appendix A.

The flow speed of the mobile phase: 0.3 mL.min⁻¹.

The temperature of the chromatographic column: 40 °C.

Temperature of the test solution: 20 °C.

Injection volume (V_i): 10 μL .

6.3 Reference conditions of mass spectra

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

Table 1 Selection parameter of ions

Aflatoxin	Parent ion	Quantization 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 appendix A.4.

6.4 Qualitative Results

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 $\pm 2.5\%$.

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 ($S/N \geq 3$), and the noise ratio of the remodeling ion chromatographic peak of the quantitative ions should be no less than 10 ($S/N \geq 10$).

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 qualification

Relative ion abundancy	> 50%	> 20% to 50%	> 10% to 20%	≤ 10
Permitted relative deviation	$\pm 20\%$	$\pm 25\%$	$\pm 30\%$	$\pm 50\%$

The target compounds are qualified 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.

6.5 Determination of test sample

According to the conditions established in 6.2 and 6.3, determine the ion intensity of M_1 in the test solution (6.1) and the series of standard solutions (4.14), and quantitate M_1 in the test sample with external standard method. For chromatogram, please see appendix A.2.

The retention time of chromatographic reference: M_1 3.23 min.

6.6 Blank test

No test sample is weighed; carry out blank experiments according to procedures in 6.4. It should be confirmed that there are no materials interfering the component to be tested.

7 Calculation and expression of results

7.1 Plotting of standard curve

Inject the series of standard solutions (4.14) in the increasing sequence of concentration to the column and detect, plot with peak area - concentration, and acquire a standard curve regression equation.

7.2 Quantitative determination

The response value of the test solution should be within the linear scope of the standard curve; if it exceeds the linear scope, it should be treated again according to 6.1 and then injected and analyzed.

7.3 Calculation

7.3.1 Quantitate the content with the external standard method, and calculate the residual volume of aflatoxin M₁ according to formula (1).

$$X = A \times (V_f / V_i) \times (1/M) \quad \dots \dots \dots (1)$$

Where,

X - the content of aflatoxin M₁ in the test sample, with a unit of μg/kg, μg Kg⁻¹;

A – the mass of aflatoxin M₁ corresponding to the chromatographic peak area of aflatoxin M₁ in the test sample, with a unit of ng;

V_i - injection volume, with a unit of μL;

V_f - the final volume of the eluent of the sample, with a unit of μL;

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 Terms and Definitions

The following terms and definitions are applicable to this standard.

9.1 Content of aflatoxin M₁

The mass content of this material determined according to this standard.

Note: the content of aflatoxin M₁ is expressed in μg/L or μg/kg.

10 Principles

When the test sample passes through the immunoaffinity column, aflatoxin M₁ will be extracted. The specific monoclonal antibodies of aflatoxin M₁ contained in the affinity column crosslink to solid support, when the sample passes through the affinity column, the antibodies selectively combine with

aflatoxin M₁ (antigen) to form antibody - antigen complex. Wash the column with water to remove impurities, then elute the aflatoxin M₁ absorbed onto the column with eluent and collect the eluent. Determine the content of aflatoxin M₁ in the eluent with high performance liquid chromatograph with a fluorescence detector.

11 Reagents and materials

Unless otherwise specified, all reagents used in this method are analytical reagent, and water is distilled water, deionized water or other water of equivalent purity.

11.1 Immunoaffinity column: it should contain antibodies to aflatoxin M₁. The maximum volume of the affinity column shouldn't be less than 100 ng aflatoxin M₁ (corresponding to 50 mL test sample with a concentration of 2 µg/ L); when the standard solution contains 4 ng aflatoxin M₁ (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 11.1.1 and 11.1.2).

11.1.1 Inspection of column efficiency

Transfer 1.0 mL aflatoxin M₁ stock solution (11.5.2) to a 20 mL tapered test tube (13.9) with a pipette (13.4). Slowly blow the fluid dry with continuous flow nitrogen gas (11.3), then dissolve the residues with 10 mL 10% acetonitrile (11.2.2) and shake heavily.

Transfer the solution to 40 mL water, mix it even, and then inject all of them to the immunoaffinity column. Use the immunoaffinity column according to the requirements in the directions. Elute aflatoxin M₁ from the immunoaffinity column after it is rinsed. Appropriately dilute the eluent, determine the content of aflatoxin M₁ combined onto the immunoaffinity column with high performance liquid chromatograph determination.

Calculate the recovery rate of aflatoxin M₁, and compare the results with the indices required in 11.1.

11.1.2 Inspection of recovery rate

Transfer 0.8 mL 0.005 µg/ mL standard working solution of aflatoxin M₁ (11.5.3) to 10mL water with a pipette (13.4), mix it even and inject all of them into the immunoaffinity column. Use the immunoaffinity column according to the directions. Elute aflatoxin M₁ from the immunoaffinity column after it is rinsed. Appropriately dilute the eluent, determine the content of aflatoxin M₁ combined onto the immunoaffinity column with high performance liquid chromatograph determination. Calculate the recovery rate of aflatoxin M₁, and compare the results with the indices required in 11.1.

11.2 Acetonitrile: chromatographic grade.

11.2.1 25% acetonitrile - water solution: Dissolve 250 mL acetonitrile (11.2) with 750 mL water (needing degassing before being used).

11.2.2 10% acetonitrile - water solution: Dissolve 100 mL acetonitrile (11.2) with 900 mL water (needing degassing before being used).

11.3 Nitrogen gas.

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

it.

11.5 Standard solution of aflatoxin M₁

11.5.1 Calibration solution

The concentration of the chloroform standard solution of aflatoxin M₁ 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 M₁.

Determine the absorbance at 340 nm - 370 nm with a spectrophotometer (13.11), 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 c_i (µg/mL) according to formula (2).

$$c_i = A \times M \times 100/\epsilon \dots\dots\dots (2)$$

Where,

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

M - 328 g/mol, molar mass of aflatoxin M₁, with a unit of g/mol

ϵ - 1995, the absorbance coefficient of aflatoxin M₁ dissolved in chloroform, with a unit of m²/mol.

11.5.2 Standard stock solution

Determine the actual concentration value of the standard solution of aflatoxin M₁ (11.5.1), 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°C and store it protecting from light. Under such conditions, the stock solution can be stable for two months; two months later, the stability of the stock solution should be verified.

11.5.3 Standard working solution of aflatoxin M₁

Take the stock solution (11.5.2) 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 on the same day of usage.

Preparation of the standard working solution of aflatoxin M₁: accurately transfer 1.0 mL stock solution (13.4.2) to a 20mL tapered test tube (13.9) with a pipette (13.4), slowly blow the solution dry with nitrogen gas (11.3), then dissolve the residues with 20.0 mL 10% acetonitrile (11.2.2), shake for 30 min and mix it even, prepare it to the standard working solution of aflatoxin M₁ 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 M₁ 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 M₁ with appropriate concentrations with the working solution. The dilution solution used is 10% acetonitrile (11.2.2)

12 Apparatus and materials

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

12.2 Vacuum system.

12.3 Centrifuge: 4000 g centrifuge force (centrifuge force $g = 1.12 \times 10^{-5} \times \text{rpm} \times \text{turning radius}$).

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

12.5 Glass beaker: 250 mL.

12.6 Volumetric flask: 100 mL.

12.7 Water bath: the temperature is controlled at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $50^{\circ}\text{C} \pm 2^{\circ}\text{C}$; with the temperature scope between 35°C and 37°C .

12.8 Filter paper.

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

12.10 High performance liquid chromatograph

12.10.1 Pulse-free pump: a pump which is suitable for constant flow of about $1 \text{ mL} \cdot \text{min}^{-1}$.

12.10.2 Injection system: an injection ring with constant or variable volume of $50 \mu\text{L} - 500 \mu\text{L}$.

12.10.3 Reversed phase chromatographic column: guard column packed with $3 \mu\text{m}$ or $5 \mu\text{m}$ octadecyl silica gel together with reversed phase materials.

12.10.4 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.

12.10.5 Recorder: with a printer or graph plotter, electronic integrator or computer data treatment system.

12.11 Spectrophotometer: with a wavelength scope of 200 nm - 400 nm and a quartz colorimetric cell with light path length of 1 cm.

12.12 Balance: accurate to 0.1 g, the smallest scale division is 0.01 g.

13 Sampling

Sampling methods are not within the scope of this standard; for recommended sampling method, please refer to ISO707 [1]

The samples received by the laboratory should have real representativeness and haven't been damaged or changed during the process of transportation and storage.

14 Analytical procedures

14.1 Summary

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

When the affinity column of a different manufacturer is used, there may be some slight difference in the preparing, washing and eluting of milk powder; operations should be carried out strictly according to the directions or requirements. Usually, the analytical procedures include: dissolve milk powder to

milk with water or salt water buffer solution, centrifuge and separate it and then inject it to the column under certain pressure (the column may need prewashing), rinse the column with water and then elute aflatoxin M₁ absorbed on the column with methanol or acetonitrile. The specified flow rate should be strictly followed.

14.2 Preparation of test sample

14.2.1 Milk

Heat the milk sample in a water bath (12.7) to 35°C - 37°C. Filter it with a filter paper (12.8) (it may need several pieces of filter paper according to circumstances), or centrifuge at 4000 g centrifuge force for 15 min. Collect at least 50 mL milk test sample, and then continue analysis according to 1.7.4.

14.2.2 Milk powder

Weigh 10g sample (accurate to 0.1 g) and place to a 250 mL beaker (12.5). Add 50 mL water which has been preheated to 50°C 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°C water bath (12.7) for at least 30 min, carefully mix even. Cool the dissolved milk powder to 20°C, transfer to a 100 mL volumetric flask (12.6), 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. Filter the milk with a piece of filter paper (12.8) or centrifuge under 4000 g centrifuge force for 15 min. Collect at least 50 mL milk test sample, and then continue to analyze it according to 14.4.

14.3 Preparation of immunoaffinity column

Connect a 50 mL single-use syringe barrel (12.1) to the top of an affinity column (11.1), and then connect the affinity column to vacuum system (12.2).

14.4 Extraction and purification of the sample

Transfer 50mL test sample (14.2.1 or 14.2.2) to a 50 mL syringe (12.1) with a pipette, adjust the vacuum system (12.2), 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. Disconnect the vacuum system, put on another 10 mL syringe barrel, add 4 mL acetonitrile (11.2), slowly push the plunger to control the flow rate to elute M₁, collect the eluent to a tapered tube (12.9); the elution time shouldn't be less than 60 s. Slowly evaporate the eluent with nitrogen gas (11.3) at 30°C until the volume V_e is within 50 μL - 500 μL (warning: if it is evaporated to dry, aflatoxin M₁ may lose). Dilute V_e 10 times with 10% water to the final volume V_f (500 μL - 5 000 μL).

Note: if a sample containing aflatoxin M₁ 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.

14.5 High performance liquid chromatograph

14.5.1 Pump

Pump acetonitrile - water solution (11.2.1) through high performance liquid phase chromatographic column at a constant flow rate. If needed (according to the model of the chromatographic column used), adjust the proportion of acetonitrile – water to ensure best separation between aflatoxin M_1 and other components.

Select the volume flow rate of acetonitrile - water solution (11.2.1) according to the chromatographic column (12.10.3) used. For a common chromatographic column (with a length of about 25 cm and an internal diameter of about 4.6 mm), best effects can be achieved at a flow rate of 1mL /min; if the internal diameter is 3 mm, best effects can be achieved at a flow rate of 0.5 mL / min.

In order to determine the best chromatographic conditions, you had better first inject negative sample extraction solution containing no aflatoxin M_1 into HPLC, and then inject mixed solution of the sample extraction solution and standard solution of aflatoxin M_1 .

14.5.2 Chromatographic performance

The linearity of the standard curve and stability of the chromatographic system need repeated check with multiple injections of a fixed amount of standard solution of aflatoxin M_1 until stable peak area and peak height are acquired. For peak area and peak height, the differences between two peaks close to each other shouldn't exceed 5%.

The retention time of aflatoxin M_1 is related with temperature, therefore, the drift of the determination system needs compensation. Determine a fixed amount of standard solution of aflatoxin M_1 from time to time, the determination results of the standard solution can be adjusted according to drift circumstances.

14.5.3 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 .

14.5.4 Chromatographic analysis and injection regimen of the eluent of the sample

Inject the eluent of sufficient volume V_i to the high performance liquid chromatograph through injection ring. Separate aflatoxin M_1 from the eluent with the same chromatographic conditions as those used for the standard solution. Inject standard solution and the eluent of the sample according to specified regimen. If a series of samples are continuously detected, it is suggested that a portion of standard solution of aflatoxin M_1 is detected every five samples.

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.

15 Calculation and expression of results

15.1 Milk

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

$$\omega_m = m_A \times (V_f/V_i) \times (1/V) \dots\dots\dots(3)$$

Where,

ω_m – the content of aflatoxin M_1 , with a unit of $\mu\text{g/L}$;

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

V_i - the volume of the sample eluent, with a unit of μL ;

V_f – the final volume of the sample eluent, with a unit of μL ;

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

The calculated result should be accurate to the third decimal place.

15.2 Milk powder

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

$$\omega_p = m_A \times (V_f/V_i) \times (1/m) \dots\dots\dots(4)$$

Where,

ω_p - the content of aflatoxin M_1 in the sample, with a unit of $\mu\text{g/kg}$;

m - the mass of milk powder in 50mL test solution (14.4), with a unit of g;

The meaning of m_A , V_f and V_i are as defined in 8.1.

Formula (3) applies to test samples which haven't been diluted; otherwise it should multiply dilution times.

The calculated result should be accurate to the third decimal place.

16 Precision

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

17 Test report

The test report should contain the following contents:

- a) All information needed to describe complete characteristics of the sample;
- b) Sampling method, if it is known;
- c) The test method adopted according to this standard;
- d) Other operation details which are not specified in this standard, operations, phenomenon and measures taken which may influence the test results;
- e) Test results;
- f) If repeatability is inspected, the final verification results;

Method 3 Immunochromatography Clean-up Combined with Fluorescence Spectrophotometry

18 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 M₁ 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 M₁ (antigen). Remove impurities on the immunoaffinity column with methanol - water (10+90), elute with methanol - water (80+20) passing through the immunoaffinity column, and add the eluent derivative with bromine solution to the fluorophotometer to determine the content of aflatoxin M1.

19 Reagents and materials

Unless otherwise specified, all reagents used in this method are analytical reagent reagents, and water is redistilled water.

19.1 Methanol (CHOH): chromatographic pure.

19.2 Sodium chloride (NaCl).

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

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

19.5 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.

19.6 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.

19.7 Quinine sulfate dihydrate ($C_2O_4H_2N_2O_2 \cdot H_2SO_4 \cdot 2H_2O$).

19.8 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.

19.9 Calibration solution for fluorophotometer: weigh 0.340 g sulphuric acid quinine ($C_2O_4H_2N_2O_2 \cdot H_2SO_4 \cdot 2H_2O$), 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 is corresponding to 0.0 µg/L aflatoxin M1.

20 Apparatus

20.1 Fluorophotometer

20.2 Centrifuge: the centrifuge force is no less than 4000 r/min

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

20.4 Aflatoxin M1 immunoaffinity column.

20.5 Pressure air pump.

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

20.7 Glass syringe.

21 Analytical procedures

21.1 Sample extraction

21.1.1 Milk

Take 50 mL milk sample, add 1.0 g sodium chloride, centrifuge at 4000 r/min for 10 min, carefully transfer the defatted milk to be analyzed from the under layer without agitating the top fat layer, filter the defatted milk with a piece of glass fiber filter paper and then store the filtrate for later use.

21.1.2 Milk powder

Weigh 5.0 g milk powder, slowly dissolve and dilute to 50 mL with 30°C - 60°C water, add 1.0 g sodium chloride, and then operate according to the procedures in 21.1.1.

21.2 Purification

Connect an immunoaffinity column to a 10 mL glass syringe. Accurately transfer 10.0 mL of the above-mentioned filtrate to a glass syringe, connect the pressure air pump with the syringe and adjust the pressure to make the solution slowly flow through the immunoaffinity column 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 (10+90) 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 (80+20) 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 and store it for later use.

21.3 Determination

21.3.1 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 0.0 μ g/L with 0.05 mol/L sulphuric acid solution as blank; adjust the reading of the fluorophotometer to be 2.0 μ g/L with fluorophotometer calibration solution.

21.3.2 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.

21.3.3 Blank test

Replace the test sample with water, and then carry out the blank test according to procedures in 21.1 - 21.3.

22 Calculation and expression of results

22.1 Milk

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

$$X_l = (c_l - C_0) \times V_l \times 10 / V \quad \dots\dots\dots (5)$$

Where,

X_l - the content of aflatoxin M_1 in the test sample, with a unit of μ g/ L;

c_1 - the concentration of aflatoxin M in the test solution read from the fluorophotometer, with a unit of $\mu\text{g/L}$;

c_0 - the concentration of aflatoxin M read from the fluorophotometer in blank test, with a unit of $\mu\text{g/L}$;

V_1 - 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;

IO - coefficient of the instrument reading

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

22.2 Milk powder

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

$$X_2 = (c_1 - c_0) \times V_1 \times IO / m \times V \quad \dots\dots\dots (6)$$

Where,

X_2 - the content of aflatoxin M_1 in the test sample, with a unit of $\mu\text{g/kg}$;

C_2 - the concentration of aflatoxin M_1 in the test solution read from the fluorophotometer, with a unit of $\mu\text{g/L}$;

C_0 - the concentration of aflatoxin M_1 read from the fluorophotometer in blank test, with a unit of $\mu\text{g/L}$;

V_1 - 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;

IO - coefficient of the instrument reading.

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

Method 4 Thin Layer Chromatography

23 Principles

After the test sample is extracted, concentrated and separated with thin layer chromatography, aflatoxin M_1 emits amethyst fluorescence under ultraviolet light (with a wavelength of 365 nm); determine the content of it according to its limit of detection when it displays fluorescence during chromatography.

24 Reagents and materials

24.1 Chloroform.

24.2 N-hexane or petroleum benzine (with a boiling range of 30 - 60°C or 60 - 90°C).

24.3 Methanol.

24.4 Benzene.

24.5 Acetonitrile.

24.6 Absolute ether or diethyl ether which has been dehydrated with sodium sulphate anhydrous.

24.7 Acetone.

24.8 Isopropyl alcohol.

For the above reagents, a reagent blank test is first carried out before the test, they can be used if they don't interfere with determination; otherwise they should be redistilled.

24.9 Silica gel G: it is used for chromatography.

24.10 Sodium chloride and sodium chloride solution (40 g/L).

24.11 Sulphuric acid (1+3).

24.12 Glass sand: they should be first treated with acid and then washed clean; they are corresponding to about 20 meshes.

24.13 Standard solution of aflatoxin M₁: standard solution of aflatoxin M₁ is prepared with chloroform, with a concentration of 10 µg/ml. With chloroform as the blank reagent, the wavelength of the maximum ultraviolet absorption peak of aflatoxin M₁ is near 357 nm; the molar absorptivity index is 19950. It should be stored in a 4°C refrigerator and be protected from light.

24.14 Standard working solution of aflatoxin M₁: the solution is prepared with chloroform, with a concentration of 0.04µg/ml. It should be stored in a 4°C refrigerator and be protected from light.

25 Apparatus

25.1 Minitype pulverizer.

25.2 Sample sieve.

25.3 Electric oscillator.

25.4 Glass concentrator.

25.5 Glass plate. 5 cm×20 cm.

25.6 Thin layer plate spreader.

25.7 Developing tank: with an internal diameter of 25 cm, width of 6 cm and height of 4 cm.

25.8 Viltalight lamp: 100 W- 125 W, with a light filter at 365 nm.

25.9 Micro syringe or hemochrome pipette.

26 Analytical procedures

The whole operation process should be completed in a dark room.

26.1 Extraction of the test sample

26.1.1 Test sample extraction and preparation table. See table 1.

26.1.2 Calculate the amount of the extracted solution according to formula (7).

$$X = 8/15 \times (90 + A + B) \dots\dots\dots (7)$$

Where,

X - extraction amount of the solution, mL;

A -water content in the test sample, mL (for cow's milk and condensed milk, the sampling amount is 30g; for cow's milk powder and cheese, the sampling amount is 15g);

B - water content, mL.

Note: the water content in the test sample should refer to Food Composition Table compiled by the Institute for Nutrition and Food Safety of the Chinese Center for Disease Control and Prevention.

Table 1 Preparation of test sample

Name of the test sample	Sample mass g	Water content, mL	Methanol content, mL	Amount of the extraction solution mL	The amount of 40g/L sodium chloride solution added mL	Concentration volume mL	Dripping volume μ L	Sensitivity of the method μ g/kg
Cow's milk	30	0	90	62	25	0.4	100	0.1
Condensed milk	30	0	90	52	35	0.4	50	0.2
Cow's milk powder	15	20	90	59	28	0.4	40	0.5
Cheese	15	5	90	56	31	0.4	40	0.5
Butter	10	10	55	80	0	0.4	40	0.5

Because the individual extraction solutions contain 48mL methanol, 39 mL water is needed to adjust the volume ratio between methanol and water to be (55+45), therefore, the volume of sodium chloride solution added (40g/L) is equal to (87 mL) subtracting the extraction solution amount (mL).

26.1.3 Cow's milk and condensed milk: weigh 30.00 g test sample which has been mixed even, place into a small beaker, and then transfer it to a 300 mL conical flask with a stopper with 90 mL methanol, and put on the stopper so as to prevent leakage. Shake for 30 min, and then filter the solution with a piece of folding fast filter paper to a 100 mL volumetric cylinder with a stopper. Collect 62 mL cow's milk and 52 mL condensed milk (respectively corresponding to 16 g test sample) extraction solution according to the above table.

26.1.4 Cow's milk powder: weigh 15.00 g test sample to a conical flask with a stopper, add 20 mL water into it, after the test sample is moistened, add 90mL methanol, then carry out the following procedures according to 6.1.2 from "Shake for 30min". Collect 59mL extraction solution (corresponding to 8g test sample) according to table 1.

26.1.5 Cheese: weigh 15.00 g test sample which has been cut into pieces and sieved through a 10-mesh round-hole sieve, mix even, place into a conical flask with a stopper, add 5mL water and 90mL methanol, carry out the following procedures according to 6.1.2 from “Shake for 30min”, and then collect 56 mL extraction solution (corresponding to 8g test sample) according to the above table.

26.1.6 Butter: weigh 10.00g test sample, place it into a beaker, dissolve the butter with 40 mL petroleum benzene and then transfer the solution into a conical flask with a stopper. Add 45 mL water and 55 mL methanol, shake for 30 min, and then transfer all of the fluid into a separating funnel. Add 1.5g sodium chloride, shake until it is dissolved, after it is laminated, collect 80 mL extraction solution (corresponding to 8g test sample) to a volumetric cylinder with a stopper according to the above table.

26.2 Purification

26.2.11 Allocation and purification with petroleum benzene: transfer the collected extraction solution to to a 250 mL separating funnel, then add a certain volume of sodium chloride solution (40 g/L) according to the category of foods (see preparation table). Add 40mL petroleum benzene, shake for 2min, after stratification, transfer the lower layer which is methanol - sodium chloride water layer to the original volumetric cylinder, pour out the upper layer which is petroleum benzene solution from the upper opening of the separating funnel and discard. Then transfer the solution in the volumetric cylinder to the original separating funnel. Then extract with petroleum benzene for 2 times, 40 mL per time, at last transfer the solution in the volumetric cylinder to the separating funnel. The test solution of butter is extracted with petroleum benzene for 2 times, 40 mL per time.

26.2.2 Allocation and extraction with chloroform: add 20 mL chloroform to the original volumetric cylinder, shake up, transfer to the original separating funnel, and then shake for 2min. **After** stratification, transfer the lower layer which is chloroform to the original volumetric cylinder, and then extract with chloroform extraction for two times, 10 mL per time, then incorporate chloroform to the original volumetric cylinder. Discard the upper layer which is methanol water solution.

26.2.3 Washing the chloroform layer with water, concentration and preparation: pour the incorporated chloroform layer to the original separating funnel, add 30mL sodium chloride solution (40g/L), shake for 30s, and allow to stand. When the suspension of the upper layer is clear, collect the lower chloroform layer to the original volumetric cylinder. Add 10g sodium sulphate anhydrous, shake and allow to stand until it is clear, filter the solution through a quantitative slow filter paper containing a little sodium sulphate anhydrous to a 100 mL evaporating dish. Extract the sodium chloride water layer with 10 mL chloroform, and filter the solution to the evaporating dish. At last, pour the sodium sulphate anhydrous onto the filter paper, wash the volumetric cylinder and sodium sulphate anhydrous with a small amount of chloroform, and then filter them into the evaporating dish. Evaporate the solution to dry in a 65°C water bath under ventilation. Transfer the residue in the evaporating dish to a concentration tube with chloroform. If there is too much residue in the evaporating dish, filter it through filter paper to the concentration tube. Concentrate the solution to less than 0.4 mL under reduced pressure and ventilation at 65 °C, wash the tube wall with a small amount of chloroform, concentrate and quantitate to 0.4 mL, and then store it for later use.

26.3 Determination

26.3.1 Preparation of silica gel G thin layer plate: the thickness of the thin layer plate is 0.3mm; activate the plate at 105°C for 2h and then store it in a desiccator for 1 – 2 days.

26.3.2 Treatment of the plate: take two thin layer plates with the size of 5 cm×20 cm, add two drops onto the baseline 3cm away from the lower end of the plate, add 10 µL standard working solution of aflatoxin M₁ at 0.8 - 1cm away from the left side on the two plates, drip a same drop at 2.8 - 3cm away from the edge of the plate (for the volume for various kinds of foods, please see table in 6.1.1), and then drip 10 µL mixed standard working solution of aflatoxin M₁ on the second drop point on the second plate. Usually place the thin layer plate on a chromatography tank filled with dry silica gel and then begin to drip, blow it dry with an air cooling machine while dripping.

26.3.3 Development

26.3.3.1 Horizontal development: Add 15mL absolute ether which has been dehydrated with odium sulphate anhydrous into the tank (add 20 g sodium sulphate anhydrous to 500mL absolute ether). Place the longer side of the thin layer plate which is close to the standard point into the tank, take it out and evaporate to dry after it has developed to the end, and then repeat the procedure.

26.3.3.2 Longitudinal development: Longitudinally develop the thin layer plate which has been horizontally developed for two times and dried with the mixed developing solvent consisting of isopropyl alcohol - acetone - benzene - N-hexane - petroleum benzine (boiling range 60 - 90°C) - chloroform (5 + 10 + 10 + 10 + 10 + 10) until the front edge is 10 - 12 cm away from the origin point, take the plate out and evaporate to dry.

26.3.3.3 Horizontal development: Horizontally develop the plate which has been longitudinally developed and evaporated with diethyl ether for 1 - 2 times; the development methods are the same as those in 6.3.3.1.

26.3.4 Results of observation and evaluation

26.3.4.1 Observe and compare the two plates under ultraviolet light. If there is the limit of detection at the point corresponding to the spot of standard substance of aflatoxin M₁ at the second spot on the second plate (the R_f values of M₁ and B₁ are 0.25 and 0.43 respectively), while there is no fluorescence spot on the same position of the first plate, then the content of aflatoxin M₁ in the test sample is below the sensitivity of the method adopted (see the table in 6.1.1).

26.3.4.2 If there is a fluorescence spot of aflatoxin M₁ on the same position of the first plate, observe whether the second spot on the second plate overlap with the spot of the standard substance, if they overlap, carry out the following quantization and validation test.

26.3.5 Dilution and quantization: If the fluorescence intensity of the fluorescence spot of aflatoxin M₁ in the test solution is consistent with that of the limit of detection (0.0004µg) of aflatoxin M₁, then the contents of aflatoxin M₁ in cow's milk, condensed milk, cow's milk powder, cheese and butter test samples are 0.1, 0.2, 0.5, 0.5 and 0.5 µg/ kg, respectively. If the fluorescence intensity of aflatoxin M₁ in the test solution is higher than that of the limit of detection, then determine the content one by one according to their intensities, estimate the number of µL needing decreased or add different µL of the solution which has been diluted, until the fluorescence intensity of the test solution is consistent with that of the limit of detection.

26.3.6 Validation test: draw a circle with a pin on the spot of aflatoxin M₁ needing validation on the qualitative or quantitative thin layer plate. Spray the spot with sulphuric acid solution (1+3), allow to

stand for 5min and then observe under ultraviolet light, if the spot of aflatoxin M₁ of the test solution emits yellow fluorescence just like the spot of the standard substance, then it can be validated that the fluorescence spot detected is aflatoxin M₁.

27 Calculation and expression of results

$$X = 0.0004 \times V_1 / V_2 \times D \times 1000 / m \quad \dots\dots\dots (8)$$

Where,

X –the content of aflatoxin M₁, µg/ kg;

V₁ – the volume of the concentrated test solution, mL;

V₂ - the volume added when there is minimal fluorescence, mL;

D –the dilution times of the concentrated test solution;

m - the mass of the test sample corresponding to the concentrated test solution, g;

0.0004 - the limit of detection of aflatoxin M₁, µg.

The determination result is truncated to an integer.

Method 5 Bidirectional Enzyme-linked Immunosorbent Assay Method

28 Principles

Utilizing the principle of enzyme-linked immunosorbent assay competition, the residual aflatoxin M₁ 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 qualification .

29 Reagents and materials

In this method, water is secondary water specified in GB/T 6682.

29.1 Bidirectional enzyme-linked immunosorbent assay kit of aflatoxin M₁, stored at 2 °C - 7 °C.

29.1.1 A series of standard solutions of flavacin M₁.

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

29.1.2.1 Antibody for flavacin M₁.

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

29.1.2.2 Enzyme - enzyme conjugates

29.1.3 Chromogenic substrate of the enzyme

30 Apparatus

30.1 Sample test tube, with a sealing cap and particles of enzyme-linked immunosorbent assay reagents inside (29.1.2).

30.2 Transfer (tube), 450 $\mu\text{L} \pm 50 \mu\text{L}$.

30.3 Enzyme-linked immunosorbent assay detection heater.

30.4 Enzyme-linked immunosorbent assay detection reader.

31 Analytical procedures

31.1 Preheat the heater (30.3) to $45 \text{ }^\circ\text{C} \pm 5 \text{ }^\circ\text{C}$, and maintain at least for 15 min.

31.2 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 (30.1), shake well to dissolve the particles of enzyme-linked immunosorbent assay reagents (29.1.2) completely.

31.3 Put the sample test tube (30.1) and the enzyme-linked immunosorbent assay detection kit (29.1) together into a preheated heater and maintain for 5 min - 6 min.

31.4 Pour all of the content in the sample test tube into the sample pool of the kit (29.1); the sample will flow through the “result display window” and to the green activation ring.

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

31.6 Maintain the kit (29.1) in the heater (30.3) for 4 min to complete color reaction.

31.7 Take out the kit (29.1), and insert the reader horizontally (30.4), operate according to the prompt on the touch screen, and carry out the assessment program of the detection results immediately. The assessment program should be completed in 10 min.

32 Calculation and expression of results

32.1 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.

32.2 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 positive¹⁾.

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

Appendix A (Informative)



Figure A.1 The scanogram of M₁ daughter ion

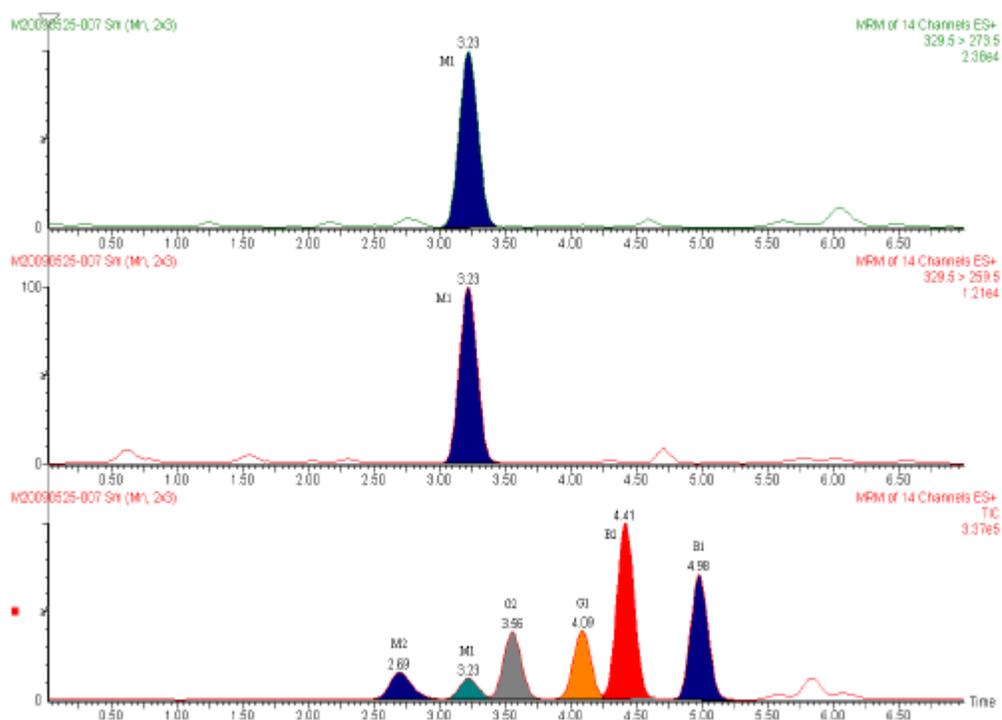


Figure A.

A.3 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.4 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 (°C)	120
Cone gas flow (L/h)	50
Desolvation temperature (°C)	350
Desolvation gas flow (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 M₁ 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 – 1 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					